Multi-species assessment of South African coal mine reclamation soils for ecosystem recovery

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DEDICATION

This thesis is dedicated to all lovers of science and the numerous individuals who provided support during the study duration.

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ABSTRACT

At present, there is no comprehensive soil quality assessment practice for the coal mining industry in South Africa. Studies have shown that belowground soil biota are crucial to soil ecosystem functioning and are sensitive bioindicators for soil quality monitoring. Due to the limitations of some previously developed methods for analysing microbial community structure, a more robust approach involving high-throughput culture-independent molecular techniques was utilised to assess post-coal-mining reclamation soils for potential ecosystem recovery, support function and potential microbial bioindicators. Also, the potential impact of soil physicochemical properties in shaping soil biotic communities were assessed. The study was conducted in three stages. Firstly, the potential contribution of soil stockpiles to post-mining reclamation success was assessed by analysing arbuscular mycorrhizal fungal (AMF) spore density and viability as well as microbial community diversity and structure. Overall, results suggest that AMF spore density in stockpiles do not differ significantly (P <0.05) from those of an unmined soil but spore viability may be affected by stockpile height. Also, variations in the microbial community structure of soil stockpiles were site-specific, but when compared to the unmined site, the microbial community structure and diversity observed in soil stockpiles were impaired. Thus, the impairment in soil microbial diversity and structure suggest post-mining reclamation success may be affected. Secondly, soil samples were collected from reclamation areas in three coal mining sites, as well as from reclamation areas of different ages (ranging from 3 years to 24 years) within a single coal mine. The samples were analysed using a combination of methods that includes community-level physiological profiling (CLPP), enzyme assays, and high-throughput sequencing of the bacterial 16S rRNA gene, fungal ITS2 and a Glomeromycotan-specific partial small subunit. The results provide evidence to support the hypothesis that indeed the microbial communities of post-coal mining soils are significantly (PERMANOVA, P < 0.05) differentiated along a temporal scale of years since reclamation as well as between unmined areas and reclamation areas. When compared to the unmined area, bacterial community richness and diversity data support that restoration is a function of time, and occurs between 15- and 19-years post-reclamation. Furthermore, relative

stability in fungal community diversity over years of reclamation compared to bacterial community diversity suggests that bacterial communities are more likely to serve as bioindicators of ecosystem restoration in the post-mining soil environments. Of all the methods, CLPP could not detect significant ($P > 0.05$) differences in microbial community richness and diversity amongst samples while enzyme activities were highly variable within-sites. The assemblages of the obligate plant symbiont, AMF, were less differentiated when compared to other microbial groups suggesting that AMF assemblages could be less suitable bioindicators of ecosystem recovery. Some genera with soil quality indicator potential such as *Acidothermus*, *Bryobacter* and *Halingium*, as well as plant-growth promotion potentials such as *Mesorhizobium*, *Bradyrhizobium and Microvirga* were relatively more abundant across soils, whereas a vast majority of other microbial species and their functions in reclamation soils are still largely unknown. Lastly, the capability of the soil to serve as a habitat to support soil biota association and functions was assessed using an ecotoxicological approach by utilising earthworms as bioindicators. Endpoints such as avoidance behaviour, growth, reproduction and mortality of earthworms were assessed. There was no evidence to suggest that the ecosystem habitat function of stockpile and reclamation soils is significantly limited compared to the Organisation of Economic Cooperation and Development's artificial control soil. Nevertheless, support functions were highest in unmined soils as determined by the earthworm avoidance behaviour test. Data generated in this study strongly supports that microbial species richness and diversity levels are restored over the years since reclamation, though community composition and structure still differ from the pre-disturbance community. Furthermore, microbial communities of reclamation soil environments are predominantly shaped by pH, phosphorus and nitrogen sources. Overall, bacterial communities are the most responsive and indicative of ecological changes during soil ecosystem restoration. In conclusion, as molecular methods are not without limitations, and because the soil ecosystem environment is governed by an interplay of factors, a comprehensive soil monitoring programme for post-mining reclamation soils in South Africa must comprise a combination of physicochemical properties and microbial community diversity indices as part of a minimum dataset. Furthermore,

a responsible stockpiling procedure which entails proper excavation and storage of topsoil, as well as the inclusion of microbial inoculants during post-mining reclamation operations, is strongly recommended. Such an approach will help improve coal-mining disturbed soil quality as well as facilitate a quicker ecosystem recovery period.

Keywords: Coal mining, ecosystem restoration, microbial diversity, soil health, ecological indicators, post-mining reclamation, arbuscular mycorrhizal fungi, bacteria.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Measuring Units

cm: centimetre

cmol kg^{-1}: centimoles per kilogram

°C: degree Celsius

E: East

g: gram(s)

g cm^{-3}: gram per centimetre cube

g kg^{-1}: gram per kilogram

h: hour(s)

Kg: kilogram

L: litre

m: metre

mg: milligram

µg: microgram

µg ml^{-1}: microgram per millilitre

µl: microliter

µM: micromole

meq 100 g^{-1}: milliequivalents per 100 grams

mM: millimolar

min: minute(s)

M: molar

ng: nanogram

nm: nanometre

N: normality/Normal

%: percentage

pmol: picomole

S: South

S (as in 16S rRNA): Sverberg unit

v/v: volume to volume

General Abbreviations

(Pty) Ltd: Property limited

AM: Arbuscular mycorrhizal

AMF: arbuscular mycorrhiza fungal

ANOVA: analyses of variance

AR: Avoidance response

ASV: Amplicon sequence variant

BD: Bulk density

C: carbon

CCA: Canonical correspondence analysis

CEC: Cation exchange capacity

CLPP: Community-level physiological profiling

D: Dominance

DNA: Deoxyribonucleic acid

E: Evenness

EC: Electrical conductivity

FDR: false discovery rate

H': Shannon-Wiener index of diversity

HCl: hydrochloric acid

HSD: Honest significant difference

ISO: International Standard Organisation

ITS2: Internally transcribed spacer 2

J´: Evenness/Pieolu's evenness

KEGG: Kyoto Encyclopaedia of Genes and Genomes

KO: KEGG Orthology

KOH: Potassium hydroxide

LEfSe: Least discriminant analysis effect size

LSD: Least significant difference

LDA: Least discriminant analyses

NGS: next-generation sequencing

NH_4: ammonium

NRF: National Research Foundation

OC: Organic carbon

OECD: Organisation of Economic Cooperation and Development

OM: Organic matter

OTU: Operational taxonomic unit

P: Phosphorus

PCoA: Principal coordinate analyses

PCR: Polymerase chain reaction

PERMANOVA: Permutational multivariate analysis of variation

PERMDISP: Permutational multivariate analyses of dispersion

RDA: Redundancy analyses

RDP: Ribosomal database project

Ref.: Reference

Recl.: Reclamation

RGR: Relative growth rate

rRNA: Ribosomal ribonucleic acid

SR: Species richness/number of OTUs

SRA: Sequence read archives

SSU: small sub-unit

USA: United States of America

VIF: Variance inflation factor

VT: Virtual taxa

CHAPTER 1:

INTRODUCTION AND PROBLEM STATEMENT

1.1 Soil Ecosystem functions and the impact of anthropogenic activities

The soil ecosystem, comprising both non-living and living matter, supports numerous interactions that are vital to the sustainability of all living organisms (Ponge, 2015; Hatfield et al., 2017; Drobnik et al., 2018). These interactions are vital to the ecosystem services, such as food production, regulation of climate and disease epidemics as well as geochemical nutrient cycling and cultural services (Figure 1.1) (Barrios, 2007; Adhikari and Hartemink, 2016). These ecosystem functions of soil are intimately related and governed by the soil physical, chemical and biological properties (Adhikari and Hartemink, 2016; Hatfield et al., 2017).

The increasing global population, as well as natural and anthropogenic disturbances, are factors that place pressure on land resources. Such factors affect the stability and sustainability of the soil ecosystem together with its services. Both natural and anthropogenic disturbances could drive soil ecosystem change, especially through their contribution to the loss of biodiversity and habitats as well as the alteration in soil nutrient cycles and climate change (NRC, 1995; Barrios, 2007; Eijsackers et al., 2017). Ultimately, these result in land degradation—the loss of soil ecosystem services and function.

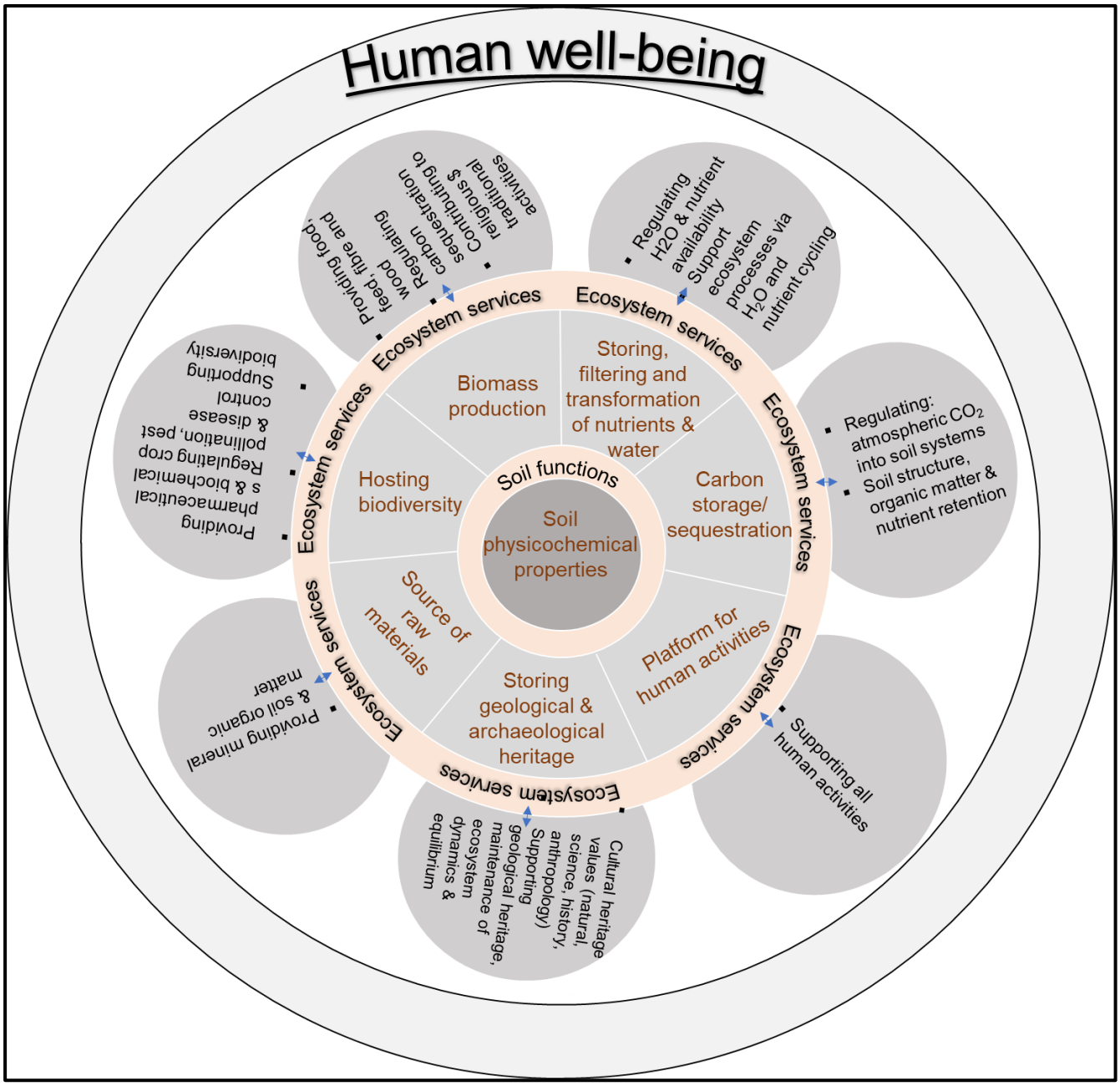

Figure 1.1: A conceptual diagram linking key soil properties to ecosystem services through soil functions for the well-being of humans (adapted from Adhikari and Hartemink, 2016).

1.2 Anthropogenic activities which impact soil ecosystems in South Africa

Anthropogenic activities which impact soil ecosystems in South Africa are diverse, cutting across the different soil ecosystem services described in section 1.1. Land use activities such as intensive farming and mining are key anthropogenic activities that drive changes in soil ecosystem

functions in South Africa (Eijsackers et al., 2017). According to Pelser and Kherehloa (2000), some of the causes of land degradation in Southern Africa include population pressure, poor farming practices and deforestation in search of new settlements and fuelwood. According to recent geospatial information, cultivated areas span 11.8% (156512 km^2) of South Africa's land mass, mining areas span 0.27% (3669 km^2), while built-up areas account for 2.90% (38887 km^2) (LRI, 2018). Although mining areas constitute a smaller fraction of South Africa's land mass, mining activities have significant impacts on the environment, including the destruction of arable land, alteration of landscapes, loss of ecosystem services and environmental pollution (Ochieng et al., 2010; Paterson et al., 2015; Carvalho, 2017).

1.3 Sustainable coal mining practices: towards the restoration of ecosystem services

Coal is one of the major mineral resources mined in South Africa (MCSA, 2019). Coal is an important domestic and export commodity and the leading contributor to South Africa's GDP (Stats SA, 2015). At present, coal accounts for over 70% of South Africa's electricity generation (MSCA, 2019). South Africa's coal deposits are predominantly located in the Highveld of the Mpumalanga province (Figure 1.2) (MCSA, 2019). Coal-mining areas are projected to span approximately 400 km^2 (EO-Miners, 2017). Most of the coal deposits are surface-mined (open-cast mining).

Open-cast mining of coal requires that the soil overburden be excavated, resulting in the alteration of soil profile and structure. The fact that most of South Africa's coal deposit lies underneath arable land generates an interesting land-use competition for coal mining and agriculture (Paterson et al., 2015). Due to the semi-arid nature of South African climate, the limited arable land and the need for food security, it is important that mining and agriculture co-exist. Such co-existence can be promoted by sustainable mining practices which include adequate pre-mining topsoil stripping and preservation in stockpiles, as well as adequate post-coal mining reclamation.

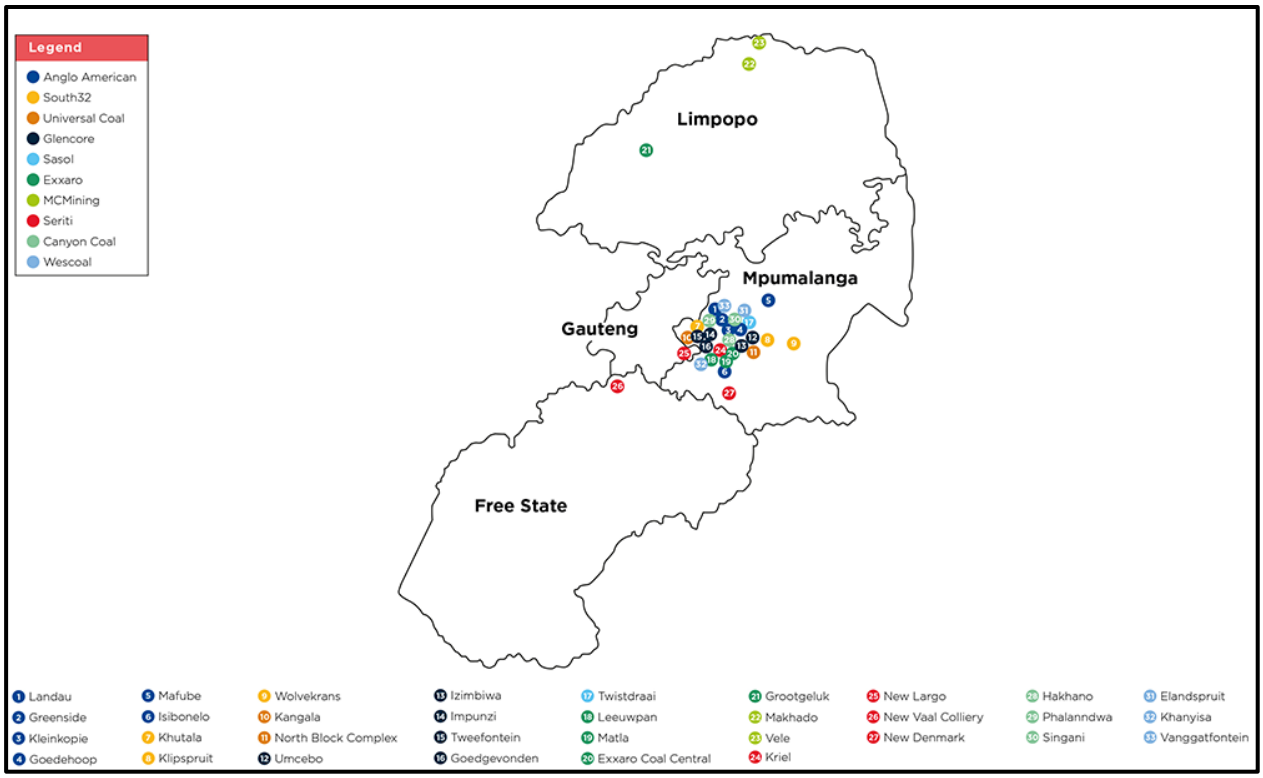

Figure 1.2: Map of coal mining areas and companies in South Africa (Source: MCSA, 2019)

Hence, there is a need for more emphasis on proper coal-mining practice aimed at a meaningful post-mining soil reclamation for arability and ecosystem stability (Wick et al., 2011; Cardoso et al., 2013). To ensure post-mining reclamation success, strict adherence to current guidelines for open cast mining is vital. These guidelines stipulate that the topsoil (the layer of the soil rich in organic matter, nutrients and microorganisms necessary to sustain crops) be salvaged and stockpiled separately from other soil layers comprising the coal overburden (Barry III, 1980). The salvaging and subsequent reapplication of topsoil during post-mining reclamation may significantly restore the pre-disturbance condition of the soil (Strohmayer, 1999). Similarly, guidelines for local post-coal mining rehabilitation have been provided in order to ensure high-end post-mining land use capability (Tanner and Möhr-Swart, 2007).

Currently, post-mining efforts are aimed at land use capability classes, namely: arable lands, pasture lands, wilderness lands and wetlands (Tanner and Möhr-Swart, 2007). In South Africa, it is unclear whether post-mining efforts are aimed at land restoration, rehabilitation or reclamation.

According to Lima et al. (2016), these concepts are closely related, often used interchangeably but should be different in terms of end goals, approach, and time scale amongst other characteristics. Restoration aims to return the pre-mining ecosystem conditions, usually an unrealistic approach. Whereas, reclamation is a more practical attempt to restore pre-existing ecosystem services or capabilities (Lima et al., 2016). On the other hand, “rehabilitation” is a managerial term, which aims at targeting a specific end-use (Tanner and Möhr-Swart, 2007; Lima et al., 2016). Because it is important to restore pre-disturbance ecosystem services in mining areas, reclamation should be a preferred approach. Therefore, the term “reclamation” is adopted in this thesis.

1.4 The essence of a comprehensive soil quality monitoring for coal mining-disturbed areas

Soil quality monitoring is important for ascertaining post-mining land use capability, ecosystem restoration as well as the appropriateness of current pre- and post-mining soil management practices. With respect to the capabilities of post-mining areas, soil quality monitoring seeks to assess the ability of the soil to support specific functions such as crop and animal production. To evaluate post-mining ecosystem restoration, soil quality monitoring indices that involve a comparison between mining-impacted areas and unmined areas (“reference”) are utilised (Lima et al., 2016). Information obtained from both land use capability and ecosystem restoration assessments provide empirical evidence which elucidates compliance with reclamation guidelines and the appropriateness of current reclamation practices. Soil monitoring outcomes may also support or inform policy decisions such as whether a mining closure certificate is to be issued.

However, defining what constitutes good quality soil is not very straightforward, given the heterogeneity and complexity of soil environments. The concept of soil health and quality refers to a combination of biological, chemical and physical properties essential for prolonged agricultural sustainability and productivity while maintaining minimal environmental disturbance

(Arias et al., 2005; Dose et al., 2015). Doran and Parkin (1994) defined soil health as “the capacity of soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health”. According to Stenberg (1999), it is imperative to define soil quality in relation to fitness for a given land use potential. Thus, the success of a post-mining soil/land rehabilitation or reclamation may be measured by the restoration of the pre-disturbance soil use capability and health, as well as ecosystem sustainability (Arias et al., 2005; Bashan and De-Bashan, 2010; Wick et al., 2011). However, current aboveground soil quality indicators such as soil depth, compaction and fertility as well as vegetation characteristics are insufficient as they fail to account for the sustainability of below-ground biological entities. The soil is a habitat to a diversity of life forms, which occupy ecological niches that are fundamental to the soil ecosystem processes and functions. Thus, soil health and/or soil quality descriptions for mining-impacted areas must also take into cognisance the living components of the soil. Such inclusion of the soil biota in soil health descriptions provides a comprehensive understanding of the state of the soil (Arias et al., 2005; Cardoso et al., 2013).

1.5 Roles of soil biota in maintaining soil ecosystem health

Soil biota contribute to ecological processes that are directly or indirectly critical to the sustainability and plasticity of the soil ecosystem processes during disturbance (Allison and Martiny, 2008; Maron et al., 2018).

Some of the ecological roles of soil biota include:

I. Biogeochemical cycling of nutrients. Through their secretion of biomolecules (e.g. enzymes), soil biota can contribute to the mineralisation of soil nutrients including carbon, nitrogen and phosphorus (Hayatsu et al., 2008; Adeleke et al., 2017).

II. Promotion of plant growth through the synthesis of biologically-active compounds such as phytohormones (Egamberdieva et al., 2017). Soil microorganisms also assist plants in scavenging for nutrients by acting as root extensions. For example, fungal hyphae of

arbuscular mycorrhizal (AM) fungi extending from colonised roots of most vascular plants (Smith and Read, 2010).

III. Improvement of soil structure through organic matter decomposition and soil aggregation. For example, the production of glomalin by mycorrhizal fungi help in soil aggregation which in turn aids soil moisture content retention (Rillig et al., 2002; Rillig et al., 2010).

IV. Generation and distribution of energy in the soil food web by acting as both primary producers and decomposers (Segovia et al., 2015; Steffan et al., 2015).

V. Driving ecosystem development by serving as pioneer organisms during ecological succession (Fitzsimons and Miller, 2010; Kikvidze et al., 2010; de Leon et al., 2016).

VI. Maintenance of soil ecosystem balance by suppressing and eliminating pathogens (Borneman and Becker, 2007; Garbeva et al., 2011).

VII. Synergistic contribution to the ecological roles of other soil fauna, including earthworms. For example, microbes in the gut of earthworms assist with the transformation of soil chemistry as the soil passes through the earthworm gut. Consequently, the soil ecosystem is transformed and enriched by the deposition of worm castings (Aira et al., 2006; Thakuira et al., 2010).

VIII. Remediation of polluted soils through biotransformation of pollutants (Suteu et al., 2013).

These and other potential contributions of soil microbial species make them essential to the soil ecosystem and other closely-related ecosystems such as aquatic ecosystems.

1.6 Soil biota as indicators of soil health: species diversity, succession and functional capabilities

Anthropogenic activities disrupt soil ecosystem balance by distorting the abundance, equitability, genetic and functional diversities of soil biota (Dose et al., 2015; Morgado et al., 2018).

Consequently, the vital ecological functions of soil biological communities are hampered. Determining the changes in genetic and functional diversities as well as species richness in the face of anthropogenic disturbances may thus serve as a measure of such impact on the soil ecosystem (Cardoso et al., 2013). The evidence of the suitability of microbes as bioindicators is supported by the direct relationship between the functional diversity of soil microbial communities and soil ecosystem resilience, as well as the exhibition of niche differentiation by microbial species in soil (Allison and Martiny, 2008; Lennon et al., 2012; Ferris and Tuomisto, 2015; Maron et al., 2018). Biological indicators such as soil microbes may reflect changes in nutrient cycling and availability as well as provide an early indication of the effectiveness of reclamation strategies (Dose et al., 2015). According to Cardoso et al. (2013), soil biota is very dynamic and more responsive to soil management and ecosystem disturbances in comparison to physicochemical properties. In addition, plant-microbial symbionts/interactions e interactions may influence plant community succession during ecosystem development (Dickie et al., 2013; de Leon et al., 2016). Such interactions and dynamism could be defined by comparing the abundance, genetic and functional diversities of microbial communities between pre- and post-disturbance states as well as comparing communities along a temporal (chronological) scale (Dickie et al., 2013). Microbial indicators are desirable because of their key ecological roles, rapid responsiveness to alterations in the soil ecosystem as well as their ability to reflect the totality of environmental variables that influence the regulation and mineralisation of soil minerals (Stenberg, 1999). Based on this premise, several studies have utilised successional changes in microbial species' diversity, abundance and functional differences for assessing the impact of anthropogenic disturbances on soil ecosystem health (Dose et al., 2015; Markowicz et al., 2015).

Generally, some notable soil biological groups that have been utilised for soil monitoring include bacteria, fungi, nematodes and annelids (Pulleman et al., 2012). On a functional scale, soil respiration, biomass, nutrient mineralisation and biologically-active secretions of soil biological communities such as enzymes can also provide indications of soil health (Stenberg, 1999; Pulleman et al., 2012). Some of the soil biota groups are discussed briefly:

1.6.1 Bacteria

Bacteria are the most abundant microbial groups within the soil. The activity of soil bacteria is important for organic matter decomposition and cycling of nutrients. Soil bacteria consist of functional groups including decomposers, mutualists, pathogens and lithotrophs (Ingham, 2000). The decomposers transform simple-carbon compounds from root exudates and plant litter into forms that can be assimilated by other (higher trophic level) soil organisms, whereas, the mutualist form a synergistic partnership with plants and contributes to soil ecological processes (Hoorman, 2016). An example of such mutualistic bacteria includes *Rhizobium* which lives in root nodules and contributes to the fixation of atmospheric nitrogen. Other free-living bacteria such as *Azotobacter*, *Azospirillum* and *Clostridium* also contribute to atmospheric nitrogen fixation (Hoorman, 2016; Raimi et al., 2017). The pathogens are responsible for the diseases of plants, while the lithotrophs or chemoautotrophs utilise energy obtained from non-carbon compounds, thereby contributing to the cycling of nutrients and degradation of pollutants (Ingham, 2000). Some of the lithotrophic bacteria include nitrifying bacteria such as *Nitrosomonas* and *Nitrobacter*, as well as sulphur oxidisers such as *Thiobacillus* (Ingham, 2000). Other bacteria, such as *Streptomyces*, contribute to disease suppression in the soil by producing bioactive compounds (Ingham, 2000). Together, these functional groups of bacteria contribute to ecological balance and processes required for soil ecosystem functioning and health.

1.6.2 Fungi

Soil fungi contribute significantly to soil biomass and organic matter accumulation in soil (Li et al., 2015). The ecological functions of soil fungi are similar to those of bacteria and include decomposition of organic materials and nutrient mobilisation (Ingham et al., 2000; Jenkins, 2005). Functional groups of soil fungi include saprophytes (or decomposers), mutualists and pathogens (Jenkins, 2005; Ingham, 2000). The decomposers break down cellulose and lignin present in plant litter to release organic acids and carbon dioxide (Ingham et al., 2000; Adeleke et al., 2017). Mutualist fungi form a partnership with plants. A common example of mutualistic fungi includes

arbuscular mycorrhizal fungi which form a symbiotic relationship with plants (Smith and Read, 2010). The pathogens are implicated in several plant diseases. Fungal hyphae bind the soil together, forming stable aggregates that help improve soil structure and soil water retention (Ingham, 2000). With the exception of pathogenic fungi, the abundance of soil fungal is linked to improvement in soil nutrient, organic matter and soil health (Stenberg, 1999).

1.6.3 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi are obligate symbionts of a large number of vascular plants (Smith and Read, 2010). The symbiosis between AM fungi and plant is mutualistic in that they assist plants with the assimilation of soil nutrients (especially phosphorus) in exchange for plant sugars (Smith and Read, 2010; Adeleke et al., 2019). Arbuscular mycorrhizal fungal symbioses are linked to plant growth, adaptation and tolerance (Barea et al., 2002), protection of plants from pathogens (Utkhede, 2006), as well as plant succession during terrestrial ecosystem development (de Leon et al., 2016). Furthermore, the production of glomalin by AM fungi contribute to soil aggregation, structure and water retention (Rillig et al., 2002). The abundance of arbuscular mycorrhizal fungal spores in the soil and extent of root colonisation with mycorrhizal hyphae are regarded as a potential indication of the soil's capacity to improve plant growth (Stenberg, 1999).

1.6.4 Earthworms

Earthworms are important soil fauna, particularly essential to several soil health processes including decomposition and stability of organic matter and soil structure (Aira et al., 2006; Thakuira et al., 2010). They are 'key species" in the soil food, occupying an important niche as soil ecosystem engineers (Thakuira et al., 2010; Pulleman et al., 2012). Their disappearance can have strong impacts on other levels of organisation of the ecosystem biological hierarchy (Pulleman et al., 2012). Hence, they are useful ecosystem bioindicators (Paoletti, 1999; Pulleman et al., 2012). Furthermore, the role of the earthworm in organic matter decomposition and nutrient cycling is dependent on earthworm-gut microbiota interactions (Aira et al., 2006; Thakuira et al.,

2010; Zhao et al., 2010). The earthworm gut is an anaerobic cavity which supports several ecological functional microbes, including nitrogen-fixing, methanogenic, nitrate-reducing and fermentative bacteria (Thakuira et al., 2010; Pass et al., 2014). These bacteria transform the chemistry of the soil as they pass through the gut (Aira et al., 2006; Pass et al., 2014), thereby transforming the soil ecosystem overall.

1.7 Recent advances in methods for studying soil microbial genetic diversity

Soil microbial diversity can be investigated using classical culture-based approaches and culture-independent approaches. The merits and demerits of the several methods under these two approaches, including plate counts, community-level physiological profiling (CLPP), phospholipid-derived fatty acid (PLFA) analyses, and molecular-based methods amongst others have been reviewed elsewhere (Kirk et al., 2004). Briefly, the accuracy of culture-based approaches is hampered by the relatively low numbers of microbial species that are currently cultivable (Kirk et al., 2004). In addition, chemotaxonomic markers such as PLFA and sole-carbon utilisation assays such as CLPP are restrictive (poor resolution) with respect to providing in-depth information on microbial community richness and functions (Kirk et al., 2004).

Although molecular-based methods are not without limitations such as PCR biases, copy number variations in marker genes and sensitivity, they can provide a robust, less laborious and rapid estimate of the genetic diversity in a given environment without the limitation associated with a culture-dependent step (Kirk et al., 2004; Zhao et al., 2011). As a note, the rapidness of such molecular methods for estimating microbial community richness of mining-impacted soils may be desirable for the coal mining industry as inference and decision can be made promptly. In recent times, advances in sequencing technologies have facilitated the detection of microbial diversity at a high throughput scale thereby providing a deeper insight into the microbial richness and diversity of a given environment (Caporaso et al., 2011; Caporaso et al., 2012; Tedersoo et al., 2014). Based on these developments, novel microbial species and their ubiquity have been discovered (Youssef et al., 2015). In addition, because sequence-based molecular methods

utilise phylogenetic markers which are universal in prokaryotes and eukaryotes, it is possible to make comparisons between species from different environments and to infer phylogenetic relationships over evolutionary time (Ludwig and Schleifer, 1994; Janda and Abbott, 2007).

1.8 Problem statement

Presently South Africa has no comprehensive soil health monitoring assessment for coal mining-impacted soils. Such soil health monitoring assessments will provide an insight into the appropriateness of current stockpiling and reclamation practices. In addition, the assessment will provide empirical evidence to support policies and ensure appropriate measures to improve current post-mining reclamation practices.

Furthermore, current aboveground indicators used in soil monitoring are not sufficiently robust. They do not account for the belowground biological components of the soil, which are particularly responsible for essential soil ecological processes including nutrient cycling and organic matter decomposition. In addition, these soil biological components contribute to suppression of plant pathogens, soil texture improvement and the overall increase in crop productivity (Arias et al., 2005; Pulleman et al., 2012; Cardoso et al., 2013). Soil biological entities are very dynamic and sensitive to soil management and ecosystem disturbances (Niemeyer et al., 2012; Stenberg, 1999). Their sensitivity to ecosystem changes makes them suitable indicators for assessing the effect of disturbances in the soil environment, monitoring soil contamination (Niemeyer et al., 2012; Maboeta et al., 2018), productivity (Stenberg, 1999), the effect of climatic variations (Pasternak et al., 2013) and different soil management practices (Figuerola et al., 2012; Cardoso et al., 2013; Dose et al., 2015). For post-mining reclamation soil environments, the diversity and dynamics of soil biological communities over a chronological time gradient may provide indications for the restoration of pre-disturbance soil biodiversity and health.

Previous studies by Claassens et al. (2006), Claassens et al. (2008) and Claassens et al. (2011) on the microbial community structure and function along a time gradient of post-coal mining

reclamations in South Africa are based on chemotaxonomic markers and physiological profiles, which are prone to low species-resolution and selectivity, respectively (Kirk et al., 2004). Very little is known about the bacterial and fungal species diversity and functional community structure in post-coal mining reclamation soils of South Africa. Compared to the previous microbial community diversity studies, recent advances in next-generation sequencing presently make it feasible to unravel microbial communities of environments at a much deeper depth and coverage (Caporaso et al., 2011; Caporaso et al., 2012; Tedersoo et al., 2014). In addition, next-generation sequencing provides an insight into the phylogenetic relatedness and potential (predicted) ecological functions of microbial communities at a high-throughput scale (Aßhauer et al., 2015; Nguyen et al., 2016). At present, there is a paucity of such high-throughput studies investigating the microbial community of post-coal mining reclamation soils (or reclaimed areas) in South Africa.

In addition to the foregoing gaps, the inclusion of ecotoxicological assessments for soils in post-coal mining reclamation areas may be necessary to elucidate the capability of post-mining reclamation soils to serve as a habitat (support function) for biocoenosis. At present, such ecotoxicity studies have been undertaken on gold and platinum mining soil environments (Maboeta et al., 2008; van Coller-Myburgh et al., 2015; Maboeta et al., 2018), with scarcely any study on post-coal-mining soil environment in South Africa till date.

Therefore, the aim of the study was to establish the relationship between potential ecosystem recovery and bioindicators during post-coal-mining reclamation of soil.

The specific objectives were to:

1. Assess the potential contribution of soil stockpile to post-coal mining reclamation soil health.

2. Determine the structural and functional differentiation of microbial communities in post-coal mining reclamation soils over a chronological gradient.

3. Investigate the potential utilisation of arbuscular mycorrhizal fungi as bioindicators of ecosystem recovery following reclamation.

4. Determine the potential dynamics in the habitat support function and ecotoxicity of coal-mining associated soils by utilising higher-class bioindicator species such as earthworm (*Eisenia andrei*).

Because stockpile soils are used during post-mining reclamation, the first objective was important in order to gain insight into the potential contribution of stockpile soil quality to post-mining reclamation soil health. Thus, the objective formed a part of a "source tracking" in order to provide a comprehensive insight into factors along the mining "process chain" that might contribute to the quality of post-mining reclamation areas/soil. Furthermore, this provides empirical evidence from which appropriate recommendations are put forward to the South African coal-mining industry. Objectives 2 and 3 were investigated on reclamation areas, while a higher-class bioindicator, earthworm, which is commonly used for monitoring the presence of pollutants and ecosystem support function was used to achieve objective 4.

1.9 Hypotheses

The hypotheses of the present study include:

(i) The microbial community structure and function in mining-impacted soils are impaired compared to unmined soils and are site-specific.

(ii) The microbial communities within a post-coal mining reclamation soil chronosequence are differentiated among reclamation soils of various ages and may differ from those of unmined reference soils.

(iii) The ability of coal-mining associated soils to support biocoenosis is limited compared to unmined soils and such support functions in reclamation areas may increase with age.

To test these hypotheses, coal mines which provided cooperation for the study were selected. The study sites were dominantly located in the coal-rich Mpumalanga Province of South Africa (Figure 1.2). Specific information on the sites used for this study is provided within the chapters.

1.10 Outline of the thesis

This thesis consists of six chapters. Chapter one provides the background for the study, the problem statements, aims, specific objectives and research hypotheses. Chapters 2, 3, 4 and 5 address objectives 1, 2, 3 and 4, respectively, and are structured in a research-based format as manuscripts for peer-review publication. Thus, some overlaps in information were unavoidable. An overview of these subsequent chapters is provided below.

Chapter 2 is titled “Relationship between microbial communities and physicochemical properties of stockpile soils: early predictors of post-mining reclamation soil health”. This chapter reports two parallel microbiological studies conducted on soil stockpiles from selected coal mining sites. This chapter provides some context for subsequent chapters on post-coal mining reclamation areas. Importantly, part of the work detailed in this chapter was performed in conjunction with another student and contributed to a master degree dissertation submitted to the North-West University (Mashigo, 2018). The work detailed in this chapter has been published as two separate peer-reviewed articles. The details of these publications are as follows:

I. **Ezeokoli, O.T**., Nwangburuka, C.C., Adeleke, R.A., Roopnarain, A., Paterson, D.G., Maboeta, M.S. and Bezuidenhout, C.C. (2019). Assessment of arbuscular mycorrhizal fungal spore density and viability in soil stockpiles of South African opencast coal mines. *South African Journal of Plant and Soil*, *36* (2): 91-99. doi: 10.1080/02571862.2018.1537011.

II. **Ezeokoli, O.T**., Mashigo, S.K., Paterson, D.G., Bezuidenhout C.C. and Adeleke, R.A. (2019). Microbial community structure and relationship with physicochemical properties

of soil stockpiles in selected South African open cast coal mines. *Soil Science and Plant Nutrition*, *65*(4), 332-341. doi: 10.1080/00380768.2019.1621667.

Chapter 3 reports findings of a multi-site study on post-coal mining reclamation areas conducted in the summer of 2016, as well as data from an intra-site study along a post-coal mining reclamation chronosequence sampled in 2017. Based on the findings from the multi-site study (in 2016) that most differences in the bacterial community are site-specific, the study was redesigned to minimise site variation but to focus on differences along a chronological scale—do microbial communities show a pattern indicative of recovery over years of reclamation? A combination of enzyme, CLPP, microbial community structure and predicted functional profile was utilised in making a comparison between reclamation areas and unmined sites. Overall, the chapter seeks to test the hypotheses that the microbial community structure and function in reclamation soil are differentiated from those of unmined soils (soil history effect) and that these differences are site-specific and reflect some patterns over different ages of reclamation.

A portion of the chapter has been accepted for publication with details as follows:

Ezeokoli, O.T., Bezuidenhout, C.C., Maboeta, M.S., Khasa, D.P. and Adeleke, R.A. (2020). Structural and functional differentiation of microbial communities in post-coal mining reclamation soils of South Africa: bioindicators of soil ecosystem restoration. *Scientific Reports*, 10: 1759. Doi: 10.1038/s41598-02058576-5.

Chapter 4 describes investigations utilising the obligate plant symbiotic fungi—arbuscular mycorrhizal (AM) fungi—as a potential indicator for soil ecosystem restoration. In this chapter, the arbuscular mycorrhizal fungal community differentiation in soil and roots of dominant vegetation collected along a post-mining reclamation soil is reported. The chapter has been published as follows:

Ezeokoli, O.T., Mashigo, S., Maboeta, M.S., Bezuidenhout, C.C., Khasa, D.P. and Adeleke, R.A. (2020). Arbuscular mycorrhizal fungal community differentiation along a post-coal mining

reclamation chronosequence in South Africa: A potential indicator of ecosystem recovery. *Applied Soil Ecology*, *147*: 103429. Doi: 10.1016/j.apsoil.2019.103429.

Chapter 5 describes investigations utilising earthworm bioassays to determine the ecosystem support functions of coal-mining impacted soils (stockpiles and reclamation soils). Such ecosystem support functions of these soils were determined based on avoidance tests, change in biomass, mortality and reproduction success. By taking into cognisance the ages of the reclamation areas, inference on the potential restoration of ecosystem support functions over the years since reclamation were drawn. The study was designed in a hierarchical order such that, if significant lethal to sub-lethal effects were observed in test subjects (*Eisenia andrei*) exposed to different soil types, further analyses of the gut microbiome of the worms were to be undertaken. Else, the investigation would be limited to tests of fitness and reproduction of earthworms. The later was true, and thus, the chapter only reports findings on the soil habitat function and fitness of *Eisenia andrei*.

Title: Utilising earthworm (*Eisenia andrei*) bioassays in assessing ecosystem support function of post-coal mining reclamation soils

Authors: **Ezeokoli, O.T**., Maboeta, M.S., Bezuidenhout, C.C., Adeleke, R.A.

Target Journal: Environmental Monitoring and Assessment

Chapter 6 provides conclusions and recommendations.

This chapter brings the findings of the study to a focus and discusses its contribution to new knowledge and implications for the coal mining industry. It also identifies gaps requiring further investigations and potential limitations of the present investigation. Furthermore, recommendations are provided towards applying knowledge obtained in the coal mining industry.

CHAPTER 2:

RELATIONSHIP BETWEEN MICROBIAL COMMUNITIES AND PHYSICOCHEMICAL PROPERTIES OF STOCKPILE SOILS: EARLY PREDICTORS OF POST-MINING RECLAMATION SOIL HEALTH

2.1 Introduction

Soil is a resource which is non-renewable on a human timescale. It plays roles that are paramount to human existence and the sustainability of other ecosystems (Faber et al., 2013). Often, this non-renewable resource is disturbed through several anthropogenic activities such as coal mining.

Most of South Africa's coalfields are in the grassland biome, which provides important ecosystem services such as provisioning services (e.g. arable use) and cultural services (e.g. recreational). Currently, coal-mining areas in South Africa are estimated to be approximately 40000 hectares (EO-Miners, 2017), while a further several thousand hectares of agricultural land are at risk of being lost to mining activities (Bench-Marks Foundation, 2014). Therefore, the rehabilitation of mined lands towards restoring pre-disturbance ecosystem services or achieving an acceptable post-mining land use capability is paramount.

For the attainment of a sustainable post-coal mining land use capability, a set of guidelines for the stripping, stockpiling and preservation of topsoil has been recommended by the Surface mining control and reclamation act of 1977 (Barry III, 1980; Wick et al., 2009). Such preservation of the topsoil is paramount because the topsoil is the most important soil horizon from an agricultural perspective since it is rich in organic matter and vital plant nutrients. It also contributes to moisture and nutrient retention (Strohmayer, 1999; Kaiser et al., 2002). However, studies have shown that the quality of topsoil is adversely affected over the long periods (in some case over several decades) in which soils are stockpiled (Johnson et al., 1991; Strohmayer, 1999). Logically, the quality of stockpiled soil is linked to the success of post-mining land reclamation because the

stockpiled soil is reapplied during reclamation and prior to revegetation (Sheoran et al., 2010). Hence, an assessment of the quality of topsoil stockpiles in South African coal mines can serve as potential early predictors of soil health during post-mining reclamation. Also, information obtained from such assessments is essential for both monitoring and recommendation. However, there is currently no comprehensive soil health assessment practice for soil stockpiles in the South African coal mining industry. A comprehensive soil health assessment for soil stockpiles is such that embodies the soil health concept and comprises the three components of soil—physical, chemical and biological components (Arias et al., 2005; Cardoso et al., 2013; Dose et al., 2015).

In recent times, the adequacy and sensitivity of soil biological/microbial parameters (bioindicators) in reflecting changes and/or state of the soil environment have been recognised (Stenberg, 1999; Niemeyer et al., 2012; Cardoso et al., 2013; Adeleke et al., 2017). The soil contains a vast microbial consortium, including bacteria and fungi, which contribute significantly to ecological processes in the soil ecosystem. Such ecological contributions include geochemical cycling of nutrients, maintaining soil nutrient status and fertility by contributing to mineralisation of nutrients essential to plant growth (Adeleke et al., 2012; Steffan et al., 2015; Adeleke et al., 2017). Unfortunately, microbial communities and their ecological functions are altered by soil management and anthropogenic practices (Dose et al., 2005) such as farming and mining (Alguacil et al., 2008; Straker et al., 2008; Xiang et al., 2014; Nkuekam et al., 2018). Similarly, soil properties and topography (Straker et al., 2007; Straker et al., 2008; Xu et al., 2017) are amongst factors which influence the microbial community of the soil ecosystem.

However, a knowledge gap still exists, especially as this relates to the microbial community diversity in soil stockpiles of South African coal mines. Furthermore, it is unclear whether the density and viability of arbuscular mycorrhizal fungal (AMF) spores are impaired in these soil stockpiles. Arbuscular mycorrhizal (AM) fungi are strict plant-symbiont of most vascular plants which are important for the uptake and modulation of essential mineral nutrients by the host plant; plant-pathogen resistance; soil-water retention and improved soil structure (Rillig et al., 2010;

Smith and Read, 2010; Adeleke at el., 2019). Therefore, the viability and colonisation of plant host by arbuscular mycorrhizal fungal spores in soil stockpiles could serve as an indirect measure for assessing topsoil quality as well as the capability of the stockpile soils to support plant growth in post-mining soil reclamation process. Such an approach could also be a valuable tool to evaluate the adequacy of current stockpiling practices.

This chapter summarises microbiological studies on soil stockpiles (Mashigo, 2018; Ezeokoli et al, 2019a, Ezeokoli et al, 2019b) which were aimed at determining the biological health of soil stockpiles based on the hypotheses that: (1) AMF spore density and viability differ between undisturbed soils and soil stockpiles of open-cast coal mines in South Africa, (2) enzyme activities and microbial diversity of topsoil stockpiles (disturbed) are impaired compared to adjacent unmined (undisturbed) soils and (3) microbial communities and enzyme activities in soil stockpiles vary across seasons. To test these hypotheses, three south African coal mines were selected for both microbial community structure and diversity studies, as well as establishing AMF spore diversity and viability. This study is important in order to provide insights into the link between stockpiling activities (pre-mining) and post-mining reclamation soil health.

2.2 Materials and methods

2.2.1 Study sites

The study sites were three opencast coal mines. The designation A, B and C are used for these three mines because of a confidentiality agreement with the mining companies. The coal deposits in these mines are bituminous thermal grade coal. Coal mining activities have been ongoing on each of these sites for at least 15 years. The distance between mine B and mine C was approximately 48 Km, while mine A was approximately 160 km away from the centre of mine B and mine C. The mines are situated at an elevation of approximately 1600 m above sea level within the coal-rich Highveld of Mpumalanga Province (24°0′–27°30′ S, 28°15′–32°5′ E), South Africa. The area experiences an annual average rainfall of 640 mm with rainfall occurring mostly in the summer (October-March) and rarely in the winter (May-July) (ARC, 2016; World Weather

Online). The natural vegetation across all sites was predominantly grass species (*Digitaria eriantha*, *Cynodon dactylon* and *Eragrostis curvula*) with an estimated basal cover of 10% – 35% across soil stockpiles. The soil stockpiles at the time of sampling were sparsely covered with natural vegetation (~10% grass and ~2% forbs cover) and have been stored for at least 5 years. While the Highveld of Mpumalanga is the biggest coal-producing area of South Africa, agriculture remains the backbone of the province (GSA, 2016), hence providing a conflicting land-use competition between coal mining and agriculture (Paterson et al., 2015). Two separate sites served as “references” for the two studies—microbial community diversity and AMF spore abundance and viability. An unmined maize field adjacent (approx. 2 km) to the coal mine A and an un-utilised unmined land adjacent to mine A (approx. 200 – 500 m distant) served as “reference” sites for the AMF and microbial community diversity studies, respectively. The plant cover of the un-utilised (no anthropogenic activity) unmined reference site was approximately 60% and predominantly grass species including *Digitaria eriantha* and *Cynodon dactylon*. The choice of the unmined maize field selected for the AMF study was due to the predominant pre-mining land use activity in the study area—maize cultivation. An agricultural field could also provide a suitable reference for ascertaining soil quality from an arability perspective.

2.2.2 Soil sampling

Soil samples were collected aseptically from randomly selected stockpiles at depths of 0-20 cm (hereafter referred to as “topsoil”) and > 20 cm (hereafter referred to as “subsoil”) using a sterile auger. The storage duration of the soil stockpiles prior to sampling was estimated at less than a year in mine A, ten to eighteen years in mine B, and seven to eight years in mine C. The age of stockpiles was not considered in the experimental design because there was also the absence of accurate data on the ages of stockpiles sampled. Also, observations made on-site indicated that soil stockpiles comprised heaps from different times and soil horizons

For the study on AMF spore density and viability, soil samples were collected from all three mines and reference site in the summer of 2013/2014 season. Whereas for microbial community

analyses, soil sampling was conducted during summer (February), winter (July), and spring (September) of 2015. For the AMF study, at least three random soil core samples (1.5 kg wet wt.) were collected per site, whereas for the microbial community study, at least five random samples were collected from each study site at the respective depths. Samples were collected in sterile bags and immediately placed on ice. Samples were appropriately stored: frozen at -70°C for microbial community analyses and -20°C for enzyme analyses. Enzyme analyses were performed within a week of sample collection.

2.2.3 Determination of physical and chemical properties of soil

Physico-chemical properties of soils, including texture, bulk density, pH (H2O), cation exchange capacity (CEC), total nitrogen (N), organic carbon (C), extractable cations (calcium, magnesium, sodium, and potassium) and available phosphorus (Bray 1) were analysed using standard methods of the Non-Affiliated Soil Analysis Work Committee (1990). Soils were ground and passed through a 2 mm sieve before analyses. Briefly, pH was determined from a 1:2.5 soil-water suspension using a pre-calibrated pH meter (pH 700, Eutech Instruments Pte Ltd, Singapore). The particle size distribution was determined by the Bouyoucos method. Cations and exchangeable cations were determined from soil ammonium acetate (1 M, pH 7.0) extracts by using Inductively coupled plasma - optical emission spectrometry (ICP-OES). Bulk density was determined by using a bulk density sampler core of known volume after overnight drying at 105°C.

2.2.4 Enumeration of AMF spore density

Spores were recovered from 100 g soils' subsamples by following the wet sieving and decanting method of Gerdemann and Nicolson (1963). AMF spores were recovered using nested sieves (500-45µm mesh pore sizes) and subsequent centrifugation in 60% sucrose gradient at 3000 rpm for 5 min. Recovered spores were washed onto 9 cm graded filter paper and enumerated under a dissecting microscope at 50X magnification.

2.2.5 Trap culturing and root colonisation assessment

2.2.5.1 Trap culturing

In practice, the viability of AMF spores in soils can be assessed through trap culturing using a host plant. A single plant species, *Zea mays* (maize), was used for assessing the viability of spores. The choice of the single plant was based on the common crop cultivated in the study area and for comparison to the unmined maize field reference. Cores of bulk soil samples collected from each study site was composited per sampling depth to obtain a total of eight (four sites by two sampling depths) bulk samples from all study sites. Precisely 500 g of soil composites were mixed with 500 g of washed sterilised river sand in 600 cm3 pots. Five replicate pots per bulk soil sample were established in a greenhouse. A randomised block design involving a two (sampling depths) X four (sites) X 5 (replications) factorial was used. Seeds of a non-transgenic maize variety ARC WE3127 were obtained, physically inspected for good quality, and treated with the fungicide apron XL (active ingredient: Metalaxyl-M and S-isomer, 33.3%) (Syngenta, South Africa) prior to planting in pots at a rate of three seeds per pot. Following germination (at two weeks), maize plants were thinned to two plants per pot. Plants were irrigated twice a day, while nutrient levels in the soils were augmented as required by applying 50 ml of a modified Hoagland solution, containing 8 mg/L phosphorus (Habte and Osorio, 2001) weekly. The greenhouse was maintained at 27°C day and 20°C night temperatures under natural lighting regimes for a total of 13 weeks during which plant shoot height, number of leaves and stem width were collected at intervals until the twelfth week. At the end of 13 weeks, plants were gently uprooted, and roots washed under running tap water. Roots for mycorrhization assessment were air-dried, while roots for DNA extraction were freeze-dried in liquid nitrogen and stored at -70°C prior to further processing.

2.2.5.2 Root staining

Roots staining was performed as described by Thorne et al. (2013). Thin portions (60 root fragments per pot) of roots were cleared in 10% KOH solution to remove cytoplasmic and cell

inclusions. Cleared roots were autoclaved at 130°C for 10 min and acidified in 2% HCl for 20 min at room temperature. Roots were then stained with trypan blue and autoclaved at 130°C for 7 minutes. Stained roots were mounted on a glass slide and assessed for colonisation by endomycorrhizae under a compound microscope at 200X magnification.

2.2.5.3 DNA-based Detection of endomycorrhizae

Precisely 0.5 g portions of roots from each experimental pot were aseptically crushed and processed for extraction of DNA using the ZR soil microbe kit (Zymo Research, Irvine, CA, USA) according to the instructions of the manufacturer. For PCR-based detection of endomycorrhizae, primers SSUmAf and LSUmAr (Krüger et al., 2009) were used. The PCR reaction mixture included 10 µl of 2x Phusion Flash master mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.5 µM of each primer, 50 ng DNA template and sterile PCR-grade water to a final volume of 20 µl. The PCR conditions were exactly as in the Krüger et al. (2009) paper. All PCRs were performed in a T100^{TM} thermal cycler (Bio-Rad Laboratories, CA, USA) in duplicates. PCR amplicons were visualised on an agarose gel to verify amplicon size (approximately 1750 bp) and to infer the presence or absence of endomycorrhizae.

2.2.6 Enzyme and microbial community analyses

Determination of beta-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) and urease (urea amidohydrolase, EC 3.5.1.5) activities were performed by the methods of Dick et al. (1996) and Kandeler and Gerber (1988). The analyses were performed on topsoil samples only because previous studies have indicated that soil enzymes activities are mostly concentrated in the topsoil (0-15 cm) region (Das and Varma, 2010). Soil samples were passed through a 2-mm sieve and oven-dried at 40°C prior to beta-glucosidase and urease activity assays of beta-glucosidase and urease activities.

For microbial community analyses, PCR-denaturing gradient gel-electrophoresis (PCR-DGGE) was used as previously described by Mashigo (2018). Briefly, DNA was extracted by using the ZR Soil Microbe DNA extraction kit (Zymo Research, Irvine, CA, USA) according to the manufacturer instruction. Thereafter, the partial bacterial 16S rRNA gene and fungal internally transcribed spacer 2 (ITS2) regions were amplified using the universal oligonucleotide primer set 341F (5′-CCTACGGAGGCAGCAG-3′)/907R (5′ CCGTCAATTCCTTTGAGTTT-3′) and ITS3 (5′-GCATCGATGAAGAACGCAGC-3′)/ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990), respectively. Each forward primer (341F and ITS3) contained a 40 bp GC-clamp at the 5' (Muyzer et al., 1993). The components and running conditions of the PCR amplifications were exactly as described previously (Mashigo, 2018). Subsequently, DGGE was performed as described previously by Roopnarain et al. (2017). Following DGGE runs, gel images were stained in 0.1% (v/v) ethidium bromide solution, visualised and analysed as described previously by Mashiane et al. (2017) while dominant bands were excised and reamplified as described previously by Ezeokoli et al. (2016a).

2.2.7 Sequencing, taxonomic and phylogenetic classification of AM fungi in roots

PCR amplicons were purified using the PureLink quick PCR purification kit (Invitrogen, Lohne, Germany) and cloned into competent *Escherichia coli* JM109 cells using the CloneJet PCR cloning kit (Thermo Fisher Scientific, MA, USA). Because the AMF colonisation observation from the root stains indicated no apparent colonisation, we suspected that the AMF diversity in PCR-positive samples is sparse. Hence, at least 15 transformants were selected per positive sample and PCR-screened for inserts. The PCR screening was performed following the instructions of the cloning kit manufacturers using primers complementary to the ends of the circular plasmid flanking the insert regions (Thermo Fisher Scientific, MA, USA). Amplicons with the right insert size following agarose gel electrophoresis were purified and sequenced (Sanger) in both forward and reverse directions.

Subsequently, continuous sequences (contigs) obtained from forward and reverse sequences (after quality inspection of electropherograms) were screened for vector sequences and chimeras by using NCBI's VecScreen (https://www.ncbi.nlm.nih.gov/tools/vecscreen/) and DECIPHER software v. 2.0 (Wright et al., 2012), respectively. For phylogenetic reconstruction, sequences along with closely related sequences available in the NCBI and MaarjAM database (http://maarjam.botany.ut.ee/) (Öpik et al., 2010), which spanned the same region as sequences obtained in this study, were selected. Publicly available sequences obtained from defined AMF cultures were selected. A consensus sequence of *Paraglomus occultum* IA072 reported in the study by Krüger et al. (2012) as "consensus 39" was also selected. All selected sequences were aligned by using MUSCLE (Edgar, 2004). Alignments were then manually edited in DAMBE software (Xia 2013) to exclude ITS1 and ITS2 regions due to alignment ambiguities as reported previously (Fahey et al., 2012; Krüger et al., 2012), yielding a 1303 bp alignment of 56 sequences which spanned the partial 3´-fragment of the SSU (18S rRNA), the entire 5.8S and the partial 5´-fragment of the LSU (28S rRNA). The edited 18S-5.8S-28S rRNA sequence alignments were used to construct a maximum likelihood phylogenetic tree in MEGA7 (Kumar et al., 2016), by using the general time-reversible nucleotide substitution model (Nei and Kumar, 2000) and gamma-distributed with invariant sites rates among sites. Branch support was assessed using 1000 bootstrap replications (Felsenstein, 1985).

For the taxonomic assignment of 18S-5.8S-28S rRNA sequences, a *de novo* clustering of sequences into operational taxonomic units (OTUs) at 97% sequence similarity was performed with the mothur software (Schloss et al., 2009) as previously described by Ezeokoli et al. (2016b). OTU representative sequences were obtained by using the mothur software sub-routine command "get.oturep". Thereafter, the obtained OTU representatives were taxonomically identified by aligning against close relatives in the GenBank sequences through the MaarjAM Glomeromycotan database (Öpik et al., 2010).

Nucleotide sequences obtained for the studies have been deposited in the National Centre for Biotechnological Information (NCBI) GenBank under the accession number ranges KY242678 to KY242704 for AMF 18S-5.8S-28S rRNA; KY985473 to KY985518 for bacterial 16S rRNA gene; and MF001318 to MF001351 for fungal ITS2 partial sequences, respectively.

2.2.8 Statistical analyses

Data (plant height, stem width, number of leaves, fresh weight and spore density) were subjected to a two-way (depth x site) analysis of variance (ANOVA) in R software (R Core Team 2013). Prior to the 2-way ANOVA, appropriate transformations were performed on data to meet normality and/or homogeneity of variance criteria. For data (number of leaves and stem width) which could not be normalised, the aligned rank transform (ART) for nonparametric factorial ANOVAs was used (Wobbrock et al., 2011). Tests for statistical significance were set at $P \leq 0.05$. The Tukey HSD post hoc test was used at 0.05 level to separate means where differences were significant in the parametric ANOVA. To test the relationship between spore density and selected l physicochemical properties of soil, the Pearson rank correlation was performed on normalised data. Test for significance of all Pearson correlation coefficients (r) was set at $P \leq 0.05$. The correlation was performed in IBM SPSS Statistics software (v. 21, IBM 264 Corporation, New York, USA).

Soil physicochemical data for each soil horizon were analysed separately by the aligned rank transform non-parametric analysis of variance (ANOVA) by using the ARTool package in R software version 3.4.0 (R Core Team 2013). Beta-glucosidase and urease activities data were subjected to a two-way (site x season) ANOVA by using R software. Prior to ANOVA, enzyme activity data were square root-transformed to near-normality and to meet homoscedastic assumption for parametric tests. After ANOVA, the Tukey honest significant difference (HSD) post hoc test was used to separate significant means at $P < 0.05$. Pearson correlation was used to test the relationship between enzyme activities (normalised by square root transformation) and soil

physicochemical properties by using the R software. Based on the outcome (i.e. significant differences, $P < 0.05$) of ANOVA, correlations were conducted across sites, as well as on a site-by-site basis. Furthermore, to test the relationship of the microbial communities with the physicochemical properties at each sampling depth, a redundancy analysis (RDA) was performed in the vegan package (Oksanen et al., 2015) of R software by using the unweighted similarity data matrix generated from the PCR-DGGE banding pattern and soil physicochemical properties. Thereafter, the environmental factors (vectors) were then fitted into the RDA model and their significance tested by permutations using the "envfit" function of the vegan package.

2.3 Results

2.3.1 Physico-chemical properties of soil

The physicochemical properties of the soil subsample for the AMF study are presented in Table 2.1. Of all parameters analysed, only sodium, potassium and particle size distribution (silt, sand and clay) were significantly ($P < 0.05$) influenced by site × depth interaction (Table 2.1 and Table 2.S1). Individual effect "site" significantly ($P < 0.05$) influenced most soil properties compared to "depth" (Table 2.S1). However, organic carbon was influenced by "sampling depth" effect and not by "site" effect (Table 2.S1). Summarily, soil texture was generally sandy-clay-loam, average pH acidic (pH 4.62-5.80), organic carbon between 0.79% and 1.53% in the topsoil, and between 0.39% and 0.78% in the subsoil. Bulk density ranged from 1.35 to 1.90 g/cm^3 with soils from the unmined reference site having the lowest bulk density highest organic carbon content, the highest sum of exchangeable cations (S-V), cation exchange capacity and highest K composition.

Similar to the soil sub-samples for the AMF spore abundance and viability study, the pH of soils collected during the summer, winter and spring seasons of 2015 were acidic (Mashigo, 2018). Furthermore, although bulk density was generally higher in stockpile soils compared to unmined reference soils, differences in selected physicochemical properties did not suggest that stockpile soils were of less quality (micronutrient levels, pH, cation-exchange capacity among others) compared to the unmined reference (Mashigo, 2018).

2.3.2 Maize performance in stockpile soils under greenhouse conditions

The interactions between factors were not significant ($P > 0.05$) for plant shoot heights at all the time intervals. However, plant shoot height (Figure 2.1) was significantly influenced by site (treatment) at week 6 ($P = 0.00188$), week 8 ($P = 0.0456$) and week 10 ($P = 0.0434$). Sampling depth did not have significant effects ($P > 0.05$) on plant shoot height. At week 6, 8 and 10, mean shoot heights of plants in mine C differed significantly from those of the control site (Tukey HSD p-adjusted value < 0.05). Number of leaves (Figure 2.S1a), stem widths (Figure 2.S1b), and fresh weights (Figure 2.S1c) were neither influenced by any of the main factors (site or depth) ($P > 0.05$) nor by interactions between factors ($P > 0.05$).

Table 2.1: Physico-chemical properties of stockpile soils for the AMF spore abundance study

	Topsoil				Subsoil			
Properties	**Reference**	**Mine A**	**Mine B**	**Mine C**	**Reference**	**Mine A**	**Mine B**	**Mine C**
pH	5.38±0.05^{a}	5.73±0.13^{a}	4.69±0.28^{a}	4.62±0.37^{a}	5.07±0.55^{a}	5.80±0.29^{a}	4.71±0.11^{a}	4.57±0.63^{a}
Na (cmol kg^{-1})	0.00±0.00^{b}	0.17±0.14^{ab}	0.05±0.01^{ab}	0.00±0.00^{b}	0.03±0.03^{ab}	0.092±0.03^{a}	0.05±0.01^{ab}	0.00^{b}±0.00
K (cmol kg^{-1})	1.09±0.04^{a}	0.21±0.07^{ab}	0.13±0.08^{ab}	0.18±0.13^{ab}	0.63±0.40^{b}	0.19±0.05^{ab}	0.08±0.03^{b}	0.05±0.03^{ab}
Ca (cmol kg^{-1})	1.47±0.28^{a}	1.96±0.66^{a}	0.45±0.30^{a}	0.76±0.73^{a}	1.21±0.09^{a}	1.63±0.08^{a}	0.40±0.11^{a}	0.95±1.18^{a}
Mg (cmol kg^{-1})	0.81±0.10^{a}	1.16±0.53^{a}	0.28±0.10^{a}	0.07±0.13^{a}	0.76±0.30^{a}	1.28±0.28^{a}	0.26±0.05^{a}	0.07±0.12^{a}
*S-V unit (cmol kg^{-1})	3.38±0.23^{a}	3.50±1.27^{a}	0.81±0.58^{a}	1.01±0.92^{a}	2.62±0.76^{a}	3.19±0.35^{a}	0.79±0.14^{a}	1.07±1.32^{a}
CEC (cmol kg^{-1})	6.00±0.76^{a}	4.78±1.30^{a}	3.42±2.46^{a}	6.07±0.55^{a}	8.88±1.34^{a}	5.37±1.46^{a}	3.27±1.72^{a}	5.13±0.51^{a}
Organic carbon (%)	1.53±0.12^{a}	0.90±1.02^{a}	1.09±1.01^{a}	0.79±0.52^{a}	0.70±0.14^{a}	0.43±0.06^{a}	0.39±0.11^{a}	0.78±0.43^{a}
$^{\beta}$Bulk Density (g cm^{-3})	1.35±0.02^{b}	1.67±0.25^{ab}	1.71±0.14^{a}	1.90±0.09^{a}	ND	ND	ND	ND
Sand (%)	60.90±1.00^{c}	74.00±5.29^{ab}	80.00±3.46^{a}	72.00±9.17^{ab}	66.85±8.35^{b}	63.20±2.68^{b}	81.20±3.03^{a}	76.00±0.00^{ab}
Silt (%)	19.95±1.65^{a}	4.67±2.31^{ab}	5.60±2.97^{ab}	4.67±1.15^{ab}	12.30±4.70^{ab}	5.20±1.10^{ab}	3.20±1.10^{b}	5.00±1.41^{ab}
Clay (%)	15.85±0.65^{ab}	21.33±5.03^{ab}	14.40±2.19^{ab}	23.33±10.07^{b}	17.60±5.00^{ab}	31.60±2.61^{a}	15.60±2.19^{ab}	19.00±1.41^{ab}
†Textural class	SaLm	SaClLm	SaLm	SaClLm	SaLm	SaClLm	SaLm	SaLm

Values are means ± SD of at least three replicates. Means with different superscript letters along columns are significantly different ($P < 0.05$) based on the interaction between site and sampling depths. ND, not determined. *S-V unit is the sum of the exchangeable cations (Ca, Mg, Na and K). $^{\beta}$Bulk density was subjected to one-way ANOVA to determine the site effects only. †Textural classes were determined from mean values for each bulk sample. SaLm, sandy loam; SaClLm, sand clay loam.

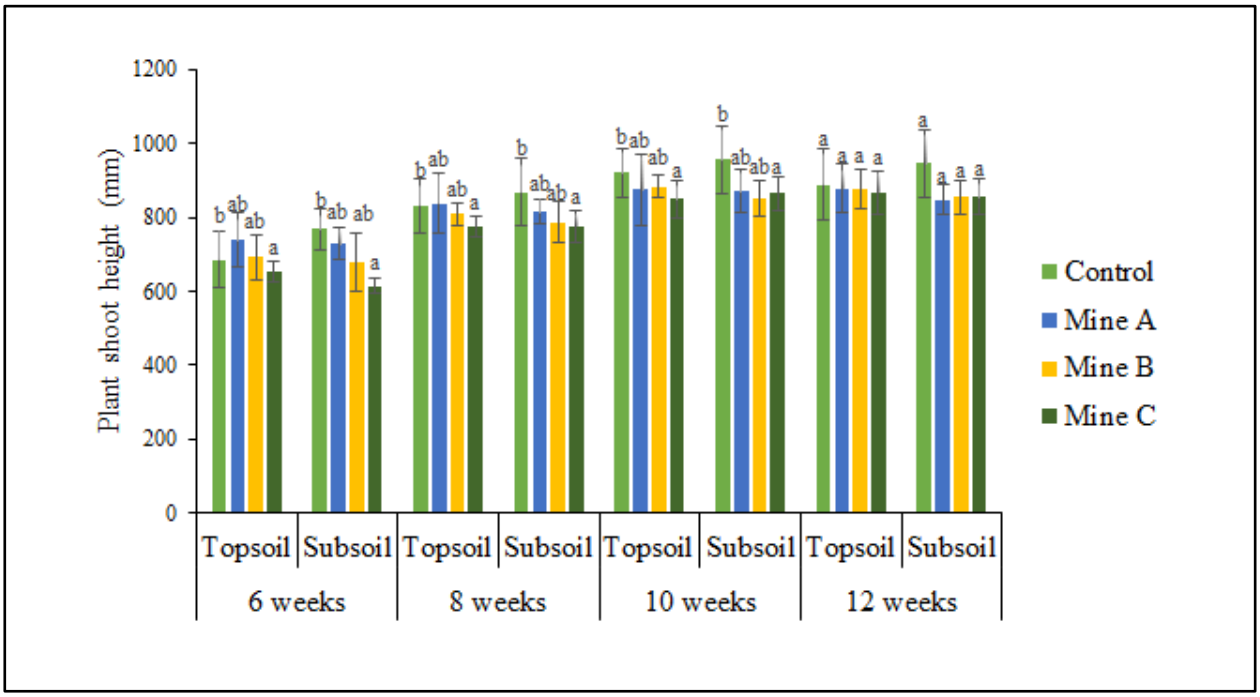

Figure 2.1: **Height of maize plants under greenhouse conditions.** Values are means of replicates (Sample size, N = 5). Error bars indicate standard deviations from the mean. The interactions between site and sampling depth are not significant ($P > 0.05$). Letters on the bars show values that are significantly different ($P < 0.05$) based on the effect of "site" only.

2.3.3 AMF spore density in stockpile soils and relationship with soil physicochemical properties

The AMF spore number per 100 g of soil is presented in Table 2.2. Spore abundance in soils was not significantly influenced ($P > 0.05$) by site and sampling depth. However, the interaction between site and sampling depth had a significant effect ($P = 0.0301$) on spore density, particularly in subsoils from the reference site. Overall, topsoil from reference sites had the highest spore density (108.7 spores/100g). In contrast, the lowest spore density (26.67 spores/100g) was observed in subsoil from the reference site. The AMF spore density in the topsoil was inversely related with (Pearson correlation) the mean pH (r = -0.336), bulk density (r = -0.588), cation

exchange capacity (CEC) (r = -0.596) and clay content (r = -0.729) of the topsoil (Table 2.3). However, only correlations between spore density and bulk density, cation exchange capacity (CEC) or clay content of the topsoil were significant ($P < 0.05$, 2-tailed test). On the other hand, the density of AMF spores in subsoils was inversely related with the mean pH, CEC and clay content of the subsoils (Table 2.3), but the relationships were not significant ($P > 0.05$).

Table 2.2: AMF spore density in soil and mycorrhiza detection in maize roots

Treatments	Site	Soil depth	†Spore density (spores 100 g^{-1} soil)	Mycorrhiza detection in maize roots	
				Root stains	‡Molecular method
1	Reference	topsoil	108.67±58.07[a]	-	+ +
2	Reference	subsoil	21.67±9.81[b]	-	-
3	Mine A	topsoil	39.67±17.03[ab]	-	+++
4	Mine A	subsoil	34.67±14.84[ab]	-	+
5	Mine B	topsoil	91.33±13.31[ab]	-	+ ++
6	Mine B	subsoil	57.67±38.52[ab]	-	+
7	Mine C	topsoil	49.00±2.00[ab]	-	+
8	Mine C	subsoil	67.50±43.50[ab]	-	+

†Spore abundance values are means ± SD of three replicates selected at random. Values on the same columns with different superscript letters are statistically different based on significant interaction effects between the site and sampling depth (Tukey HSD, $P < 0.05$). ‡Based on PCR amplification of the SSU-5.8S-LSU rRNA subunits using primers SSUmAf and LSUmAr. Positive PCR amplification (Mycorrhiza detected) per treatment replicates were ranked into three categories as + present in only one of the five trap culture replicates per treatment; ++, present in only two of the five trap culture replicates per treatment; and +++, present in 3 of the five trap cultures replicates per treatment. -, no mycorrhiza observed in roots of maize trap cultures.

2.3.4 Colonisation of maize roots by AM fungi

Microscopic examination of stained root sections did not reveal fungal structures indicative of mycorrhizae in all samples (Table 2.2). However, DNA-based detection revealed the presence of

AMF species in roots of maize plants in all but the subsoil from the control sample (Table 2.2). Overall, results (number of replicates per treatment positive for AMF root colonisation) of DNA-based detection showed that AMF root colonisation was more associated with topsoil (above 20 cm) samples than subsoil (below 20 cm) samples.

Table 2.3: Pearson rank correlations of number of AMF spores (spores 100 g^{-1} soil) with some physicochemical properties

Soil depth		Spore Abundance	Soil properties				
			pH	Bulk density (g cm^{-3})	CEC (meq 100 g^{-1})	Clay content (%)	Organic C (%)
Topsoil							
	Pearson Correlation coefficient (r)	1	-0.336	-0.588*	-0.596*	-0.729**	0.639*
	P-value		0.286	.044	0.041	0.007	0.025
	N	12	12	12	12	12	12
Subsoil							
	Pearson Correlation	1	-0.252	ND	-0.301	-0.057	0.050
	P-value		0.429	-	0.341	0.859	0.879
	N	12	12	-	12	12	12

**Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed). ND, not determined. CEC, cation exchange capacity.

2.3.5 Molecular Identification of AM fungi

Following PCR amplification, endomycorrhizae was detected in 12 of the 40 root samples (Table 2.2). Although we screened over 200 transformants from all clone libraries, only in 27 transformants were the cloned rRNA insert sequences successfully obtained. These sequences clustered into five operational taxonomic units (OTUs) and were all closely related to the defined AMF culture *Paraglomus occultum* (Figure 2.2 and Table 2.S2). Phylogenetic reconstruction of sequences further confirms the close associations to the genus *Paraglomus* (Figure 2.2). However, the bootstrap values for tree topology strongly indicate that a number of these OTUs belong to distinct clades which do not include any presently defined cultures of the *Paraglomus* taxa (Figure 2.2).

2.3.6 Overview of enzyme activities and microbial community structure

Beta-glucosidase and urease activities were significantly influenced by interactions between site and season (Figure 2.3). Based on a comparison of the number of significant factor interactions amongst treatments, beta-glucosidase activity appeared to be more sensitive to environmental factors than urease activity. Beta-glucosidase activity in stockpile soils was mostly higher than those of the reference soils suggesting a higher biological activity in the stockpiled soils in response to the availability of higher (compared to unmined soils) organic carbon in most of the soil stockpiles (Mashigo, 2018).

Across sites, beta-glucosidase activity was only positively correlated with C: N in mine A (Pearson's correlation coefficient, $r = 0.91$, $P = 0.013$) (data not shown), while urease activity was significantly correlated with the sum of exchangeable cations (S-V) only in the reference site (Pearson's correlation coefficient, $r = 0.57$, $P = 0.021$) (data not shown). Based on PCR-DGGE profiles of partial bacterial 16S rRNA gene diversity and fungal ITS2 sequences, bacterial and

fungal diversities of unmined soil were higher than those of stockpiles in the topsoil horizon

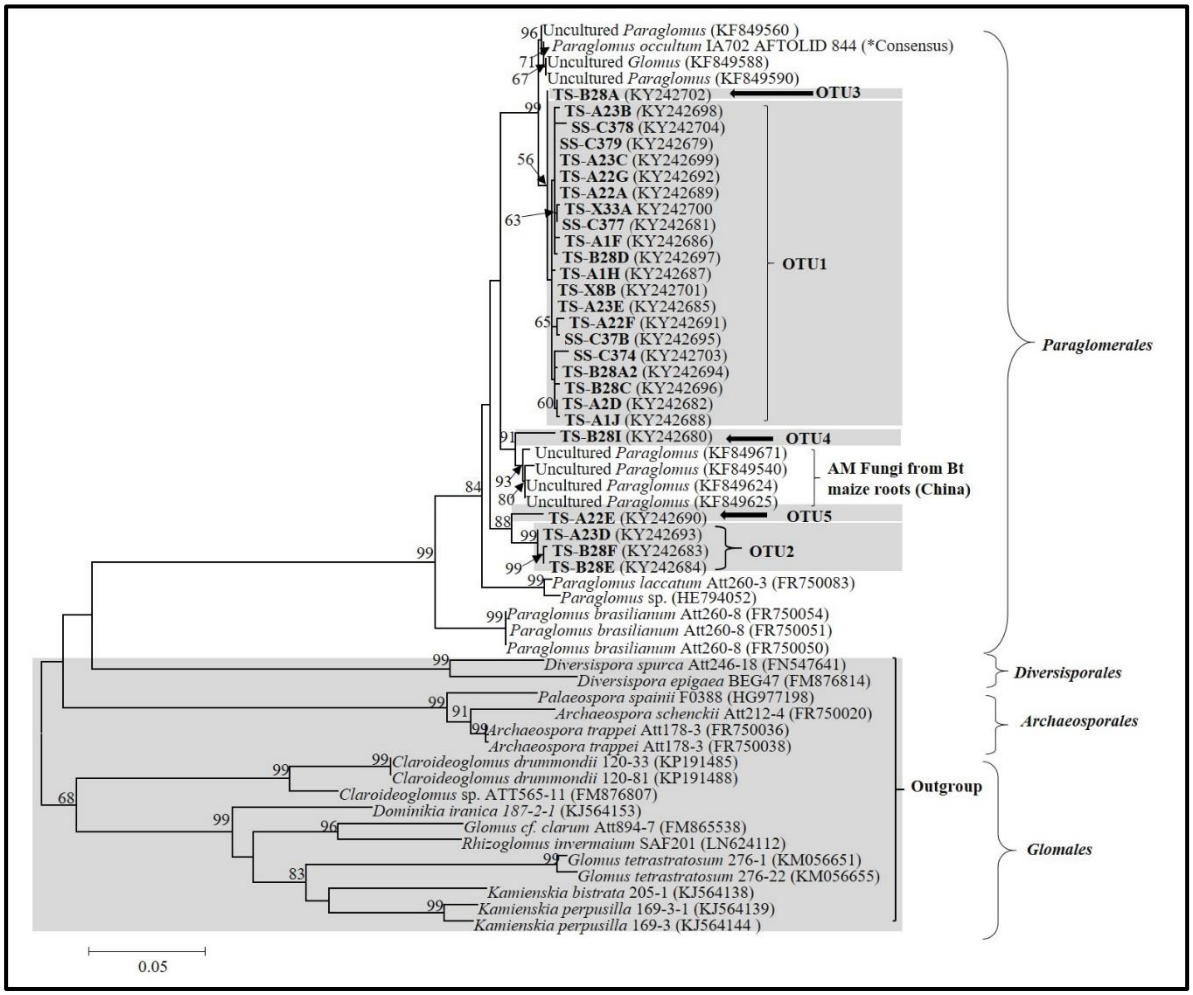

Figure 2.2: Maximum likelihood tree showing the phylogenetic association of AM fungi with reference database sequences based on 18S-5.8S-28S rRNA gene sequences. Bootstrap support values for branches less than 50% are not shown. Sequences beginning with TS and SS represent sequences from topsoil and subsoil. A, B, C or X (reference site) after hyphens represent study sites. GenBank Accession numbers are in parenthesis. *Paraglomus occultum* IA702 AFTOLID 844 is a consensus sequence obtained from sequence accession numbers DQ322629, AY997069 and DQ27387 reported as “consensus 39” in the study by (Krüger et al., 2012).

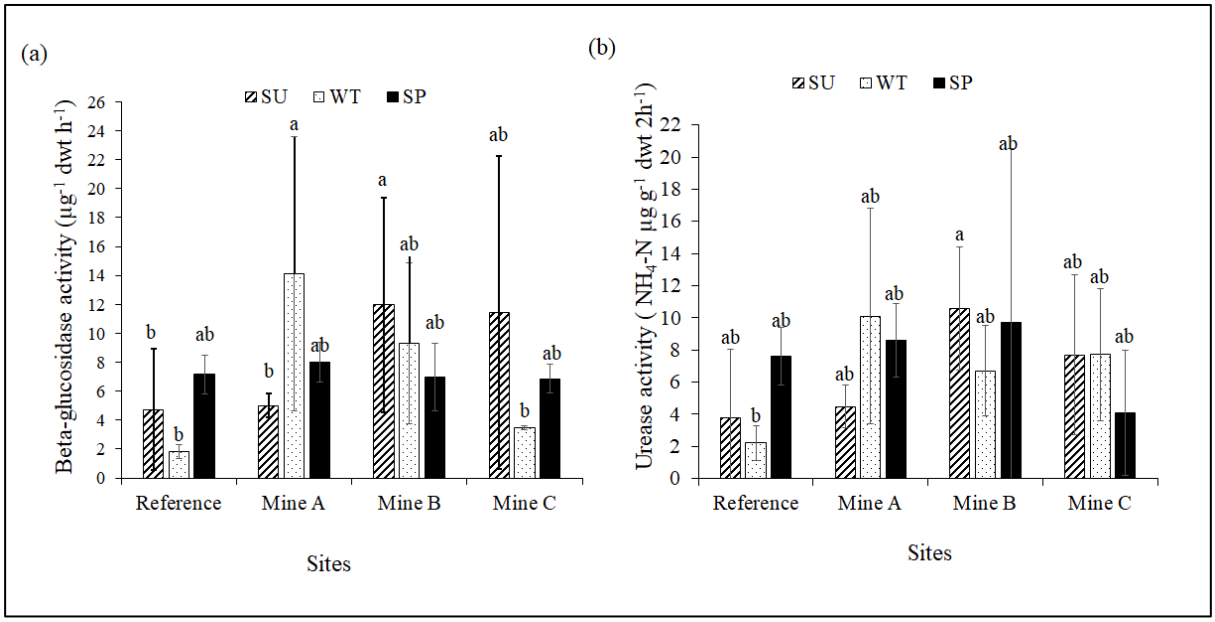

Figure 2.3: **Mean enzymatic activities in soils.** (a) Beta-glucosidase activity. (b) Urease activity. Sample size (N) ≥ 5. Bars with different letters are significantly different based on the effect of interactions between sites and season (Tukey HSD, $P < 0.05$). Error bars are standard deviations from means. SU, summer; WT, winter; SP, spring (adapted with modifications from Mashigo (2018)).

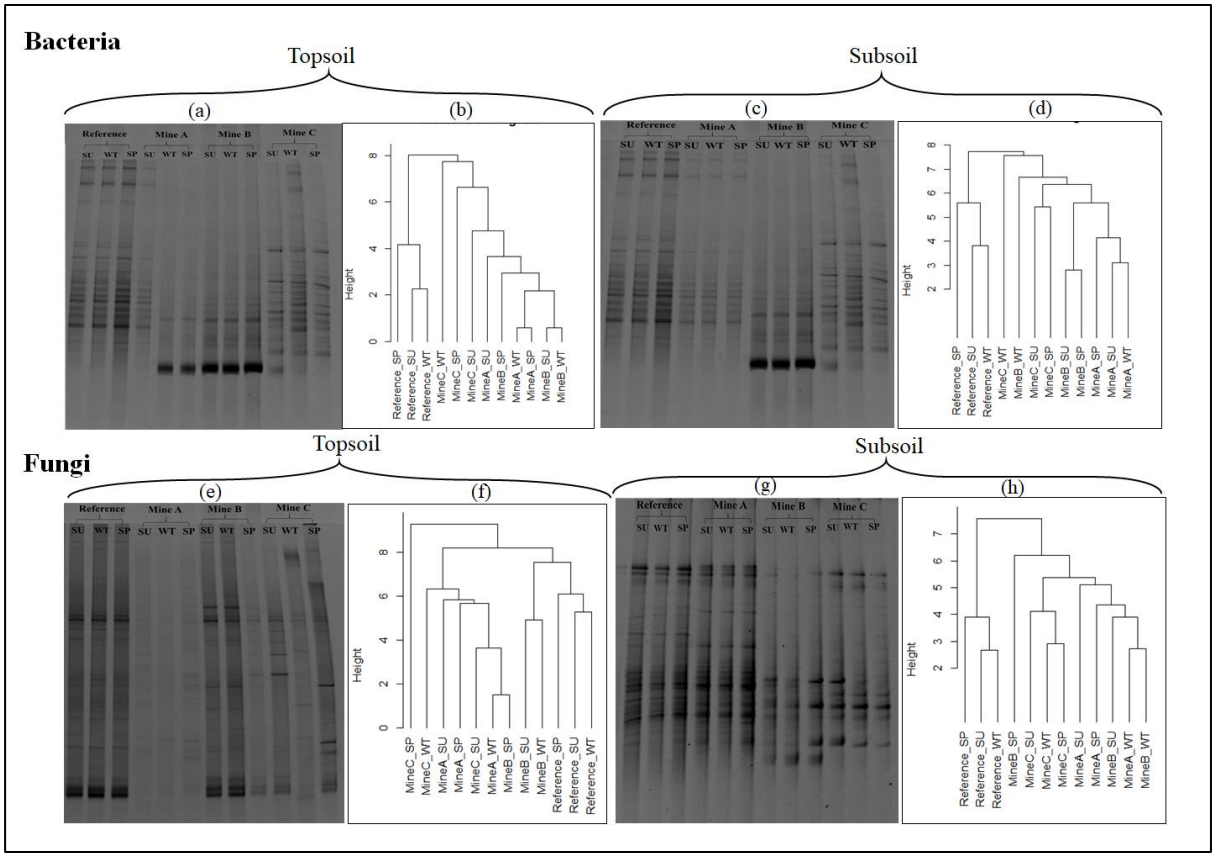

Figure 2.4: PCR-DGGE gel image and weighted hierarchical cluster dendrogram of microbial communities in soils. (a-b) Bacterial 16S rRNA gene diversity in topsoil samples. (c-d) Bacterial 16S rRNA gene diversity in subsoil samples. (e-f) Fungal ITS2 gene diversity in topsoil. (g-h) Fungal ITS2 gene diversity in subsoils. SU, summer; WT, winter; SP, spring See also Figure 2.S2 for unweighted hierarchical cluster dendrogram (adapted with modifications from Mashigo (2018)).

In the topsoil, the least microbial diversity was observed in mining site A: with bacterial diversity lowest in the spring while fungal diversity was lowest in winter. Across seasons, the soil microbial diversity did not vary greatly within the unmined reference soil (Figure 2.3 and Table 2.S3), whereas varying levels of differences were observed in bacterial and fungal diversities amongst seasons in each of the coal mining sites (Table 2.S3). Based on the associations depicted by the weighted hierarchical cluster dendrograms in Figure 2.4, the microbial community structure of the unmined reference soil was dissimilar to those of stockpiles in all three seasons. Within the reference site, summer and winter microbial community structures were more similar compared

to the spring season in both topsoil and subsoil (Figures 2.4b, 2.4d, 2.4f and 2.4h). While microbial communities were mostly clustered based on-site, close associations were observed in microbial community structure between some sites, such as the subsoil fungal community structures of mine A and mine B in winter (Figure 2.4h).

2.3.7 Taxonomic delineation of OTUs obtained from dominant bacterial and fungal PCR-DGGE bands

Precisely 44 bacterial and 33 fungal sequences were successfully (without ambiguous nucleotide base positions) obtained after sequencing the partial 16S rRNA gene and ITS2 sequences, respectively, from dominant bands excised from the PCR-DGGE gels. After OTU clustering at 97% 16S rRNA gene or ITS sequence similarity, seven bacterial and nineteen fungal OTUs/species were obtained, respectively (Table 2.4). Taxonomically, the bacterial OTUs spanned 2 phyla namely Firmicutes (comprising four OTUs) and *Proteobacteria* (comprising three OTUs) and comprised species of *Bacillus* (three phylotypes), *Pseudomonas* (two phylotypes), *Azomonas* (one phylotype) and *Lysinibacillus* (1 phylotype) (Table 2.4 and Supplementary Figure 2.S3). All 19 fungal OTUs belonged to the Ascomycota phylum and included species of *Alternaria*, *Aureobasidium*, *Austroplaca*, *Cenangium*, *Claviceps*, *Curvularia*, *Dendroclathra*, *Diaporthe*, *Fusarium*, *Helotiales*, *Macrohilum*, *Neoscytalidium*, *Phialocephala*, *Phoma*, *Pyrrhospora*, *Thyronectra and Valdensinia* (Table 2.4 and Figure 2.S4).

Table 2.4: Taxonomic delineation and economic importance of phylotypes obtained from dominant PCR-DGGE bands

‡Closest relative	Potential importance in an agroecosystem (Reference)
Bacteria	
Bacillus gaemokensis	Unknown
B. zhangzhouensis	Unknown
B. amyloliquefaciens	Non-pathogenic, plant-growth promoter (Gül et al., 2008; El-Daim et al., 2014)
Pseudomonas paralactis	Unknown
Azomonas macrocytogenes	Nitrogen fixation (Page and Collinson, 1987)
Lysinibacillus macroides	Potential bio-control agent, plant-growth promoter (Xiang et al., 2017)
Pseudomonas matsuisoli	Unknown
Fungi	
Aureobasidium pullulans	Endophyte, bio-control agent of blue mould in Rocha pear (Ferreira-Pinto et al., 2006)
Phoma herbarum	Endophyte, herbicide (Neumann and Boland 2002; Vikrant et al., 2006)
Fusarium fujikuroi	Plant pathogen; *Bakanae* disease of rice (Carter et al., 2008)
Claviceps purpurea	The ubiquitous pathogen of cereals and grasses (Tudzynski and Scheffer 2004)
Alternaria tenuissima	Plant pathogen: leaf spot of *Aloe barbadensis* (Vakalounakis et al., 2016)
Austroplaca soropelta	Unknown
Pyrrhospora arandensis	Unknown
Alternaria petroselini	Plant pathogen: leaf blight of fennel (Bassimba et al., 2012)
Curvularia trifolii	Plant pathogen: leaf spot in Berseem clover (Khadka 2016)
Neoscytalidium dimidiatum	Plant pathogen: etiological agent of cancer, shoot blight and fruit rot of almond (Nouri et al., 2018)
Dendroclathra lignicola	Unknown
Helotiales sp.	Symbiotic root endophyte (Zijlstra et al., 2005)
Macrohilum eucalypti	Endophyte: phylloplane of *Eucalyptus* (Swart, 1988)
Phialocephala humicola	Endophyte: promote the growth of tomato seedlings (Mahmoud and Narisawa, 2013)
Valdensinia heterodoxa	Plant pathogen: etiological agent of leaf blight in highbush blueberry (Nekoduka et al., 2012)

‡Closest relative	Potential importance in an agroecosystem (Reference)
Phoma sp.	Endophyte. Plant pathogen: leaf spot on *Schisandra chinensis* (Strobel et al., 2011; Choi et al., 2014)
Diaporthe foeniculina	Plant pathogen: stem and shoot cankers on sweet chestnut (Annesi et al., 2016)
Cenangium acuum	Plant saprophyte: Plant litter decomposition (Millar, 1974)
Sordariomycetes sp.	Endophyte: mineralisation of nutrients (Khan et al., 2017)

‡Closest relative denotes sequence with the best match in the Ezbiocloud database (https://www.ezbiocloud.net/). Due to the use of only partial 16S rRNA gene or ITS2 sequences, the identities of OTUs to species taxa level are not definitive but provided here for a discussion of potential ecological functions.

2.3.8 Association between microbial communities and soil physicochemical properties

The RDA model for the species-environmental relationship for topsoil as depicted in the biplot of Figure 2.5a is not significant ($P = 0.725$), suggesting that physicochemical properties in the topsoil did not influence the microbial community of the topsoil in general (Table 2.S4). In contrast, the RDA model depicted in Figure 2.5b for the relationship between microbial communities and the physicochemical properties of the subsoil is significant ($P = 0.011$). Specifically, in the subsoil, pH, organic carbon, total nitrogen and phosphorus contents of the subsoil significantly ($P < 0.05$) influenced the microbial community of the subsoil (Table 2.S4). In the fitted model, the correlation of the first two axes with the silt content is significant ($R^2 = 0.566$, $P = 0.027$), suggesting that the silt content was a predictor of topsoil microbial community. Whereas, in the subsoil, total nitrogen ($R^2 = 0.544$, $P = 0.039$) and phosphorus ($R^2 = 0.559$; $P = 0.030$) were significant ($P < 0.05$) predictors of the soil microbial community structure.

Furthermore, the RDA biplot of Figure 2.5 suggests that the microbial community of the topsoil and subsoil in the reference and mine C sites are least influenced by physicochemical parameters. However, in mine A and mine B, selected physicochemical properties influenced seasonal variations in the soil microbial communities. For example, pH and cation exchange capacity of the topsoil strongly influenced the microbial community of mine A in summer (Figure

2.5a), while in winter and spring, phosphorus and total nitrogen content of soil influenced microbial communities of mine A. The biggest predictor of the microbial communities in mine B subsoils during winter and spring is phosphorus (Figure 2.5b).

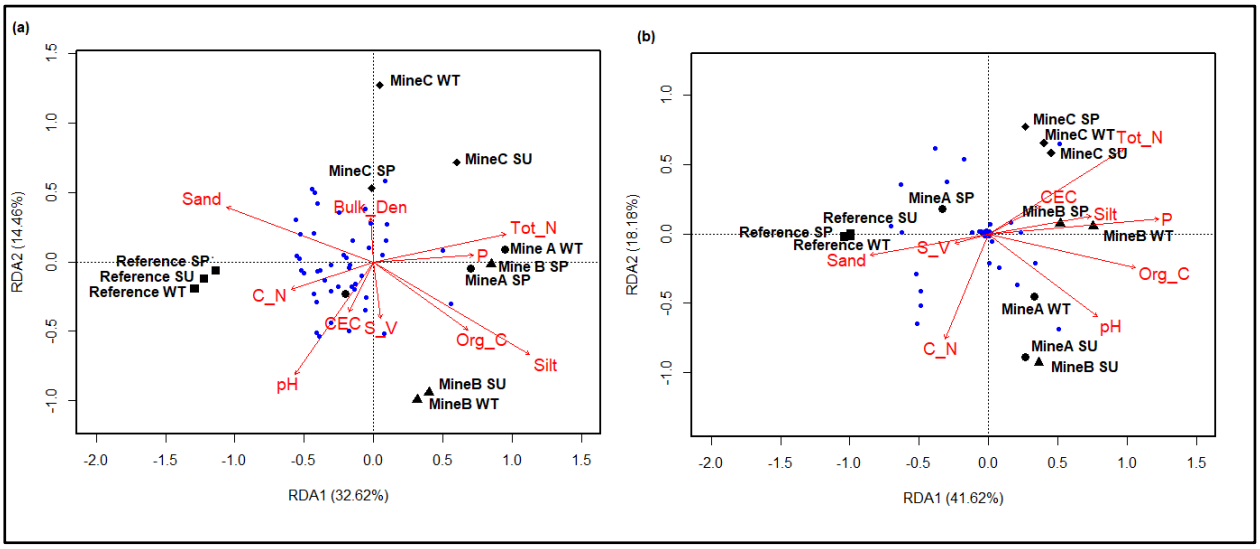

Figure 2.5: Redundancy analysis (RDA) biplot showing the relationship between soil physicochemical properties and unweighted PCR-DGGE profile of microbial communities in soils. (a) Topsoil. (b) Subsoil. In both RDA plots, blue dots are indicative of "microbial species" in the context of combined (per soil horizon) number of different bands in the bacterial and fungal PCR-DGGE image of Fig. 2. Wherever present in site names, "SU", "SP" and "WT" denotes summer, spring and winter samples. See also Table 2.S4 or the significance of the environmental factors in the RDA model.

2.4 Discussion

With the increasing demand for arable land and the need to ensure environmental sustainability, it has become necessary that mining activities be conducted according to best practices which promote meaningful post-mining land use. The storage duration and condition of the topsoil stockpile are important parameters, which could influence the important soil physical, chemical and biological attributes (Abdul-Kareem and McRae 1984; Wick et al., 2009). In the present study, the microbial community of selected soil stockpiles were investigated with reference to soils from

unmined areas. Furthermore, the number of recovered AMF spores, as well as the establishment of maize root-AMF symbioses were utilised as potential bioindicators of the soil capability to support plant growth since AMF species are generally considered beneficially to plants.

2.4.1 Physicochemical properties of soil stockpiles

With respect to the hypothesis of microbial community study, the pattern of differences observed in the physicochemical properties during (except for the total exchangeable cations in the topsoil horizon) of soils between unmined/reference site and stockpiles did not clearly suggest that stockpiled soils were in less adequate physicochemical conditions. However, the differences appeared to be site-specific and variable across seasons (Mashigo, 2018). The acidic pH of soil stockpiles may be attributed to leaching of basic cations, as well as from the oxidation of sulfide minerals and acid mine drainage throughout soil storage in stockpiles (Katzur and Haubold-Rosar, 1996; Pietrzykowski, 2014). Hence, for post-mining utilisation of such acidic soils in agricultural, liming of soils to optimum pH values specific for crops will be required. Furthermore, the high bulk density of the stockpile soils indicates higher compaction levels, which may affect root development. Hence, measures to reduce compactions during storage of soils in stockpiles are required

Ghose (2004) reported drastic alterations in the quality of topsoil dumps generated during opencast coal mining and that such alteration in soil quality increased with duration of storage. The high bulk density in some soil stockpiles soils may indicate high soil compaction attributable to heavy machineries, such as those used during the stripping and stockpiling process (Shrestha et al., 2005). Within a post-mining revegetation context, such high bulk density soils may pose restrictions on the growth of deep-rooted plants (Ghose, 2004; Shrestha et al., 2005; Mensah et al., 2015). In the soil microbial community study, a higher organic carbon in coal mine stockpiles compared to the reference site were observed.

2.4.2 Arbuscular mycorrhizal fungal spore density

The generally observed higher numbers of AMF spores in topsoil (compared to subsoil) may be attributed to spore dispersal limitations (Dumbrell et al., 2010). AMF spores are commonly dispersed in soils by the wind (Warner et al., 1987), as well as by soil macrofauna (Mangan and Adler 2002). Therefore, it is expected that spore numbers will be limited by vertical depth in soils. In a study by Bellgard (1993), topsoil was found to harbour twice as many AMF spores as the subsoil. Furthermore, elevated soil temperatures commonly associated with increasing soil depth may contribute to spore degradation and reduction in spore density in the deeper subsoils. In a study by Zhang et al. (2016), spore density was found to be negatively correlated with elevated temperatures.

The number of spores (108 spores/ 100 g soil) recovered from the reference site is comparable to values of total AMF spore abundance reported for protected and unprotected woodland systems in Kenya (Muchane et al., 2012) as well as in the topsoil of conventionally tilled and fertilized farming systems (Verzeaux et al., 2017). A higher number of spores were recovered from the control soil than in the coal mine stockpile soils. A similar observation was reported between undisturbed soils and topsoil stockpiles by Harris et al. (1989). The lower recovery of AMF spores in stockpile soils may be due to edaphic factors of the stockpiles such as moisture availability and length of storage duration of the soil stockpiles amongst others (Miller et al., 1985). Also, the observed non-significant differences between the number of AMF spores recovered from the control and the mining sites is similar to the observation of Straker et al. (2007) who observed no significant differences in the AMF spore densities in slime dams and surrounding soils of gold and uranium mines. Similar observations have been observed between iron mining areas and surroundings (Teixeira et al., 2017). However, Straker et al. (2008) observed significant differences in the number of AMF spores recovered between rhizosphere of grasses in slim dams and surrounding areas. Miller et al. (1985) observed that a combination of storage duration and moisture of soil stockpiles influenced the accuracy of predictive models on the survival dynamics of AMF spores in topsoil stockpiles.

Indeed, soil biological species are influenced by alterations in soil structure, land topography and vegetation types, as is the case with the topsoil stockpiling processes and mining in general (Jansa et al., 2006; Straker et al., 2007; Schalamuk and Cabello, 2010; Teixeira et al., 2017). For example, the significant negative correlation between bulk density and spore density suggest that soil compaction levels may affect AMF spore survival in stockpiles. Hence, from the preceding discussions, recommended best soil stockpiling practices, which will promote the survival of AMF spores, may include minimising the duration of stockpile storage and the reduction in the use of heavy equipment over topsoil stockpiles in order to minimise compaction levels.

As a note on the minimal effect on agronomic performance of plants observed in this study, we hypothesise that the regular supplementation of soils with equal amounts of nutrients might have masked any initial differences in soil chemical composition and consequently plant biomass. However, the reason for the observed significant differences in the shoot heights between plants grown in the control soil and soils from mine C is unclear.

2.4.3 Root colonisation and taxonomic diversity of AMF propagules

This study employed a combination of classical root staining and molecular methods to assess the establishment of AMF colonisation in a single plant (maize)-trap culture. The classical root stains did not detect any evidence of mycorrhizae in all root sections examined. However, AMF-specific nuclear rRNA was detected in root tissues, thereby suggesting mycorrhization. The disparity in results between both classical staining and DNA-based methods has been previously observed (Morton and Dirk, 2001). Mycorrhizae of certain AMF lineages, including *Paraglomus* spp. (the only species we detected in this study), stain weakly or not at all with commonly used stains (Morton and Dirk, 2001; Redecker et al., 2003; Błaszkowski et al., 2012). Also, it is possible that the fungicide used for surface sterilization of the maize seeds may persist in the maize plant and kill-off arbuscular mycorrhizal fungi during trap culturing. Because nuclear material of the AM fungi may persist long after AMF propagules have degraded in the plant, they may be detected by PCR amplification. The foregoing statements may explain the disparity observed between

results of root stains and rRNA sequence analysis in this study. In a general context, this shortcoming of classical mycorrhizal detection amongst other shortcomings may lead to false conclusions in mycorrhization assessments and experiments (Redecker et al., 2003; Krüger et al., 2009; Krüger et al., 2012). Hence, mycorrhization studies should combine morphological and molecular approaches. Nevertheless, the observation of AMF colonisation of roots in mostly topsoil by the DNA-based method appears to corroborate the observation of higher numbers of spores in the topsoil. Logically, it will seem that the higher numbers of AMF spores in soils will increase the likelihood of obtaining a higher number of viable AMF spores and subsequently, a higher level of root colonisation.

The five operational taxonomic units (OTUs) detected in maize root tissues belonged to the genus *Paraglomus*. The genus *Paraglomus* is distinguished from the genus *Glomus* (the largest AMF genus) based on rRNA phylogenetic affiliation (Morton and Dirk, 2001). The type species of the *Paraglomus* genus include *P*. *occultum*, *P*. *brasilianum*, *P*. *lacctum* and *P*. *Majewskii* (Morton and Dirk, 2001; Błaszkowski et al., 2012). *Paraglomus* species have been detected in several regions of the world. However, certain species have so far been reported in only certain regions (Błaszkowski et al., 2012). At present, reference rRNA sequences of defined *Paraglomus* cultures spanning the SSU-5.8S-LSU are few in the GenBank. Hence, the phylogenetic association of sequences obtained in this study with the publicly available reference sequences of the Paraglomus taxa, clearly indicate that most of the sequences obtained in this study belong to distinct clades and are thus different from presently defined *Paraglomus* species. Presently, there is a need for a strong consensus regarding the classification of AM fungi (Redecker et al., 2013). Such consensus will be important for the accurate classification of the growing number of environmental AMF sequences to species taxonomic level by using reference culture sequences (Krüger et al., 2012).

Although deeper sequencing efforts (than that achieved through the cloning step conducted in this study) are required for an adequate characterisation of the AMF community in the trap

cultures, the observation of sequences phylogenetically related to currently defined *Paraglomus* taxa is an interesting finding. Ideally, the detection of a single species in all root mycorrhization would prompt a few hypotheses. These hypotheses will include, firstly, the occurrence of host specificity. Host specificity (the preference of AM fungi to associate with specific plant types) is commonly reported (Smith and Read 2010; Torrecillas et al., 2012). For example, certain species of the genera *Glomus* are reported to demonstrate host specificity to herbaceous plants (Torrecillas et al., 2012). Similarly, Spruyt et al. (2014) observed that there was a higher mycorrhization preference for *Tamarix usneoides* compared to other rehabilitation plants on mine wastes in South Africa. Secondly, studies have shown that the diversity of AM fungi in soils under conventional agriculture (tillage systems, monocropping systems) may be sparse (Alguacil et al., 2008; Moebius-Clune et al., 2013; Xiang et al., 2014). Thirdly, given the high numbers of spores enumerated in these soils, a single species mycorrhization may suggest that many of these spores are degraded and hence non-viable. In hindsight, the utilisation of multiple (or other) host species in the trap culturing may be necessary in order to limit the effect of host specificity in undermining the true richness of viable AMF spores. Alternatively, other methods for assessing spore viability, such as the utilisation of tetrazolium chloride (INT) reduction (Welersbye-Witkowski and Straker, 1997) may be adopted. As stated earlier, there is a likelihood of significant underrepresentation of AMF species by the cloning approach employed in this study. Therefore, further studies employing the use of high-throughput sequencing on next-generation sequencing platforms may help elucidate the AMF diversity in these roots at a deeper depth and coverage.

2.4.4 Beta-glucosidase and urease activities in stockpile soils

The observed higher beta-glucosidase activity in stockpile compared to unmined soils is similar to the findings of Claassens et al. (2012) who observed that the beta-glucosidase activity in coal discard and asbestos sites had a higher maximum value compared to reference sites. Similar observations of higher urease activities in stockpile soils may be attributed to the observed relatively higher total nitrogen present in most of the stockpile soils (Mashigo, 2018). Generally,

urease activity correlates with soil nitrogen, especially when a source of urea is available (Meyer et al., 2015).

According to Das and Varma (2010), enzyme activity in soil ecosystems is mostly dependent on organic carbon levels, soil type, soil composition and microbial community diversity and function. For example, numerous authors have reported that beta-glucosidase activity is sensitive to soil pH and management practices (Tabatabai and Dick, 2002; Makoi and Ndakidemi, 2008; Meyer et al., 2015). In contrast, Meyer et al. (2015) observed that beta-glucosidase and urease activities correlated significantly with soil carbon, NO_3^- and pH but that the correlations between beta-glucosidase and soil carbon, NO_3^- and pH were weaker compared to correlations with urease. Furthermore, standard deviations from the mean beta-glucosidase and urease activities were higher in soil stockpiles than in reference soils, suggesting higher intra-site variations within coal mines. Similarly, Claassens et al. (2012) observed higher variations in enzyme activities on disturbed sites (coal discard sites) compared to undisturbed reference sites. The higher variation may be attributable to the mixtures of soil horizons (physically observed on-site) and intra-site variations in ages of soil stockpiles which was not considered in the design of this study (due to of lack of comprehensive information on stockpile ages).

2.4.5 PCR-DGGE banding pattern and diversity indices of stockpile microbial communities

The observed highest number of different bands (“species richness”) in the topsoil and subsoil of reference soils compared to stockpile soils at all three sampling seasons (Table 2.S3), suggests soil disturbances during stockpiling impair microbial diversity. Similar observations in the microbial diversity of disturbed and undisturbed sites have been previously reported (Fresquez et al., 1984; Harris et al., 1989; Harris et al., 1993). The variation of microbial communities in response to environmental conditions is well documented (Habekost et al., 2008; Baldrian et al., 2010; López-Mondéjar et al., 2015). 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. The changes in microbial diversity across seasons may be due to the different supply of nutrients (resource availability) as a consequence of certain seasonal (environmental) factors such as temperature and moisture (Rasche et al., 2011; Fekete et al., 2012). In the soil ecosystem, these factors influence the allocation of photosynthates to the soil by roots of primary producers, the inputs of fresh litter and above- or below-ground biomass production (López-Mondéjar et al., 2015). Invariably, the alteration in plant-derived exudates and biomass influences microbial community structure and composition of the soil and may give rise to niche partitioning of microorganisms.

2.4.6 Taxonomic diversity (phylotypes) and potential ecological functions of stockpile microbial communities

The biodiversity of soil microbial communities is vital to the sustainability of soil ecosystem functioning. The detection of specific phylotypes at only certain depths (i.e. topsoil or subsoil) suggests a vertical niche differentiation of species in the soil stockpiles (Hansel et al., 2008). Generally, vertical and seasonal variations in nutrient and physical parameters predispose the selection (selective pressure) of species with different adaptability (e.g. adaptation to energy and nutrient limiting conditions) and capabilities (e.g. nutrient cycling) along the soil physicochemical differentiation profile (Hansel et al., 2008; Rasche et al., 2011; López-Mondéjar et al., 2015; Stone et al., 2015). Niche differentiation of species in the soil ecosystem is important for the sustainability of soil health through biological processes that regulate, amongst others, nutrient cycling (Lennon et al., 2012).

Although the partial 16S rRNA gene sequences used for taxonomic identification of bacterial OTUs may not be sufficient to delineate sequences into the species taxonomic rank, reference to the closest relative at the species level was made in other to facilitate discussions towards potential functional roles of these species in the soil and for comparison purposes within the scientific literature. The observation of dominant phylotypes of the Firmicutes and Proteobacteria phyla agrees with observations made in previous soil microbial ecology studies (Lennon et al.,

2012; López-Mondéjar et al., 2015). The dominance of *Bacillus* spp. in these soils suggest that these species are well-adapted to the prevailing nutrient-limiting conditions of the soils and are likely contributors to ecosystem processes in these soils. Phylogenetically similar species of *Bacillus* (taxonomic clade) detected in soil stockpiles have been associated with mining soils (Jamal et al., 2016; Oladipo et al., 2018). *Bacillus* spp. are functionally diverse and play roles in the mineralisation of plant-derived materials, plant-growth promotion, humus, pesticides and hydrocarbons in the soil (Garbeva et al., 2003; Mandic-Mulec and Prosser, 2011). For example, strains of *B*. *amyloliquefaciens* have been shown to promote yields in tomato plants (Gül et al., 2008) as well as, improve the tolerance of wheat to heat stress (El-Daim et al., 2014). Other phylotypes whose strains are known to have ecological relevance in the agroecosystem include *Lysinibacillus* (e.g. *L*. *macroides*) and *Azomonas* (e.g. *A*. *macrocytogenes*) (Table 2.4). Similarly, some *Pseudomonas* spp. are able to demineralize coal (Singh et al., 2016), degrade polychlorinated biphenyl and polycyclic aromatic hydrocarbons (Nam et al., 2014; Bello-Akinosho et al., 2016), perform soil ecological functions (Naseby and Lynch 1999), promote plant-growth (Wicaksono et al., 2017) and may also participate in the remediation of metal-contaminated soils (Wasi et al., 2013; Oladipo et al., 2018).

Known plant pathogens dominated the fungal diversity of stockpile soils (Table 2.4), thereby suggesting that these soils may serve as reservoirs for economically important plant pathogens. Hence, within the context of post-mining land use, especially for agriculture, the use of these soils may have implications on the productivity of certain crops if adequate disease management practices are not implemented. In contrast, close relatives of the fungi *Aureobasidium pullulans*, *Phialocephala humicola*, *Phoma herbarum* and *Sordariomycetes* sp. detected in soil stockpiles study have been reported to have beneficial importance to industry and agriculture (Neumann and Boland, 2002; Ferreira-Pinto et al., 2006; Mahmoud and Narisawa, 2013) (Table 2.4). Thus, stockpile and reference soils may also serve as a source for the bioprospection of such industrially important and phytobeneficial fungal strains.

2.5 Conclusion

AMF spore numbers were higher in the topsoil of unmined soils than in topsoil of stockpiles. This suggests that stockpiling soil disturbances influence the density of AMF spores in soil stockpile. Furthermore, the density and viability of AM fungal spores were mostly higher in the “topsoil” depth (0-20 cm) and inversely related to soil bulk density. Thus, it is recommended that soil stockpiling is conducted with minimal disturbance to the topsoil structure and that the use of heavy equipment over topsoil stockpiles be minimised to prevent soil compaction, which is linked to AMF spore survival.

While there were no clear differences between the physicochemical properties of soil stockpiles and unmined sites, the differences in enzyme activities (mostly beta-glucosidase) and microbial diversity between soil stockpiles and unmined sites suggests that biological components of the soil are quite sensitive to soil management practice and/or disturbance. The impaired (compared to an unmined site) microbial diversity and community structure observed in stockpiles may have negative implications for soil biological processes driven by microbes, especially processes that are critical for nutrient mobilisation or cycling, food web structure and overall ecosystem sustainability. In addition, several microbial species detected in both reference and stockpile soils are closely related to characterised phytobeneficial species. Thus, these soils may serve as sources for the bioprospection of microbes of both agricultural and industrial applications

More importantly, because stockpile soils are utilised for post-coal mining reclamation, the alteration observed in microbial communities of soil stockpiles in comparison to reference sites may be carried over to reclamation areas. Consequently, the health of reclamation soils may be impaired. Such impairment in microbial community richness and associated poor soil health may constitute an increase in the duration of ecosystem recovery and ultimately land use capability of post-mining reclamation soil/areas. Hence, further studies are needed to investigate the microbial community richness and structure in post-coal mining reclamation soils.

CHAPTER 3:

UTILISING STRUCTURAL AND FUNCTIONAL DIFFERENTIATION OF MICROBIAL COMMUNITIES FOR ASSESSING ECOSYSTEM RESTORATION IN POST-COAL MINING RECLAMATION SOILS

3.1 Introduction

The soil ecosystem supports numerous interactions between living and non-living matter. These interactions are vital to the soil's ecological processes and key ecosystem services (Drobnik et al., 2018; Ferris and Tuomisto, 2015). However, anthropogenic disturbances of the soil ecosystem through agriculture, mining and other land use activities, negatively affect these vital interactions (Morgado et al., 2018; Ezeokoli et al., 2019b).

Although relevant post-mining reclamation guidelines exist in South Africa for restoring mined-outlands to acceptable conditions (Tanner and Möhr-Swart, 2007), appropriate comprehensive soil quality assessment tools for monitoring post-mining reclamation areas are still lacking. Such soil quality assessment tools will help elucidate the adequacy of current reclamation practices and could provide insights into the potential restoration of ecological roles in mining-impacted areas. At present, several above-ground monitoring indicators for soil quality, including vegetation cover, erodibility, and compaction levels have been proposed and utilised on post-coal mining reclamation sites (Paterson et al., 2019). However, these above ground indicators do not provide a comprehensive assessment of soil health given that the soil ecosystem is multidimensional in nature—with respect to its biological, physical and chemical components (Arias et al., 2005). In recent years, the suitability of soil biota as soil health indicators has received much attention (Arias et al., 2005; Stenberg, 1999; Dose et al., 2015). Notably, the use of soil microbial indicators has been proposed for soil quality monitoring because diversity and structure of microbial community are sensitive to natural or anthropogenic disturbances (Arias et al., 2005; Dose et al., 2015; Nkuekam et al., 2018; Stenberg, 1999). The suitability of microbes as bioindicators is further supported by the direct relationship between the diversity of soil microbial communities and soil

ecosystem function (Kennedy and Stubbs, 2006; Allison and Martiny, 2008; Ferris and Tuomisto, 2015; Maron et al., 2018b), as well as the key roles of soil microorganisms in nutrient cycling, plant growth promotion, ecological succession and energy flow in soil ecological food webs (Hayatsu et al., 2008; Frouz et al., 2013; Dose et al., 2015; Markowicz et al., 2015; Steffan et al., 2015; Adeleke et al., 2017). For these reasons, several studies have investigated soil microbial community composition and function in anthropogenically- and naturally-disturbed environments (Claassens et al., 2008; Claassens et al., 2012; Dose et al., 2015; Markowicz et al., 2015) with the aim of determining the potential impact of such disturbances on soil health in terms of soil processes and function.

Few studies (Claassens et al. 2006; Claassens et al., 2008; Claassens et al., 2011) have explored the microbial community functions and structure along a time gradient of post-coal mining reclamations within South Africa. Yet, very little is known about the microbial species diversity and functional community structure in post-mining reclamation soils of South Africa. Furthermore, compared to previous microbial community diversity studies, recent advances in sequencing technologies now make it feasible to unravel microbial communities of environments at a much deeper depth and coverage (Caporaso et al., 2010; 2012; Tedersoo et al., 2014; van Wyk et al., 2017). In addition, these techniques also provide a glimpse into potential ecological functions of microbial communities at a high-throughput scale (Aßhauer et al., 2015). An in-depth study of the microbial ecology of coal mining soil environments will help unravel species diversity of reclamation soils for bioprospection purposes, and more importantly, for identifying potential biomarker species for monitoring soil ecosystem recovery and health post-mining disturbance. At present, such in-depth microbial community studies on post-coal-mining reclamation sites are however sparse.

In the previous chapter (Chapter 2), it was observed that the microbial community structure in stockpiles is impaired compared to those of unmined areas. It is unclear whether such impairment in microbial communities persists in reclaimed areas and over many years since reclamation.

Hence, the present study investigated the diversity and potential ecological functions of microbial communities in selected post-coal-mining reclamation soils in South Africa. The study investigated two major hypotheses. Firstly, the bacterial community structure and function in reclamation soils are impaired compared to unmined soils (soil history effect), and such impairment is site-specific. Secondly, the microbial communities of post-coal mining soils are differentiated along a temporal scale of years since reclamation. The first hypothesis was tested on three coal mining sites located in the coal-rich Mpumalanga province of South Africa, while the second hypothesis was investigated along a post-mining chronosequence within a specific mining site (company). Here, microbial community structure and potential function were investigated by utilising a combination of physiological (enzyme assays and/or carbon substrate utilisation pattern) assays and next-generation sequencing (NGS) of the bacterial 16S rRNA gene and fungal internally-transcribed spacer 2 (ITS-2) region. Furthermore, the potential function and ecological guilds of microbial communities were predicted using the bacterial 16S rRNA gene and fungal ITS-2 sequences, respectively.

3.2 Materials and methods

3.2.1 Study site selection, description and design

For this study, the selection of sites was mostly influenced by the cooperation of coal mining companies at specific times as well as the availability of reclamation areas ranging over several years. A total of three active opencast coal mining sites located in the high-veld Emalahleni district (29° 3' 36"S, 25° 52' 12"E) of Mpumalanga province, north-eastern South Africa were utilised for the microbial community study (Figure 3.1). All the study sites are within the Grassland Biome of South Africa (Ferrar and Lötter, 2007). Due to a confidentiality agreement, these coal mining sites are herein referred to as site X, Y and Z (Figure 3.1). Site Y is approximately 67 km to the south of site X; site Z is approximately 32 km to the south of site X, while site Z is approximately 41 km to the north of site Y.

With the exception of mine Z, these sites differed from those included in the study reported in Chapter 2. Mine Z was the same as the mine C utilised for the study on stockpiles in Chapter 2 but renamed herein and in subsequent chapters for convenience sake. For clarity, changes in site selection (compared to those in chapter 2) were mostly due to sustained (or unstained) cooperation of the coal mining companies and/or availability of reclamation areas. The altitude of the study area ranged from 1400 – 1600 m above sea level. The annual maximum and minimum temperature for the Mpumalanga province is 31°C and 10°C, respectively, while total annual rainfall is 938 mm (World Weather Online). Monthly average maximum and minimum temperature of the study area is 24±3°C and 15±3°C, respectively, while average precipitation and humidity are 12.5±10.27 mm and 54 ±5 %, respectively (World Weather Online). Although the Highveld of Mpumalanga is the largest coal-mining region of South Africa, agriculture remains a key component of the province since the soils are highly arable (BFAP, 2012; GSA, 2016). Post-mining land reclamation is therefore paramount for the restoration of pre-disturbance conditions or other acceptable high-end land-use capabilities such as pasture lands for animal grazing. The procedure for reclamation in all three sites was according to the local guidelines. Briefly, different soil horizons—overburden, subsoil and topsoil (each stockpiled separately pre-mining)—are replaced in the same order, soil amelioration and fertilization are applied prior to seeding with mixed vegetation species (commonly *Eragrostis tef*, *Eragrostis curvula*, *Digitaria eriantha*, *Cynodon dactylon* and *Chloris gayana*) (Claassens et al., 2006). However, over several years of revegetation, a dominating vegetation type appears on reclamations sites (Van Eeden, 2010; Claassens et al., 2012). *Eragrostis* species dominated all reclamation areas. Herein, reclamation refers to land prepared and revegetated as described above. In addition, the ages of the reclaimed lands are the period between revegetation and time of sampling.

Over the course of the study, sampling was conducted in 2016 and 2017. In the initial sampling in 2016, a recently (<1 yr.) reclaimed area was sampled in mine X (hereafter referred to as ReclX), while a 1.5-year-old reclaimed site was sampled in mine Y (hereafter referred to as ReclY). In mine Z, a reclamation area of 18 yr. was sampled (hereafter referred to as ReclZ). In 2017, within

mine Z, a chronosequence of reclaimed areas, comprising five different ages were established (Figure 3.2) to minimise site-specific influences observed from the analysed results obtained in 2016. The reclamation lands were reclaimed in the year 2014 (3 years old at the time of sampling), 2006 (11 years old), 2002 (15 years old), 1998 (19 years old) and 1993 (24 years old). Herein, these reclamation areas are abbreviated as ReclZ-3, ReclZ-11, ReclZ-15, ReclZ-19 and ReclZ-24 for the 3, 11, 15, 19- and 24-years old reclamation areas, respectively (Figure 3.2). The sampling information is summarised in Table 3.1.

For clarity, the sampling in 2016 was designed to test the first hypothesis of the study while the sampling in the subsequent year (2017) was aimed at testing the second hypothesis of the study.

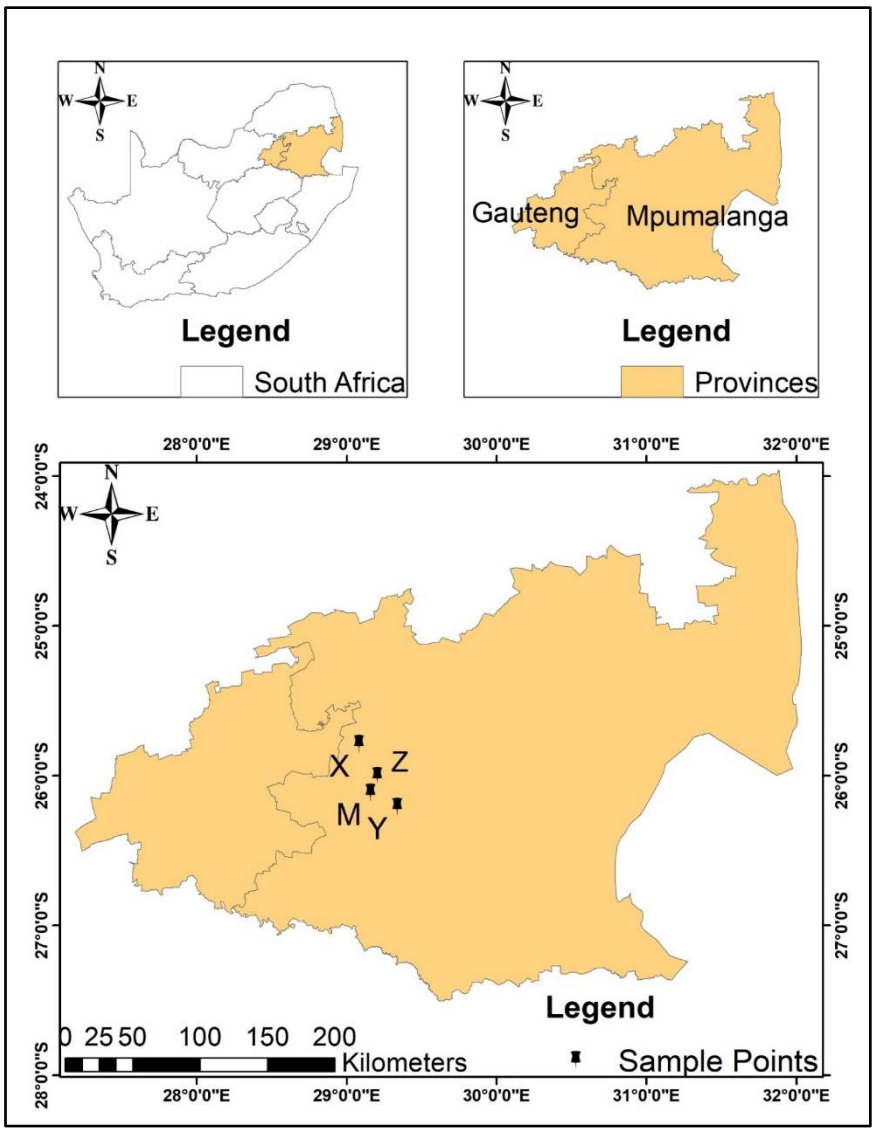

Figure 3.1: **Map showing coal mining companies sampled for the overall study on reclamation areas.** For the microbial community analysis, only mines X, Y and Z were sampled. Mines X, Y and Z were sampled in 2016, while additional samples were collected along a chronosequence in mine Z in 2017 (See also Table 3.1). Mine M was sampled in 2018 for the ecotoxicology study reported in Chapter 5.

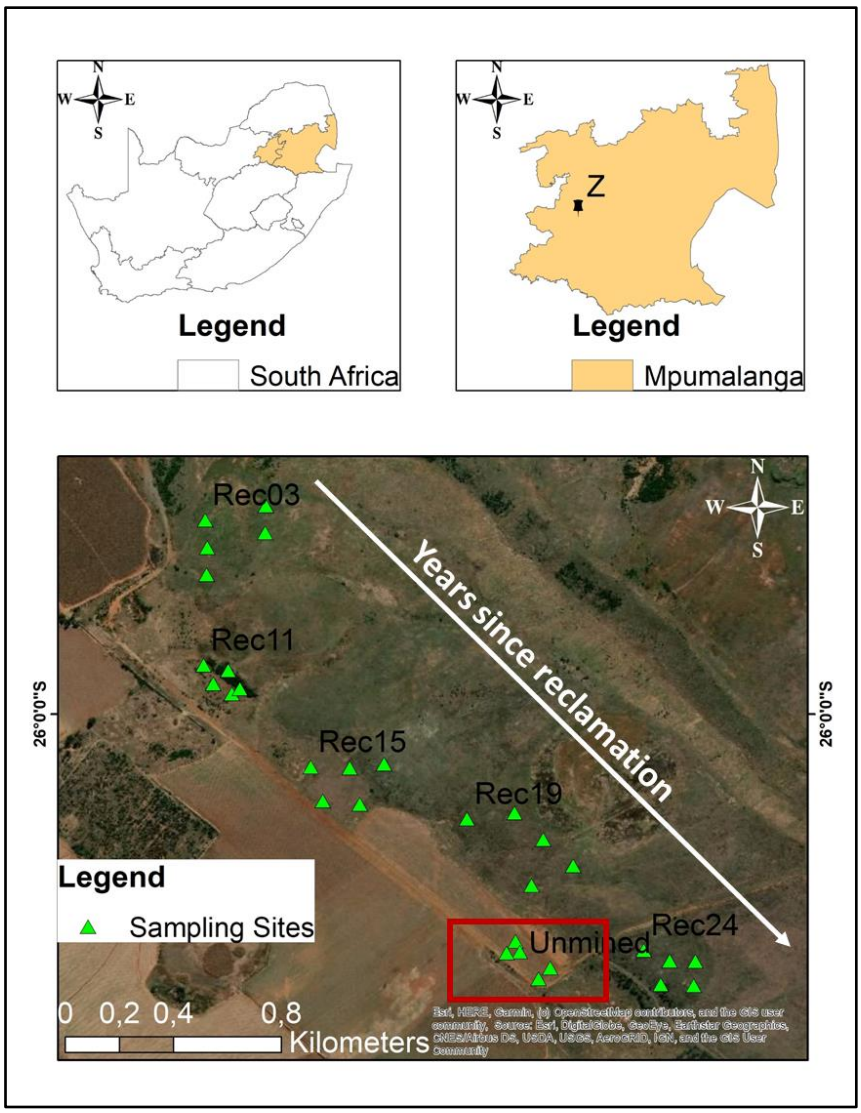

Figure 3.2: **Map showing sampling areas in mine Z sampled in 2017.** Each green triangle represents only one end of each transect along which samples were collected. Rec03 (or ReclZ-3), 3 years since reclamation; Rec11 (or ReclZ-11), 11 years since reclamation; Rec15 (or ReclZ-15), 15 years since reclamation; Rec19 (or ReclZ-19), 19 years since reclamation; Rec24 (or ReclZ-24), 24 years since reclamation; Unmined (or unm-Z), unmined maize field. ReclZ-19 is the same area sampled in 2016 (i.e. ReclZ).

Table 3.1: Description of sampling sites for the study

*Sampling year	Site code	Description	Site/Mine	Year of seeding/stockpiling (†age in years)	[1]No of bulk samples	Notes
2016	ReclX	Reclamation area	X	2015 (0.5)	3	No activity since reclamation/revegetation.
	RefX	Unmined “reference” area	X	-	5	An area outside mining company X. No current land use activity. Likely prone to other anthropogenic influences.
	ReclY	Reclamation area	Y	2014 (1.5)	5	No activity since reclamation/revegetation.
	RefY	Unmined “reference” area	Y	-	3	Same notes as for RefX above.
	ReclZ	Reclamation area	Z	1998 (18)	5	No activity since reclamation/revegetation.
	RefZ	Unmined “reference” area	Z	-	5	Same notes as for RefX above.
2017	ReclZ-3	Reclamation area	Z	2014 (3)	5	No activity since reclamation/revegetation.
	ReclZ-11	Reclamation area	Z	2006 (11)	5	No activity since reclamation/revegetation.
	ReclZ-15	Reclamation area	Z	2002 (15)	5	No activity since reclamation/revegetation.
	ReclZ-19	Reclamation area	Z	1998 (19)	5	No activity since reclamation/revegetation. Same area as ReclZ sampled in 2016.
	ReclZ-24	Reclamation area	Z	1993 (24)	5	No activity since reclamation/revegetation.
	Unm-Z	Unmined area	Z		5	A post-harvest maize field at the time of sampling.

†Age at the time of sampling. -, undated. See also Figures 3.1 and 3.2. *Additional sampling information conducted in 2018 are provided separately in Chapter 5. [1]Each bulk sample is a composite of five soil cores collected at 10 m intervals along a 40 m transect or at points of a cross. A total of three to five transects or crosses were used per site. No of bulk sample differed due to sampling strategy for representative sample collection per sampled area.

In 2016, soil samples were collected from non-mining impacted- and non-farming areas (no evidence to suggest so at the time of sampling) adjacent to each coal mine as a reference. The designations RefX, RefY, and RefZ are used herein for such unmined “reference” areas in mine X, Y, Z, respectively (Table 3.1). Although the unmined sites sampled in 2016 were not impacted by mining, other anthropogenic activities such as road construction, farming and grazing may have occurred. This is because the mining areas span large areas and are near urban areas. Also, typical for pre-mining land areas within this coal-rich region, unmined areas (or areas marked for future mining) are used for agriculture, notably maize farming. Following the analyses of the samples collected from the reference sites in 2016, data evidence (standard deviations from mean values) showed very high variability and hinted on possible influences of stochastic factors on the reference sites. Thus, during the single-mining company soil sampling of 2017, an unmined area, cultivated during the prior planting season (September 2016 to May 2017) was sampled as the reference site (herein designated “unm-Z”) (Table 3.1).

3.2.2 Sampling and vegetation cover estimation

In 2016, soil sampling was conducted in the autumn (April-May). Whereas in 2017, samples were collected in the winter (July). In each site, bulk soil samples were collected from the 0-15 cm depth using a sterile auger. Based on-site dimensions and for obtaining a representative sample, bulk soil was sampled from sites either along (at 10 m intervals) three to five parallel 40-m transects placed 100 m apart or from five points of five systematically positioned (at least 100 m apart) crosses (see supplementary Figure 3.S1 for the two sampling schematics). For both sampling designs, soil cores (1 kg each) collected along transects (Figure 3.S1b) or at points of each cross (Figure 3.S1a) were composited. Each composite sample from each transect or crosses served as sample replicates. Soil sampling in 2017 along a soil chronosequence was performed using the transect method (Fig. 3.S1b). Composites were divided into sub-samples, passed (with the exception of soil samples for DNA analysis) through a 2-mm sieve, and immediately stored for respective analyses: frozen at -70°C for DNA based microbial community analyses, frozen at 20

°C for enzyme and physiological profiling and analysed within a week of collection, and at room temperature prior to physicochemical analyses.

For vegetation cover estimation, standard methods were followed. Briefly, the plant cover, including foliar cover, basal cover, and plant litter of each reclamation land/area was estimated using a one-square metre quadrant placed at the beginning, centre and end of each transect (Figure 3.S1a) or the centre of the crosses (Figure 3.S1b).

3.2.3 Selected soil physicochemical analyses

Selected physicochemical properties were analysed using standard methods. Briefly, pH was determined from a 1:2.5 soil suspension in 1 N KCl by using a pre-calibrated pH meter (pH 700, Eutech Instruments Pte Ltd, Singapore). Bulk density (BD) was determined using a bulk density sampler of known core volume after overnight drying at 105°C. Exchangeable bases were determined from ammonium acetate (1 M, pH 7) soil extracts using Inductively coupled plasma - optical emission spectrometry according to the method of Schollenberger and Simon (1945), while the anions phosphate (PO_4^--P) and nitrate (NO_3^--N) were determined by the method of Sonneveld and van ven Ende (1971). Ammonium (NH_4^+-N) was determined using the ammonia-selective electrode method (Banwart et al., 1972). Organic carbon (OC) was determined by the Walkley-Black method (Walkley and Black, 1934), while the Bouyoucos method was followed to determine particle size distribution (Bouyoucos, 1962).

3.2.4 Community-level physiological profiling (CLPP) of soil microbial communities

Carbon substrates utilisation pattern in a 96-well Biolog EcoPlate (Biolog Inc., Hayward, CA, USA) was used to determine microbial community richness and evenness in soil (Garland and Mills, 1991; Habig et al., 2018). For CLPP, 10 g of soil sample was suspended in 90 ml of sterile distilled water and shaken on a rotary shaker at 250 rpm for one hour. The supernatant was further diluted (1:100 in sterile H_2O), inoculated (150 µl) into the wells and incubated at 28°C for 7 days, during which optical density (at wavelength 590 nm) measurements were taken twice daily to determine

the average colour development within each well (Habig et al., 2018). Optical density values were normalised prior to computing Shannon-Wiener index of diversity (*H´*) and evenness index (*J´*) based on the different number of substrates utilised and relative intensity as described by Habig et al. (2018). Because differences in CLPP-based microbial community diversity indices between soil sample groups (site and soil history groupings) in 2016 were not significant, further analyses were not performed on the samples collected in 2017.

3.2.5 Determination of soil enzyme activities

Enzyme assays were used to estimate the functional activities of soil microbial communities. The enzymes beta-glucosidase, acid- and alkaline- phosphatases and urease are involved in the mineralisation of carbon, phosphorus and nitrogen, respectively (Eivazi and Tabatabai, 1977; Tabatabai and Dick, 2002). Additionally, for the soil samples collected in 2017 (it was not possible to determine dehydrogenase activity on the 2016 samples), determination of soil dehydrogenase activity was performed in other to determine the overall soil microbial activity in post-coal mining reclamation areas and unmined reference soil. Beta-glucosidase, acid- and alkaline-phosphatases) were determined as described by Dick et al. (1996) while urease activity was determined according to Kandeler and Gerber (1988). Dehydrogenase activity was determined using iodonitrotetrazolium violet-formazan solution as described by Von Mersi and Schinner (1991). Soil enzymatic activities were computed from extrapolations made from the standard curves of appropriate references (Kandeler and Gerber, 1988; Dick et al., 1996).

3.2.6 High-throughput sequence analyses

3.2.6.1 DNA extraction

DNA was extracted directly from 0.25 g of soil sample using the Power Soil DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The integrity of the extracted DNA was verified on 0.5% agarose gel electrophoresis. Furthermore, the concentration of extracted DNA was quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) and normalised to equimolar concentrations (5 ng/µl) using 0.1M Tris-HCl (pH 8.5) as indicated in the

Illumina MiSeq 16S metagenomic sequencing library preparation protocol (Illumina, n.d.). DNA was stored at -20°C prior to PCR amplification.

3.2.6.2 16S rRNA gene and ITS2 library preparation

Bacterial and fungal communities were investigated using next-generation sequencing on the Illumina MiSeq platform. In the 2016 multi-site sampling, only bacterial communities were investigated. Whereas, the 2017 study along the chronosequence in mine Z involved analyses of both bacterial and fungal communities. The inclusion of fungal communities in 2017 was to investigate, in addition to bacterial communities, how fungal communities are differentiated along the site-specific chronosequence. Compared to bacterial communities, the diversity and ecological guilds of fungal communities in such soil ecosystems are generally underexplored.

For bacterial and fungal community analyses, the partial 16S rRNA gene (hypervariable V3-V4 region) (approx. 460 bp) and internally transcribed spacer 2 (ITS2) regions (approx. 290 bp) were used as the marker regions, respectively. For amplification of the partial bacterial 16S rRNA gene, primers 341F (5-CCTACGGGNGGCWGCAG-3) and 805R (5'-GACTACHVGGGTATCTAATCC-3') primers (Klindworth et al., 2013) were used, whereas for the amplification of the fungal ITS2 region, primers ITS3 (5'-CAHCGATGAAGAACGYRG-3') and ITS4 (5'-TTCCTSCGCTTATTGATATGC-3') (Tedersoo et al., 2014; White et al., 1990). Illumina forward (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and reverse (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) overhang adapters were attached to the 5-end of forward and reverse primers, respectively (Illumina, Inc., CA, USA). The PCR amplification and library preparation were exactly as described in the Illumina library protocol (Illumina, n.d.). Briefly, each 25 PCR reaction included 12.5 µl of 2 x KAPA HiFi HotStart ready mix (KAPA Biosystems, Massachusetts, USA), 0.2 µM of each forward and reverse primers, 12.5 ng DNA template and PCR-grade water. The PCR conditions for 16S rRNA gene was an initial denaturation of 95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final elongation step at 72°C for 5 min. For amplification of the ITS2 region, the PCR conditions

were an initial denaturation of 95°C for 3 min, 15 cycles of 95°C for 30 s, 53°C for 30 s, 72°C for 30 s, 15 cycles of 95°C for 30 s, 65°C for 30 s, 72°C for 30 s, and a final elongation of 72°C for 5 min. The integrity of PCR amplicons was verified on 1.5% agarose gel electrophoresis prior to further library preparation steps, including PCR clean-ups, index PCR, library quantification, normalisation, denaturing and loading onto the Illumina flow-cell (Illumina Inc, CA, USA). A paired-end (2 x 300 bp) sequencing of the partial 16S rRNA gene and ITS region was performed on the Illumina MiSeq sequencer using the Nextera v3 kit (Illumina, Inc., San Diego, CA, USA) at the Agricultural Research Council-Biotechnology Platform, Pretoria, South Africa.

3.2.7 Bioinformatics

3.2.7.1 16S rRNA gene analysis

Sequence reads were demultiplexed and trimmed of barcodes using the MiSeq Reporter software (Illumina Inc, San Diego, CA, USA). Demultiplexed reads were quality checked using FastQC (v. 0.11.5, Babraham Bioinformatics, UK). Thereafter, quality trimming was performed using Trimmomatic software (v. 0.36) (Bolger et al., 2014), eliminating poor quality trailing and leading nucleotide sequences from both forward and reverse reads. Reads with an average quality score (Phred, Q) less than 20 and read length less than 250 bp were further eliminated. For assembly, quality-trimmed forward and reverse reads were assembled and filtered for ambiguous bases ("N") and spurious length (assembled read length 420 bp ≥ L ≤ 466 bp) by using the Simple Bayesian algorithm and a threshold of 0.7 in PANDASeq software (v. 2.10) (Masella et al., 2012). Operational taxonomic units (OTUs) were clustered at 97% 16S rRNA gene or ITS2 sequence similarity in QIIME software (v. 1.9.1) (Caporaso et al., 2010) by using both "open reference picking" (for taxonomic diversity analyses) and "closed reference picking" (for predicted functional profiling or ecological guild) strategies against the SILVA rRNA database (release 132) (Quast et al., 2013) with usearch61 reference (Edgar, 2010; Edgar et al., 2011) which eliminate chimeras. The OTU count tables (for closed and open reference OTU picking strategies) were depleted of singletons and non-target phylotypes (e.g. archaea, and unassigned to domains) prior to single

rarefaction (normalisation) to an even depth across samples. Thereafter, alpha and beta diversity analyses were performed in QIIME software and/or R software (v. 3.5.3) (R Core Team, 2013).

3.2.7.2 ITS2 sequence analyses

Fungal ITS sequences were analysed using the automated PIPITS pipeline for ITS sequences (Gweon et al., 2015). PIPITS accept demultiplexed pair-end reads as input and outputs an OTU count table and a Ribosomal database project (RDP) (Cole et al., 2014) taxonomic table in three steps. Firstly, the paired-sequence reads are joined across overlapping regions and quality-filtered using PEAR (Zhang et al., 2014) and FASTX-Toolkit (Gordon and Hannon, 2010), respectively. Secondly, the ITS2 region of the quality filtered merged reads are extracted (i.e. none ITS2 flanking sequences are removed) using ITSx (Bengtsson-Palme et al., 2013). In the last step, sequences are binned into OTUs at 97% similarity, depleted of chimeras using the UNITE UCHIME reference data set (Kõljalg et al., 2013; Nilsson et al., 2015) and then taxonomically assigned using the RDP Classifier (Wang et al., 2007) against the UNITE fungal ITS reference dataset (Kõljalg et al., 2013; Gweon et al., 2015). The resulting chimera-free OTU count tables were depleted of singletons and rarefied to an even depth across samples prior to computing alpha and beta diversity measures.

3.2.8 Nucleotide sequence accession numbers

All sequence reads for 16S rRNA gene and ITS2 generated for the work reported in this chapter are available in the sequence read archives (SRA) of the National Centre for Biotechnological Information under the SRA accession number PRJNA526293 (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA526293).

3.2.9 Prediction of the functional metagenomic profile of bacterial communities and ecological guilds of fungal OTUs

The functional metabolic profile (enzyme-coding genes) of soil bacterial community was predicted by using the Tax4Fun package (Aßhauer et al., 2015) of R software. Tax4Fun transforms the SILVA-based OTU count table into functional or metabolic profiles by using normalised 16S rRNA copy numbers and a set of pre-computed metabolic reference profiles based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) (Aßhauer et al., 2015). The functional prediction utilising 16S rRNA genes was performed in terms of the KEGG Orthology (KO) terms. To investigate if specific soil bacterial community functions are influenced by site and soil history, we focused on a subset of the KO terms involved in the metabolism of key soil nutrients such as carbon, sulphur, phosphorus and nitrogen. Ecological guilds of fungal species were determined using FUNGuild (http://www.stbates.org/guilds/app.php; Nguyen et al., 2016). FUNGuild utilises available records of the ecological guild and trophic modes of fungal species to parse or assign ecologically meaningful categories to OTUs/species present in the OTU count table (generated by PIPITS) (Nguyen et al., 2016).

3.2.10 Statistical analyses

Except stated otherwise, all statistical analyses were performed in R software (v. 3.5.3). Data (physicochemical properties, and physiological-based) was transformed to meet the normality and homogeneity of variance criteria for parametric tests by applying either $\log_{10}$, square-root or sin transformations. Where normality could not be achieved through transformation, non-parametric tests were used for the statistical comparison. Significance for all tests was set at probability (P) < 0.05.

For the multi-site study of 2016, statistical analyses of physiological and bacterial diversity data were performed under a nested mixed effect model. Because it was not possible to obtain replicate sites with the same age of reclamation within sites, the experiment design did not include age as a main factor but rather as a random variable. For normally distributed data, a linear mixed

model with restricted maximum likelihood estimation method was performed in the “lm4” package of R (Bates et al., 2007). Whereas, for non-normally distributed data, a generalised linear mixed model with a Gaussian log-link function and a penalised quasi-likelihood estimation method was performed using the “glmmPQL ()” function in the “MASS” package of R software. In the mixed models, “site” and “history” (reclamation vs. unmined) were set as fixed factors while age (coded as a categorical variable in which same values were assigned to unmined soils and different values assigned for each reclamation site) was set as a random variable nested within “history”. Model assumptions were confirmed by inspecting residual plots, while pair-wise posthoc tests were performed by using the “Tukey” adjustment in the “emmeans” package (v. 1.3.5.1) of R software. For the 2017 within-site sampling, statistical analysis was performed using either a one-way ANOVA or the non-parametric one-way Kruskal-Wallis H test depending on normality distribution of data.

The relationship between soil physicochemical parameters and physiological data (enzymes, carbon substrate utilisation-based microbial diversity indices) were tested by Pearson correlation on pairs of normalised data, while Spearman rank correlation was used where one of the pair of continuous variables did not assume a normal (Gaussian) distribution after transformation efforts. Multivariate analyses of microbial communities (97% 16S rRNA gene similarity OTUs), were based on relative proportions of OTU counts within a sample. The relative proportions were further log-transformed (log (x) + 1, only where x > 0) (Anderson et al., 2006) by using the “decostand ()” function in the vegan package (v.2.54) (Oksanen et al., 2019) of R software. Visualization of community structure in multivariate space was performed using a non-metric dimensional scaling and an unweighted pair-group method with arithmetic mean (UPGMA) by using the “metaMDS ()” and “hclust ()” functions in R software.

Test for differences between sample groups in multivariate space was performed on the Bray-Curtis dissimilarity using permutational multivariate analysis of variance (PERMANOVA). Permutational test for homogeneity of multivariate dispersions (PERMDISP) was also performed

to test homogeneity of multivariate dispersions of microbial species within groups. Post hoc test for significant PERMANOVA ($P < 0.05$) were further performed using the "pairwiseAdonis ()" function (Martinez, 2019) in the vegan package. To detect differentially abundant phylotypes between soil sample groups, the Linear Discriminant Analysis (LDA) Effect size (LEfSe) (Segata et al., 2011) was performed. Significantly different phylotypes [Mann-Whitney U test (for two groups, reclamation versus reclamation for 2016 sampling) or Kruskal-Wallis (greater than two groups, for 2017 sampling), $P < 0.05$, least discriminant analyses (LDA) score > 2] between sample groups were further visualized in an annotated cladogram using the GraPhlAn software (Asnicar et al., 2015) or as bar plots using the web-based Microbiome Analyst tool (www.microbiomeanalyst.ca) (Dhariwal et al. 2017).

Lastly, to elucidate which soil physicochemical parameters best explain variations in microbial community composition across sites and along the chronosequence, a canonical correspondence analysis (CCA) was performed using an automatic stepwise model selection ("ordistep ()") in the vegan package. For CCA, log-transformed physicochemical data and bacterial species composition relative counts (>1% at the genus-level) were used. Non-significant environmental variables with high multicollinearity (Variance inflation factor > 10) to one or more variables were excluded from the final plot.

3.3 Results

3.3.1 Soil physicochemical properties across sites and within post-mining reclamation chronosequence

The texture of reclamation soils was largely characterized as sandy-clay-loam, while the reference soils were characterized as sandy-loam (Table 3.2). On the average, pH ranged from 4.41 to 5.86 in reclamation soils, and from 4.52 to 7.22 in reference soils. Organic matter (OM) ranged from 3.18 - 3.84% in reclamation soils and from 4.20 to 9.07% in reference soils while cation exchange capacity (CEC) ranged from 3.88 - 5.84 cmol (+) kg^{-1} in reclamation soils, and from 4.46 to 11.83 cmol (+) kg^{-1} in reference soils (Table 3.2). Pair-wise comparisons revealed significantly (Tukey

HSD, $P < 0.05$) higher bulk density (BD) in reclamation soils compared to respective unmined soils at all sites. Significant (Tukey HSD, $P < 0.05$) differences in pH were only observed between reclamation soil and reference soils on site Y. Differences in NO_3^--N, PO_4^{3-}-P, K, Cl^-, electrical conductivity (EC) and OM between reclamation and unmined soil were not significant (Tukey HSD, $P > 0.05$) in all sites (Table 3.2), whereas, differences in Ca, Mg CEC, particle size were significant (Tukey HSD, $P < 0.05$) between reclamation soil and reference soils at one or more sites. Generally, reclamation soils were more acidic, more compacted (inferred from BD measurement), lower in OM, CEC and EC compared to their respective unmined reference soils (Table 3.2).

Physico-chemical and plant cover estimations for reclamation areas sampled along the chronosequence in site Z (in 2017) are presented in Table 3.3. Textural classification of reclamation soils was largely sandy-clay loam soils, with the oldest reclamation site (ReclZ-24) and unmined reference soil (unm-Z) categorized as sandy-loamy (Table 3.3). ReclZ-15 was characterized as loamy fine sand (Table 3.3). The mean pH of soils was acidic (4.03 – 4.89) in both reference and unmined soils (Table 3.3). With the exception of bulk density and foliar cover, all soil physicochemical parameters and plant cover estimations were significantly different (Fisher's LSD, $P < 0.05$) (Table 3.3). In particular, the nitrate (NO_3^--N) content was significantly higher in the unmined reference compared to all reclamation areas. Of all reclamation areas, ReclZ-24 had significantly ($P < 0.05$) lower chemical properties compared to the unmined area in terms of pH, N-forms (NO_3^--N and NH_4-N), P, K, Ca and Mg (Table 3.3). Similarly, ReclZ-15 had significantly lower pH, N forms (NO_3^--N and NH_4-N) K, Ca and Mg content compared to the unmined area. Peak values of most minerals across the chronosequence were observed in the sandy clay loam soils of ReclZ-19 (Table 3.3).

Table 3.2: Selected physicochemical properties of soil samples collected from site X, Y and Z in 2016

Soil properties	Site X		Site Y		Site Z	
	Recl. (0.5 yr.)	Ref.	Recl. (1.5 yr.)	Ref.	Recl. (18 yr.)	Ref.
pH	4.41±0.07[a]	4.52±0.79[a]	5.86±0.25[b]	7.22±0.22[a]	5.36±0.35[a]	5.19±0.74[a]
Moisture (%)	0.56±0.10[a]	4.52±6.29[a]	1.37±0.29[b]	2.65±0.01[a]	1.06±0.23[a]	0.68±0.08[b]
OM (%)	3.77±1.31[a]	5.10±1.91[a]	3.84±0.77[a]	9.07±3.85[a]	3.18±0.47[a]	4.20±1.11[a]
BD (g cm^{-3})	1.79±0.06[a]	1.45±0.10[b]	1.88±0.03[a]	1.68±0.09[b]	1.76±0.18[a]	1.37±0.19[b]
EC (mS/m)	4.01±0.63[a]	4.59±1.89[a]	5.12±1.52[a]	3.91±0.63[a]	4.467±2.96[a]	4.63±2.47[a]
Cl^- (mg kg^{-1})	1.05±0.28[a]	1.42±0.94[a]	0.94±0.32[a]	1.06±0.07[a]	1.04±0.49[a]	0.88±0.72[a]
NO_3^--N (mg kg^{-1})	2.03±0.47[a]	3.74±6.26[a]	0.48±0.24[a]	1.97±1.40[a]	0.73±0.14[a]	1.49±2.07[a]
NO_2^--N (mg kg^{-1})	0.06±0.02[a]	0.08±0.11[a]	0.01±0.02[b]	0.29±0.16[a]	0.05±0.03[a]	0.26±0.57[a]
PO_4^{3-}-P (mg kg^{-1})	0.16±0.08[a]	0.10±0.03[a]	0.02±0.03[a]	0.09±0.07[a]	0.05±0.09[a]	0.18±0.32[a]
Na (mg kg^{-1})	3.22±0.40[a]	4.05±1.42[a]	15.01±10.4[a]	6.67±1.84[a]	2.07±0.88[a]	7.04±8.77[a]
K (mg kg^{-1})	22.88±1.58[a]	40.56±23.35[a]	79.27±13.3[a]	75.27±14.43[a]	77.30±17.09[a]	69.50±19.5[a]
Ca (mg kg^{-1})	42.80±10.82[b]	176.48±126.0[a]	322.85±74.2[a]	1371.90±7.5[b]	237.08±45.47[a]	226.8±120.3[a]
Mg (mg kg^{-1})	10.76±2.22[b]	37.01±20.27[a]	147.03±35.5[b]	358.32±138.2[a]	57.89±32.05[a]	43.39±22.6[a]
CEC (cmol (+) kg^{-1})	5.84±0.42[a]	7.11±3.44[a]	5.42±1.31[b]	11.83±2.66[a]	3.88±0.91[a]	4.46±0.85[a]
Sand (%)	77.33±1.15[a]	74.80±6.42[a]	69.5±1.91[b]	76.00±2.0[a]	72.80±5.02[a]	76.40±1.67[a]
Silt (%)	2.67±1.15[b]	9.20±5.40[a]	6.5±1.00[a]	8.00±0.0[a]	5.20±1.79[a]	5.20±1.10[a]
Clay (%)	20.00±2.00[a]	16.00±1.41[b]	24.00±1.68[a]	16.00±2.0[b]	22.00±3.74[a]	18.40±2.2[a]
Textural class	SaClLm	SaLm	SaClLm	SaLm	SaClLm	SaLm

Values are mean ± SD (N ≥3). Recl.; Reclamation. Textural class: Sa, Sand; Lm, Loam; Cl, Clay, EC, electrical conductivity; CEC, cation exchange capacity; OM, Organic matter; BD, Bulk density. Different superscript alphabet letters for each between pairs of reclamation and reference soils per site are significantly different based on the parametric independent sample t-test or the non-parametric Mann-Whitney U test.

Table 3.3: Selected physicochemical properties and vegetation cover across a post-coal mining reclamation chronosequence and unmined soils in site Z

Site (Age)	Reclamation Chronosequence					Reference (unm-Z)
	ReclZ-3 (3 yr.)	ReclZ-11 (11 yr.)	ReclZ-15 (15 yr.)	ReclZ-19 (19 yr.)	ReclZ-24 (24 yr.)	
pH (KCl)	4.12±0.06^{cd}	4.39±0.37^{ab}	4.16±0.10^{bc}	4.69±0.41^{a}	4.03±0.04^{d}	4.89±1.030^{a}
NO_3-N (mg kg^{-1})	0.15±0.12^{bc}	0.28±0.14^{b}	0.14±0.09^{bc}	0.18±0.14^{bc}	0.06±0.08^{c}	4.49±2.64^{a}
NH_4 -N (mg kg^{-1})	3.75±1.26^{ab}	3.86±0.90^{ab}	3.23±0.50^{bc}	3.79±0.99^{ab}	1.96±0.31^{c}	4.62±0.83^{a}
Organic C (%)	0.52±0.105^{bc}	0.47±0.22^{c}	0.63±0.09^{abc}	0.66±0.10^{ab}	0.62±0.09^{abc}	0.80±0.24^{a}
P (Bray 1) (mg kg^{-1})	5.17±2.32^{bc}	3.80±1.30^{c}	13.80±12.90^{ab}	8.60±4.62^{abc}	5.00±0.82^{bc}	35.60±20.33^{a}
K (mg kg^{-1})	59.33±14.25^{b}	62.80±18.19^{b}	36.80±6.53^{c}	85.40±18.47^{a}	54.00±12.03^{bc}	103.20±27.69^{a}
Ca (mg kg^{-1})	133.50±38.29^{bc}	176.80±63.11^{b}	139.20±58.43^{bc}	339.20±76.90^{a}	96.50±28.45^{c}	350.40±228.00^{a}
Mg (mg kg^{-1})	34.33±14.53^{b}	44.00±15.57^{b}	27.60±16.32^{b}	68.40±10.29^{a}	29.00±7.39^{b}	69.60±23.73^{a}
Na (mg kg^{-1})	0.50±1.22^{ab}	2.20±2.17^{a}	1.20±1.64^{ab}	2.40±1.34^{a}	2.25±1.50^{b}	0.00±0.00^{b}
†S-value (cmol(+)/kg)	1.11±0.33^{bc}	1.42±0.49^{b}	1.02±0.44^{bc}	2.49±0.44^{a}	0.87±0.22^{c}	2.59±1.36^{a}
BD (gcm^{-3})	1.63±0.07^{a}	1.58±0.22^{a}	1.53±0.17^{a}	1.62±0.06^{a}	1.49±0.11^{a}	1.61±0.15^{a}
Sand (%)	75.50±5.05^{bc}	77.00±3.46^{b}	85.40±3.58^{a}	68.80±4.66^{c}	77.75±3.86^{b}	79.40±6.84^{b}
Silt (%)	3.00±1.10^{ab}	2.00±1.22^{b}	2.00±0.71^{b}	4.40±0.55^{a}	3.25±0.96^{ab}	3.80±1.30^{a}
Clay (%)	21.50±4.04^{ab}	21.00±2.92^{ab}	12.60±3.21^{d}	26.80±4.66^{a}	19.00±3.46^{bc}	16.80±6.53^{cd}
Soil texture	SaClLm	SaClLm	LmFiSa	SaClLm	SaLm	SaLm
Basal cover (%)	47.40±7.52.02^{a}	28.11±12.38^{b}	29.17±20.10^{b}	24.55±6.88^{b}	31.15±9.822^{b}	-
Litter (%)	28.38±18.88^{ab}	34.44±16.47^{ab}	19.17±12.81^{ab}	16.1±10.64^{b}	1.77±1.09^{c}	-

Foliar cover (%)	65.13±21.42[a]	53.89±22.86[a]	45.00±21.68[a]	47.00±17.02[a]	58.85±16.85[a]	-

-, not determined. Sample size, N = 5. Values (means ± standard deviation) with different superscript alphabet letters across rows are significantly different ($P < 0.05$) based on the parametric Tukey's HSD or non-parametric Fisher's least significant difference. †S-value, sum of extractable Ca, Mg K and Na. BD, Bulk density; Texture: SaClLm, Sandy Clay Loam; LmFiSa; Loamy Fine Sand; SaLm, Sandy Loam.

3.3.2 Community-level physiological profiles (CLPP) and enzyme activities across sites and soil history

Differences in CLPP-based soil microbial community diversity (Shannon-Wiener index, *H´*) and evenness (species proportionality) were not significantly different amongst treatments (Wald chi-square test, $P > 0.05$) (Table 3.4). However, the highest mean *H´* was observed in site X while the lowest *H´* was observed in site Z. However, *H´* increased with increasing age of reclamation, with greater *H´* obtained in the older reclamation site (ReclZ) compared to the adjacent unmined soil (Table 3.4).

Soil beta-glucosidase activity, urease activity, alkaline- and acid- phosphatases activities were significantly influenced (Wald chi-square test, $P < 0.05$) by cross-level interaction of the fixed factors (Table 3.4). Overall, lower activities of β-glucosidase activity, urease activity, alkaline- and acid- phosphatases were observed in reclamation soils compared to corresponding adjacent reference soils in each site (Table 3.4), with the most recently re-vegetated soil (ReclX) having the significantly (Wald chi-square test, $P < 0.05$) lowest soil enzyme activities among the reclaimed soils except for Alkaline-phosphatase activity (Table 3.4). In site Y, the average activities of beta-glucosidase, acid phosphatase and urease were approximately four, fourteen and three times higher, respectively, in the reference soil compared to those of ReclY soil (Table 3.4). Furthermore, high intra-site variations (standard deviations) were observed in enzyme activities, especially for the phosphatases (Table 3.4). The trend in the mean values of all enzyme activities amongst reclamation soils was in the increasing order of ReclX < ReclY < ReclZ (Table 3.4), suggesting that enzyme activities are directly related to the age of reclamation sites.

Correlations between carbon utilization-based microbial diversity indices and soil-physicochemical properties were not significant ($P > 0.05$) (Table 3-5). However, positive correlations were observed between beta-glucosidase activity and Na content (Spearman rank correlation coefficient $r = 0.55$, $P = 0.001$) and between beta-glucosidase activity and Ca content

(r = 0.413, P = 0.045) (Table 3.5). In contrast, a significant negative correlation was observed between NO_3^- and soil urease (r = -0.45, P = 0.027). All other correlations between soil biological parameters and physicochemical properties were not significant (P > 0.05) correlations (Table 3.5).

Table 3.4: CLPP-based diversity and enzyme activities

Site	Soil group (Sample size)	CLPP-based diversity		Enzyme activities			
		H'	*J´*	ß-glucosidase (p-nitrophenol µg/g/h)	Alkaline-phosphatase (p-nitrophenol µg/g/h)	Acid-phosphatase (p-nitrophenol µg/g/h)	Urease (NH_4-N µg/g/2h)
Site X	ReclX (N=3)	1.81±1.08^{a}	0.62±0.33^{a}	66.18±18.86^{c}	148.59±54.60^{b}	869.95±153.65^{c}	5.02±1.07^{c}
	RefX (N=5)	1.86±0.51^{a}	0.79±0.08^{a}	457.14±64.42^{ab}	182.87±39.09^{b}	1603.13±48.97^{a}	21.25±7.44^{abc}
Site Y	ReclY (N=5)	1.68±0.58^{a}	0.73±0.21^{a}	175.66±74.66^{bc}	155.17±73.16^{b}	1186.49±233.67^{bc}	16.44±5.56^{bc}
	RefY (N=3)	1.91±0.20^{a}	0.69±0.07^{a}	693.83±130.37^{a}	2142.69±633.53^{a}	1437.06±378.83^{ab}	45.42±25.38^{a}
Site Z	ReclZ (N=5)	1.37±0.89^{a}	0.61±0.30^{a}	529.79±113.28^{a}	226.16±110.03^{b}	1640.53±102.21^{a}	28.95±7.26^{ab}
	RefZ (N=5)	1.36±0.72^{a}	0.60±0.28^{a}	494.92±270.51^{ab}	256.77±142.08^{b}	1536.95±170.33^{ab}	27.42±12.36^{abc}

Values (mean ± SD) followed by different superscript letters across columns are significantly different (Wald chi-square test, $P < 0.05$) based on interactions between fixed factors “site” and “history” in the mixed linear model. Differences in sample sizes are due to different sampling designs. H', Shannon-Wiener index of diversity; J´, Evenness.

Table 3.5: Correlation coefficient (r) for the association between soil physicochemical properties and physiological (enzyme activities and CLPP-based microbial diversity) data

Physico-chemical properties	Correlation coefficient (*r*)					
	Beta-glucosidase	Alk-P	Acid-P	Urease	*H'*	*J'*
pH	0.40	0.65**	0.10	0.64**	0.22	0.00
Moisture	0.44*	0.37	0.28	0.44*	0.16	0.05
Organic matter	0.06	0.19	-0.02	0.25	0.09	-0.10
Bulk Density	-0.34	-0.13	-0.38	-0.27	-0.10	-0.24
EC	-0.30	-0.44*	-0.34	-0.26	-0.22	-0.31
Cl-	-0.09	-0.32	-0.27	-0.18	-0.40	-0.09
NO_3^--N	0.02	0.18	-0.08	-0.30	0.25	0.17
NO_2^--N	0.33	0.42	-0.08	0.35	0.48*	0.20
PO_4^{3-} -P	-0.25	-0.20	-0.06	-0.43	-0.24	-0.17
Na	-0.33	0.08	-0.53**	-0.05	0.19	0.02
K	0.46*	0.21	0.42*	0.60**	-0.10	-0.19
Ca	0.56**	0.51**	0.07	0.68**	0.14	-0.13
Mg	0.35	0.48*	0.06	0.518*	0.09	-0.09
CEC	0.16	0.27	-0.36	0.10	0.29	0.06
Sand	-0.08	-0.18	0.15	-0.13	-0.25	-0.24
Silt	0.34	0.17	0.17	0.32	0.02	0.04
Clay	-0.43*	-0.37	-0.27	-0.26	-0.45*	-0.33

*Correlation is significant at 0.05 probability level. ** Correlation is significant at the 0.01 probability level. Acid-P, Acid phosphatase, Alk-P, Alkaline phosphatase.

3.3.3 Enzyme activities and relationship with soil physicochemical properties along a post-coal mining soil reclamation chronosequence

With the exception of acid phosphatases (Figure 3.3b) and alkaline phosphatase (Figure 3.3c), enzyme (beta-glucosidase, urease, and dehydrogenase) activities across the post-coal mining reclamation chronosequence in Figure 3.2 were significantly different ($P < 0.05$) (Figure 3.3). Significantly higher ($P < 0.05$) urease activity was observed in the unmined soil compared to soils of the reclamation areas, whereas, beta-glucosidase activities of ReclZ-11, ReclZ-15 and ReclZ-24 were significantly lower than those of the unmined area (Figure 3.3a). Dehydrogenase activity in ReclZ-15 was significantly different from those of the unmined area (Figure 3.3e). The highest variation in alkaline-phosphatase was observed in the unmined area and may be related to high variation (standard deviation) in its P content compared to other soil (Table 3.3). Additionally, the significantly highest urease activity in the unmined area may be related to the generally higher N-forms in the unmined soil compared to other soils (Table 3.3 and Figure 3.3).

All the enzyme activities were significantly ($P < 0.05$) correlated with organic C and K (Table 3.6). Other soil properties which were significantly correlated with most (four of the five) of the enzyme activities included silt content, P, Ca and Mg. pH was positively correlated with alkaline phosphatase ($r = 0.565$) and dehydrogenase ($r = 0.398$) activities. Bulk density was significantly correlated ($r = -0.45$) with only alkaline phosphatase activity, while nitrate was significantly positively correlated ($r = 0.399$) with urease solely (Table 3.6).

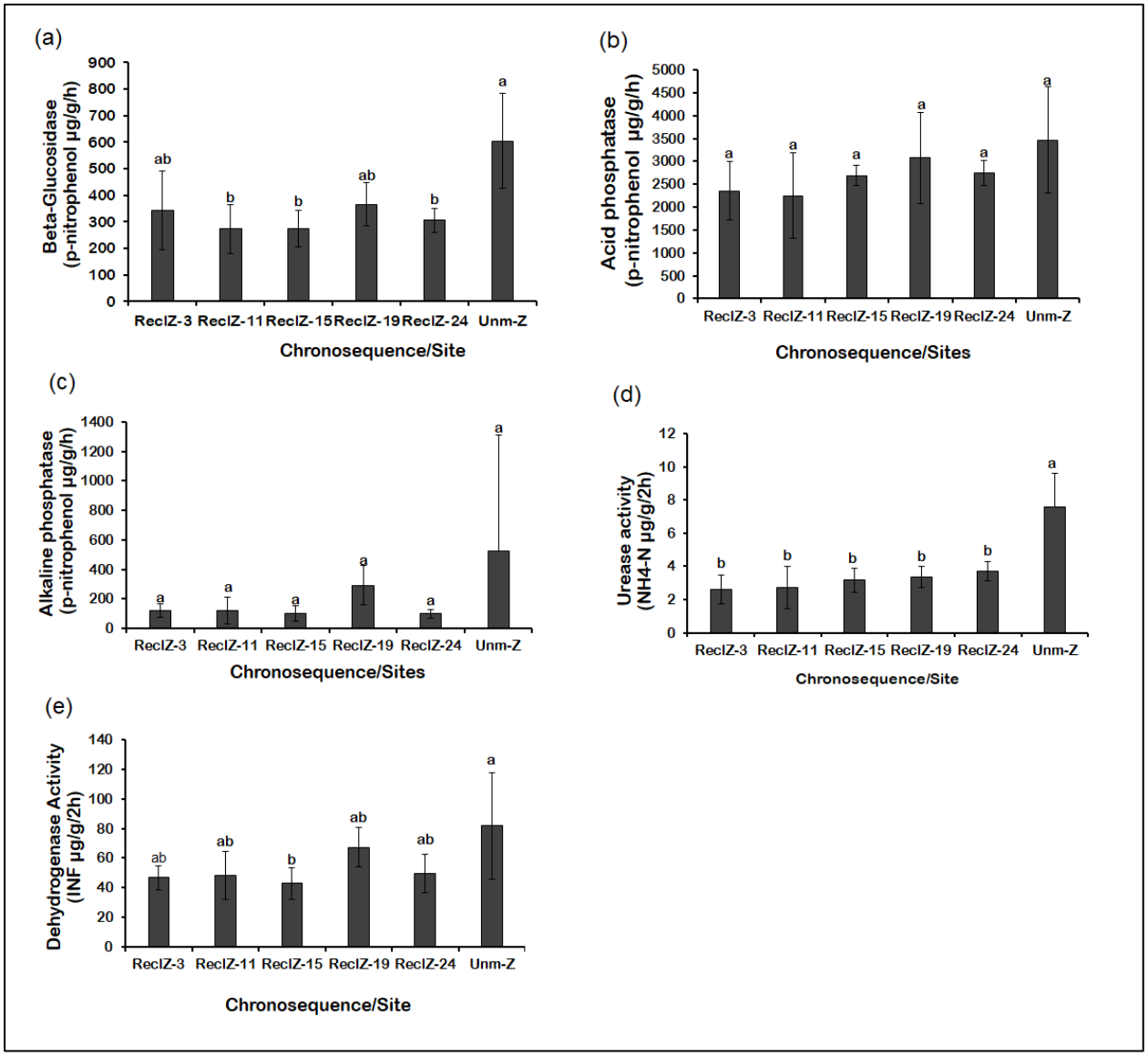

Figure 3.3: **Enzyme activities in post-mining soil chronosequence and unmined site. (a) Beta-glucosidase.** (b) Acid-phosphatase. (c) Alkaline-phosphatase. (d) Urease. (e) Dehydrogenase. Bars (mean ± SD) with different superscript letters are statistically different (ANOVA, $P < 0.05$).

Table 3.6: Correlation coefficient for the relationship between enzyme activities and physicochemical properties of soil across reclamation chronosequence and unmined soils

Physicochemical properties	Correlation coefficient (rho)				
	Beta-glucosidase	Alk-P	Acid-P	Urease	Dehydrogenase
pH	0.32	0.57**	0.05	0.20	0.40*
Organic C	0.69**	0.44*	-0.68**	0.71**	0.70*
BD)	-0.19	-0.45**	-0.01	-0.03	-0.23
NO_3^--N	0.30	0.21	-0.11	0.40**	0.22
NH_4-N	0.28	0.19	0.08	0.21	0.34
P (Bray 1)	-0.54**	0.35	-0.54**	-0.54**	0.46*
Na	-0.21	0.31	-0.05	-0.18	0.03
K)	0.63**	0.58**	0.31*	0.48**	-0.77**
Ca	0.57**	0.70**	0.16	0.42*	0.71**
Mg	0.57**	0.75**	0.16	0.42*	0.73**
Sand	-0.07	-0.24	0.04	-0.10	-0.25
Silt	0.43*	0.45*	0.31	0.42*	0.64**
Clay	-0.02	0.17	-0.11	-0.20	-0.13

Correlations were based on the parametric Pearson or the non-parametric Spearman rank correlations, depending on the normality of the bivariates. Acid-P, Acid phosphatase, Alk-P, Alkaline phosphatase. *Correlation is significant at the probability level of 0.05; **Correlation is significant at the probability level of 0.01.

3.3.4 Diversity and community structure of soil microbial operational taxonomic units (OTUs)

3.3.4.1 OTU diversity and structure across Sites and soil-history interaction

After rarefaction to even depth of 19 500 sequences (see rarefaction curve in Figure 3.S2), the number of OTUs common to site-pairs of reference and reclamation soils was highest in site Z (Figure 3.S3). Lowest OTU richness and diversity were observed in the most recently reclaimed ReclX soil, whereas ReclZ had the highest species richness and diversity amongst the reclamation sites (Figure 3.4). Based on a mixed linear model, differences in OTU richness

(Figure 3.1a), Chao1 richness estimation (Figure 3.4b) and phylogenetic diversity (Figure 3.4d) are significant (Wald chi-square test, $P < 0.05$) only between sites (main effect of "site" averaged over soil history), with OTU richness and phylogenetic diversity values of site X significantly lower (Wald chi-square test, $P < 0.05$) than those of other sites (Figure 3.4a and Figure 3.4d). The interactions between fixed factors for Shannon-Wiener index of diversity is significant (Wald chi-square test, $P < 0.05$) (Figure 3.4c).

In multivariate space, differences between bacterial community structure (97% 16S rRNA gene similarity OTUs) were differentiated between reclamation (ReclY) and unmined soil (RefY) in site Y, but less differentiated in site X and site Z (Figure 3.5). Between reclamation soils, the bacterial community structure of ReclY and ReclZ were closely similar but jointly less similar to those of ReclX (Figure 3.5A). The community differentiation pattern observed in multivariate space (Figure 3.5A) is also supported by the hierarchical cluster dendrogram shown in Figure 3.5b. For the whole bacterial community dataset, permutational analyses of variance (PERMANOVA) revealed that the interactions between "site" and "soil history" effects (R^2 = 14.8%, P = 0.001) are significant. Whereas for the pair-wise comparisons, only differences between ReclY and RefX are significant (PERMANOVA R^2 = 51.5%, $P < 0.026$; PERMDISP P = 0.111) (Figure 3.5) (Table 3.S1). Overall, these trends in bacterial community structure suggest the influence of chronological age-related factors in driving differences among reclamation areas as well as the influence of other confounding factors that may be specific to each site or sampled area.

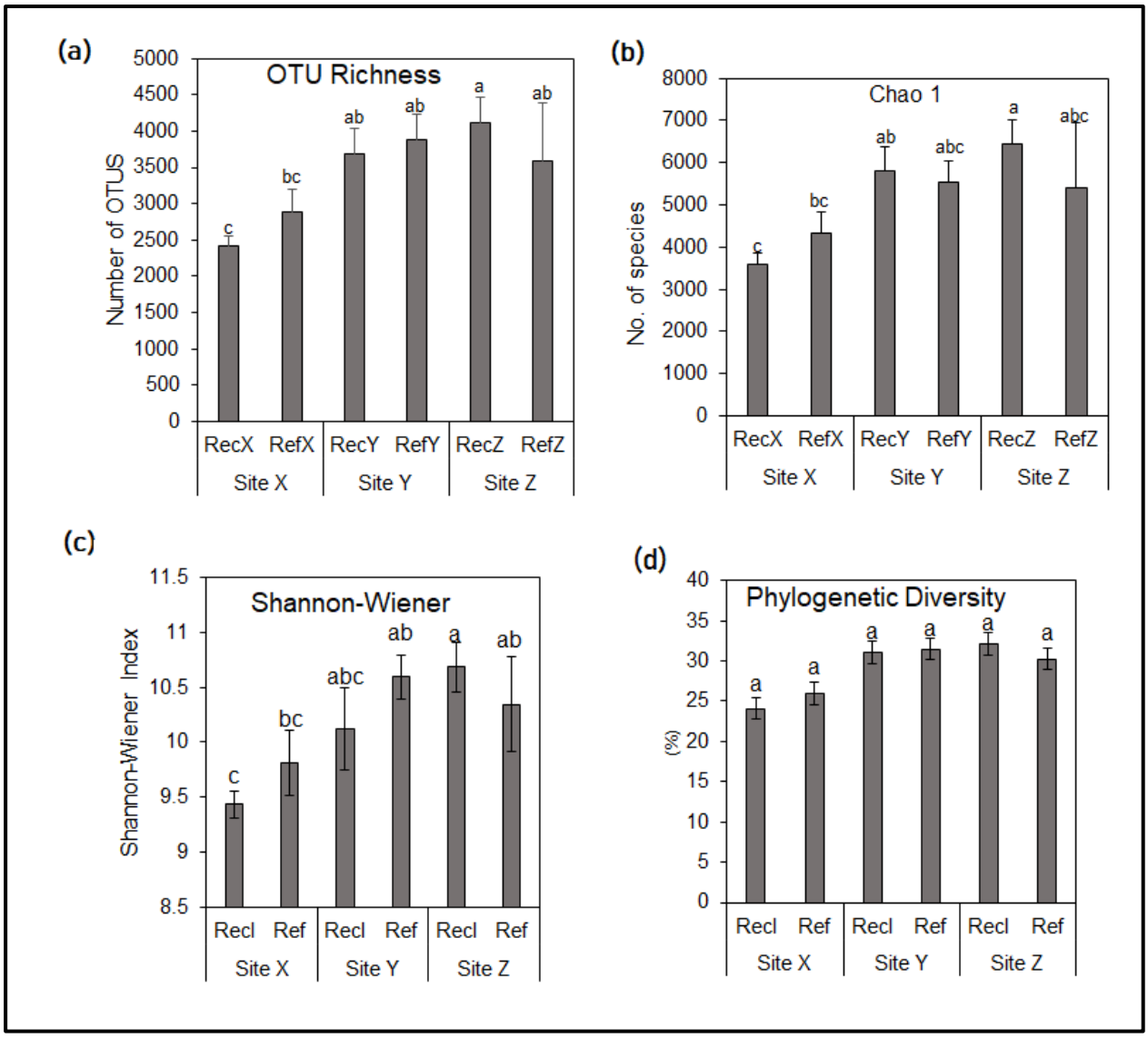

Figure 3.4: **16S rRNA-based OTU diversity indices.** (a) OTU richness. (b) Chao 1 richness estimate. (c) Shannon-Wiener index of diversity. (d) Phylogenetic diversity based on PD whole tree. Values with different superscript letters are significantly different (Wald chi-square test, $P < 0.05$) based on the interaction effect in a mixed linear model. The main effect "site" is significant (Wald chi-square test, $P < 0.05$) for OTU richness, Chao1 and phylogenetic diversity.

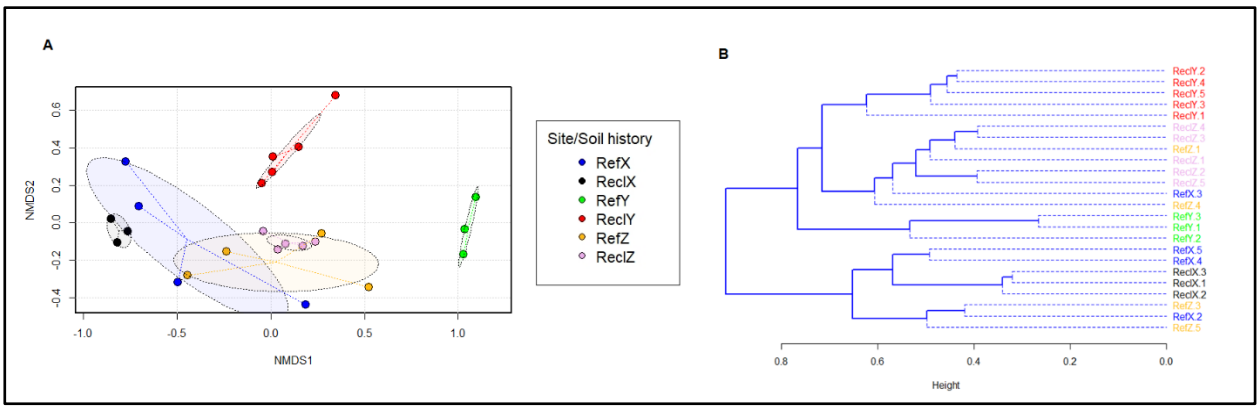

Figure 3.5: Bray-Curtis dissimilarity between bacterial communities (97% 16S rRNA gene similarity). (A) Non-metric dimensional scaling (nMDS) plot. (B). UPGMA hierarchical cluster dendrogram. Dotted lines in the nMDS plot show the distance of every sample to its group centroids in multivariate space, while ellipses show 95% confidence intervals (standard error) in multivariate space around group centroids. The stress of the nMDS plot is 0.067 (also see Figure 3.S5). Differences in multivariate space are significant for site and history interactions (PERMANOVA R2 = 14.8%, P = 0.001). See Table 3.S1 for pair-wise PERMANOVA test for reference and reclamation soils per site. nMDS and UPGMA cluster dendrograms were constructed by using the vegan (v. 2.5.5) and dendextend (v. 1.12.0) packages of R software (https://cran.r-project.org/), respectively.

3.3.4.2 OTU diversity and community differentiation along a post-coal mining reclamation chronosequence

Following rarefaction of bacterial and fungal sequences to an even depth of 7600 and 45600 sequences, respectively (Figure 3.S4), the number of observed OTUs, estimated OTU richness (Chao1) and Shannon-Wiener index of diversity (*H*') were significantly (Kruskal-Wallis, $P < 0.05$) different for the bacterial communities amongst sites (Figure 3.6). In terms of the bacterial communities, the unmined reference had the highest species richness and *H'* index compared to the reclamation areas (Figures 3.6a and 3.6b). Furthermore, bacterial species richness (observed OTUs and Chao1) and diversity in the reclamation areas ReclZ-3, ReclZ-11 and oldest reclamation area, ReclZ-24, were significantly different from those of the unmined reference

(Figure 3.5a). Overall, the trend in bacterial species richness in the reclamation areas indicated an increase over years since reclamation until a climax in ReclZ-19 (19-year-old site). In contrast, the fungal species richness and diversity were highest in ReclZ-19 compared to other sites (including the unmined area). However, the differences in these metrics amongst sites were not significant (Kruskal-Wallis, $P > 0.05$) (Figures 3.6c and 3.6d).

In multivariate space, the unmined soil was clearly differentiated from reclamation soils in terms of their microbial (bacterial and fungal) community composition and structure (Figure 3.7). The PCoA plots (Figure 3.7) provide clear insights into the pattern of shifts in the microbial community composition and structure within the reclamation area over chronological time. The microbial community structure in post-coal mining sites remain largely similar (overlapped) during the first 3 - 11 years of reclamation, changes significantly by the fifteenth year and thereafter remain stable within 15 to 19 years of reclamation (Figure 3.7). Furthermore, the homogeneity (closer associations) of sample points in Figure 3.7 suggests that within the soil landscape, fungal communities (Figures 3.7a and 3.7b) are more stable (less divergent) compared to bacterial communities (Figures 3.7c and 3.7d). Based on PERMANOVA, differences in multivariate space are significant ($P < 0.01$) for both microbial community composition and structure (Table 3.S2), with differences in the bacterial and fungal communities of ReclZ-11 and ReclZ-3 solely not significant (FDR-adjusted, $P > 0.05$) (Table 3.S2).

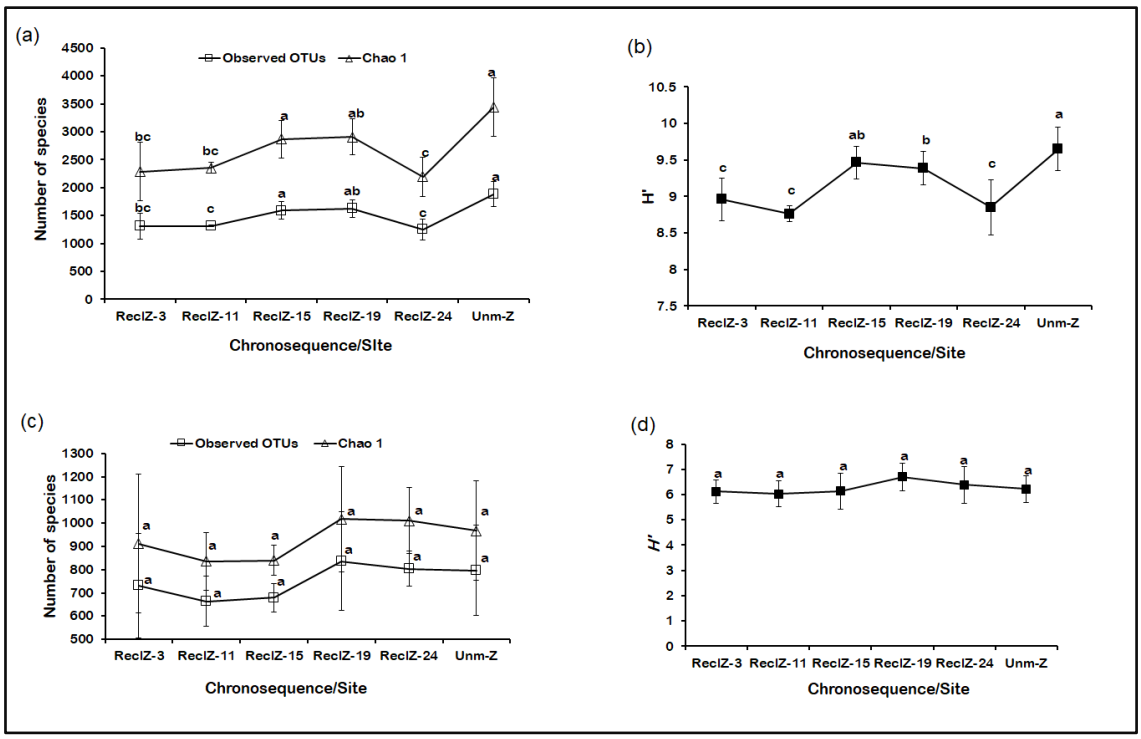

Figure 3.6: Alpha-diversity indices of bacterial and fungal diversity in post-coal mining soil reclamation chronosequence. (a) Bacterial observed 16S rRNA gene OTUs and Chao1 richness estimation. (b) Shannon-Wiener index (*H'*) of 16S rRNA gene diversity (c) Fungal ITS2 observed OTUs and Chao1 Richness estimator (d) Shannon-Wiener index (*H'*) of ITS2 sequence diversity. Values with different superscript letters are significantly different (Fisher's LSD, $P < 0.05$).

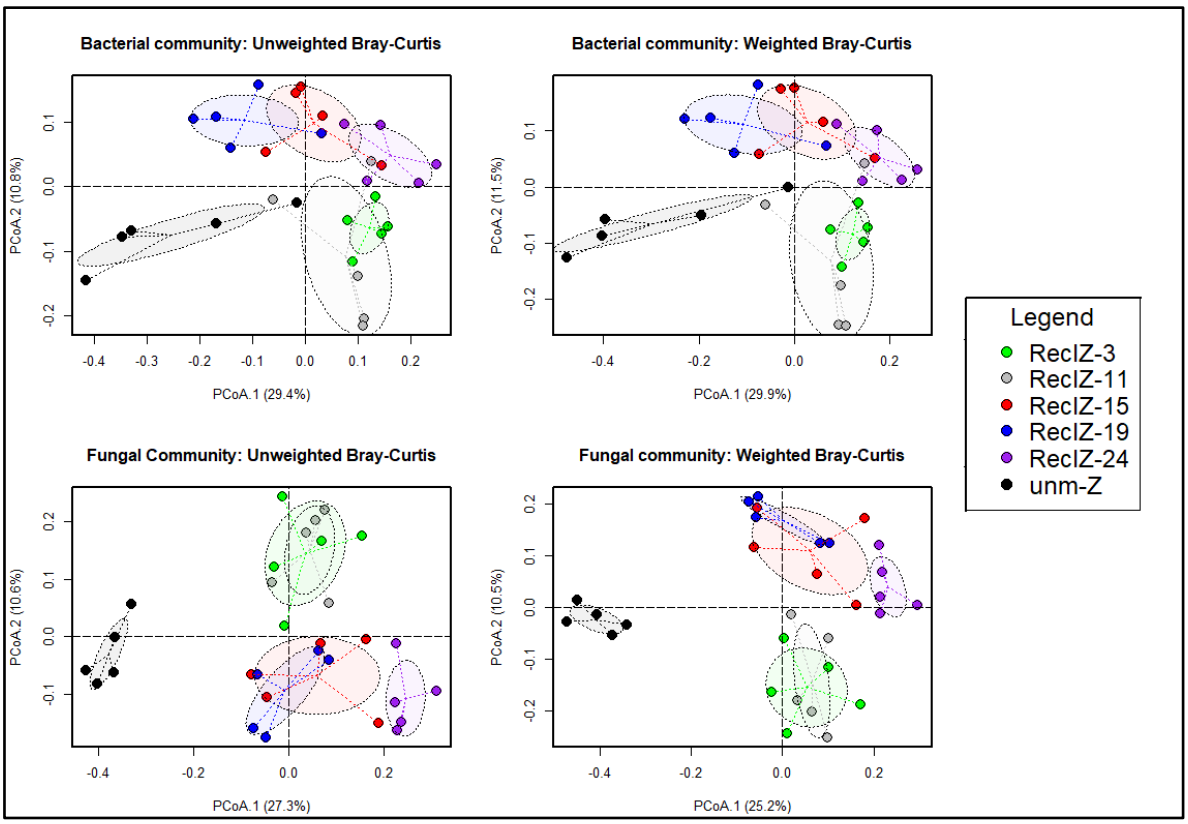

Figure 3.7: Principal coordinate analysis plot of the Bray-Curtis dissimilarity among post-coal mining soil chronosequence and unmined area. (a) Unweighted bacterial community (b) Weighted bacterial community. (c) Unweighted fungal community. (d) Weighted fungal community. Dotted lines (ordispider) in PCoA plots show the distance of every sample to its group centroids in multivariate space, while ellipses show 95% confidence intervals in multivariate space around group centroids for each reclamation area or unmined area. Differences in multivariate space are significant (PERMANOVA, $P < 0.001$) and within-group homogeneity true (PERMDISP, $P > 0.05$).

3.3.5 Dominant and differentially abundant phylotypes between reference and reclamation soils

Taxonomically, the dominant (≥ 1% relative abundance on the average) classifiable OTUs belonged to 14 phyla and 24 genera (Figure 3.8). Proteobacteria, Actinobacteria, Chloroflexi, Acidobacteria, Planctomyces, Verrucomicrobia and candidate phylum division WPS-2 were among the most relatively abundant phyla across sample groupings. The relative abundance of Firmicutes exceeded 1% only in soils from site Z (Figure 3.8A). A large proportion (30 – 48%) of 16S rRNA gene sequence were unclassified at the genus taxonomic rank (data not shown). Of the classifiable phylotypes, the genera *Acidibacter*, *Acidothermus*, *Bacillus*, *Bradyrhizobium*, *Burkholderia-Caballeronia-Paraburkholderia*, *Candidatus Udaeobacter*, *Candidatus Xiphinematobacter*, *Conexibacter* and *Sphingomonas* were relatively abundant across soils (Figure 3.8B). *Acidothermus*, *Sphingomonas* and *Candidatus Udaeobacter* were relatively most abundant in both soil types for sites X, Y and Z, respectively, (Figure 3.8B).

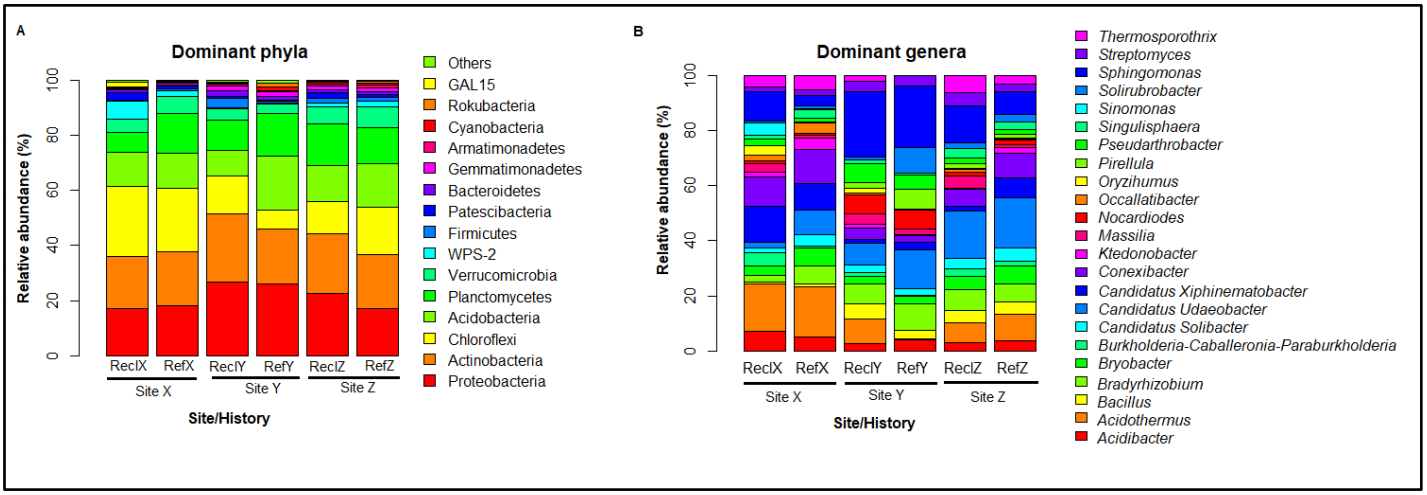

Figure 3.8: The average relative abundance of dominant (>1 %) phylotypes per site. (A) Phylum. (B) Genus. Phylotypes with less than 1% relative abundance and those unclassified at the genus taxonomic rank are excluded.

A total of 194 discriminative features (Mann-Whitney U test, $P < 0.05$, Linear discriminant analysis (LDA) score > 2) were identified between reclamation and reference soils following a sample-wide LDA Effect size (LEfSe) analysis (data not shown). The top 100 discriminative features are shown in Figure 3.9. Of these, the classifiable genus-level features differentially more abundant in reclamation soil compared to references soil include *Jatrophihabitans*, *Massilia*, *Oryzihumus*, *Segetibacter*, *Sphingomonas, Streptomyces* and *Terrabacter* (Figure 3.9 and Table 3.S3). Whereas, *Solirubrobacter* and *Pedomicrobium* were differentially more abundant in reference soil compared to reclamation soils (Figure 3.9 and Table 3.S3).

In the context of this study, phyla Planctomycetes and Candidate phylum WPS-2 were discriminant (false discovery rate (FDR)-adjusted $Q < 0.3$, LDA score > 2,) between reference and reclamation soils in both sites X and Y (Figure 3.S6A and 3. S6B), with Planctomycetes more abundant in reference soils and Candidate phylum WPS-2 more abundant in reclamation soils at both sites X and Y. The top 15 (ranked by *P*- values) differentially abundant (FDR-adjusted $Q <$ 0.1 or 0.3, LDA score > 2) features at the genus taxonomic rank for site X and Y are shown in Figure 3.10. In site X, *Sinomonas*, *Burkholderia-Caballeronia-Paraburkholderia*, *Oryzihumus*, *Rhodanobacter* and *Mucilaginibacter* were differentially (FDR-adjusted $Q < 0.1$, LDA score > 2) more abundant in ReclX compared to RefX, whereas *Bradyrhizobium*, *Bryobacter*, *Mycobacterium*, *Crossiella*, *Pseudolabrys*, Ellin6055 (Family: *Sphingomonadaceae*) and *Gemmata* were differentially more abundant in RefX compared to ReclX (Figure 3.10A). In site Y, RB41 (Family: *Pyrinomonadaceae*), *Solirubacter*, *Pedomicrobium* and *Dongia* were differentially more abundant in ReclY compared to RefY, whereas, *Acidothermus*, *Bacillus*, *Conexibacter*, *Gemmatimonas*, *Massilia*, 7703 (Family: *Ktedonobacteraceae*), *Streptomyces*, Ellin6067 (unclassified Betaproteobacteria) and FCPS473 (Family: *Ktedonobacteraceae*) were differentially more abundant in RefY compared to ReclY (Figure 3.10B). Unlike site X and Y, no differentially abundant *P*-> 0.05, LDA score > 2) phylotypes were observed between reclamation (ReclZ) and reference (RefZ) soils in site Z (data not shown).

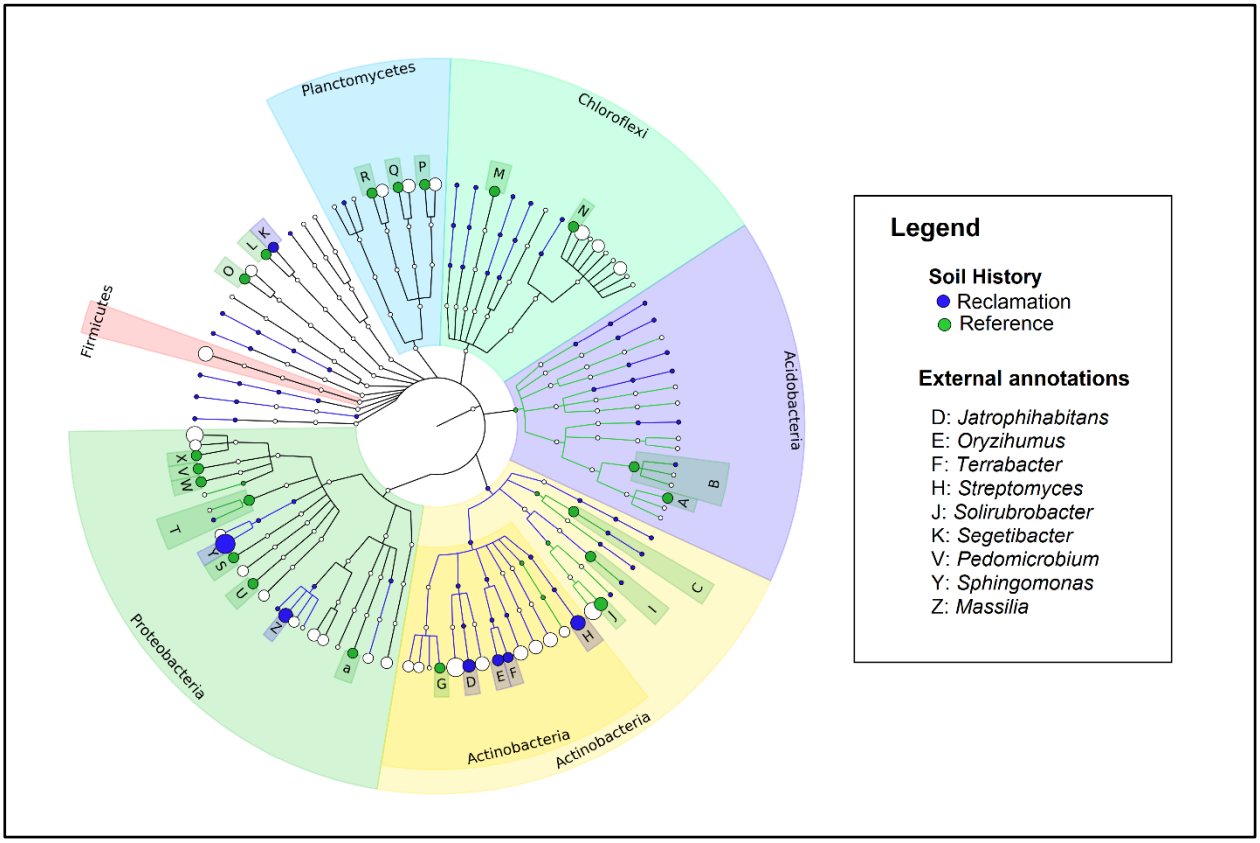

Figure 3.9: Cladistical representation of the top 100 differentially abundant (LDA >2.0, Mann-Whitney U test, *P* < 0.05) features amongst soil bacterial communities. The features are ordered based on relative abundance. Rings (from inner to outer rings) 1, 2, 3, 4, and 5 represent phylum, class, order, family and genus taxonomic ranks. Only annotations for discriminant features classifiable at the genus taxonomic rank (ring 5) are shown in the legend. See supplementary Table S3.3 for the FDR-adjusted P-values for the genus-level discriminative features. Cladogram was constructed using GraPhlAn software (v. 0.9.9).

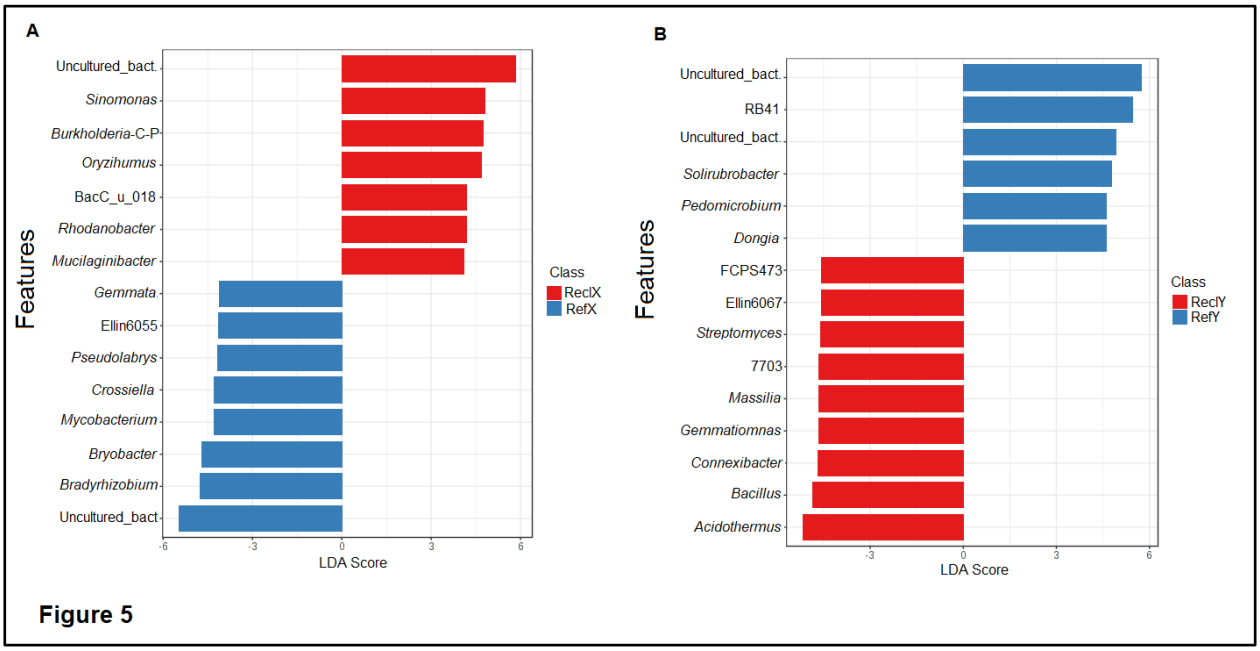

Figure 3.10: Differentially abundant genera between reclamation and reference soils. (A) Top 15 discriminant (LDA score > 2.0, FDR-adjusted *P*-value < 0.1) genera in Site X. (B) Top 15 discriminant (LDA score > 2.0, FDR-adjusted *P*-value < 0.3) genera in Site Y. Differential abundance and bar plots were determined and generated, respectively using Linear Discriminant Analysis (LDA) Effect size (LEfSe) via the web-based Microbiome Analyst tool (www.microbiomeanalyst.ca). Burkholderia-C-P, *Burkholderia-Caballeronia-Paraburkholderia*; Uncultured_bact., Uncultured bacteria.

3.3.6 Diversity and dynamics of dominant bacterial phylotypes across a post-coal mining chronosequence and unmined soil

The phylum Actinobacteria was relatively dominant (approx. 34 - 57% of sequences) throughout the reclamation soil chronosequence and unmined soil in site Z (Figure 3.11), and accounted for a majority of the potential biomarker phylotypes (Figure 3.11b). Other relatively abundant (> 1% total abundance) bacteria phyla included Acidobacteria, Chloroflexi, Deinococcous-Thermus, Planctomycetes and Proteobacteria (Figure 3.11a). Of the phyla presented in Figure 3.11a, Verrumicrobia, Proteobacteria, Parubacteria, Deinococcus-Thermus, Bacteriodetes and

candidate phylum FBP were significantly ($P < 0.05$) differentially abundant across sampled areas. The Verrumicrobia phylum was significantly different ($P < 0.05$) across soils, with significantly higher ($P < 0.05$) abundance observed in ReclZ-19 compared to the soils of younger reclamation areas (ReclZ-3 and ReclZ-11) and unmined site (unm-Z) (Figure 3.11a). In contrast, Proteobacteria was significantly lower in ReclZ-15 compared to younger reclamation areas (ReclZ-3 and ReclZ-11) and the unmined area, reflecting a pattern of decreasing abundance in later years (15 years after reclamation and upwards) compared to in the earlier stages after reclamation (Figure 3.11). Similarly, candidate phylum FBP was significantly higher in younger reclamation soils compared to older reclamation areas ReclZ-15 and ReclZ-19.

Across the chronosequence and unmined soils, 44 bacterial genera were differentially abundant (Kruskal-Wallis $P > 0.05$, LDA > 2.0) with *Jatrophihabitans*, *Amycolatopsis*, *Streptomyces*, *Roseiflexus*, *Mycobacterium*, and *Pseudonocardia* were amongst the relatively most abundant phylotypes in ReclZ-3, ReclZ-11, ReclZ-15, ReclZ-19, ReclZ-24 and um-Z, respectively (Figure 3.11b). Although dynamics (increase or reduction) in bacterial diversity were observed across the reclamation chronosequence and unmined area (Figure 3.12), only the relative abundances of *Actinoplanes* and *Bryobacter* genera were significantly different across the sampled areas (Figure 3.12). While the relative abundance of *Actinoplanes* was significantly higher in the youngest reclamation area (ReclZ-3) compared to ReclZ-15 and ReclZ-19, its abundance was not significantly different from those in ReclZ-11, ReclZ-24 and unm-Z soils (Figure 3.12 and Table 3.S4) suggesting fluctuating trends in the abundance of *Actinoplanes* across years since reclamation. Whereas the relative abundance of *Bryobacter* was significantly ($P < 0.05$) higher in ReclZ-24 than in ReclZ-19 and unm-Z (Figure 3.12 and Table 3.S4).

Based on the highest average relative abundances of phylotypes at the genus taxonomic rank (Figure 3.12), the bacterial succession pattern was: *Jatrophihabitans* and *Sphingomonas* dominated the early stages of reclamation (ReclZ-3), *Amycolatopsis* and *Singulisphaera* dominated at 11 years (ReclZ-11), *Varibacter*, *Streptomyces* and *Bradyrhizobium* were most

dominant at 15 years, *Chloroacidobacteria* RB41 at 19 years, while *Acidothermus*, *Crossiella* and *Mycobacterium* dominated at 24 years since reclamation (ReclZ-24) (Figure 3.12). *Pseudonocardia*, *Micromonospora* and *Nocardioides* were most dominant in the unmined maize field soil (unm-Z) (Figure 3.12).

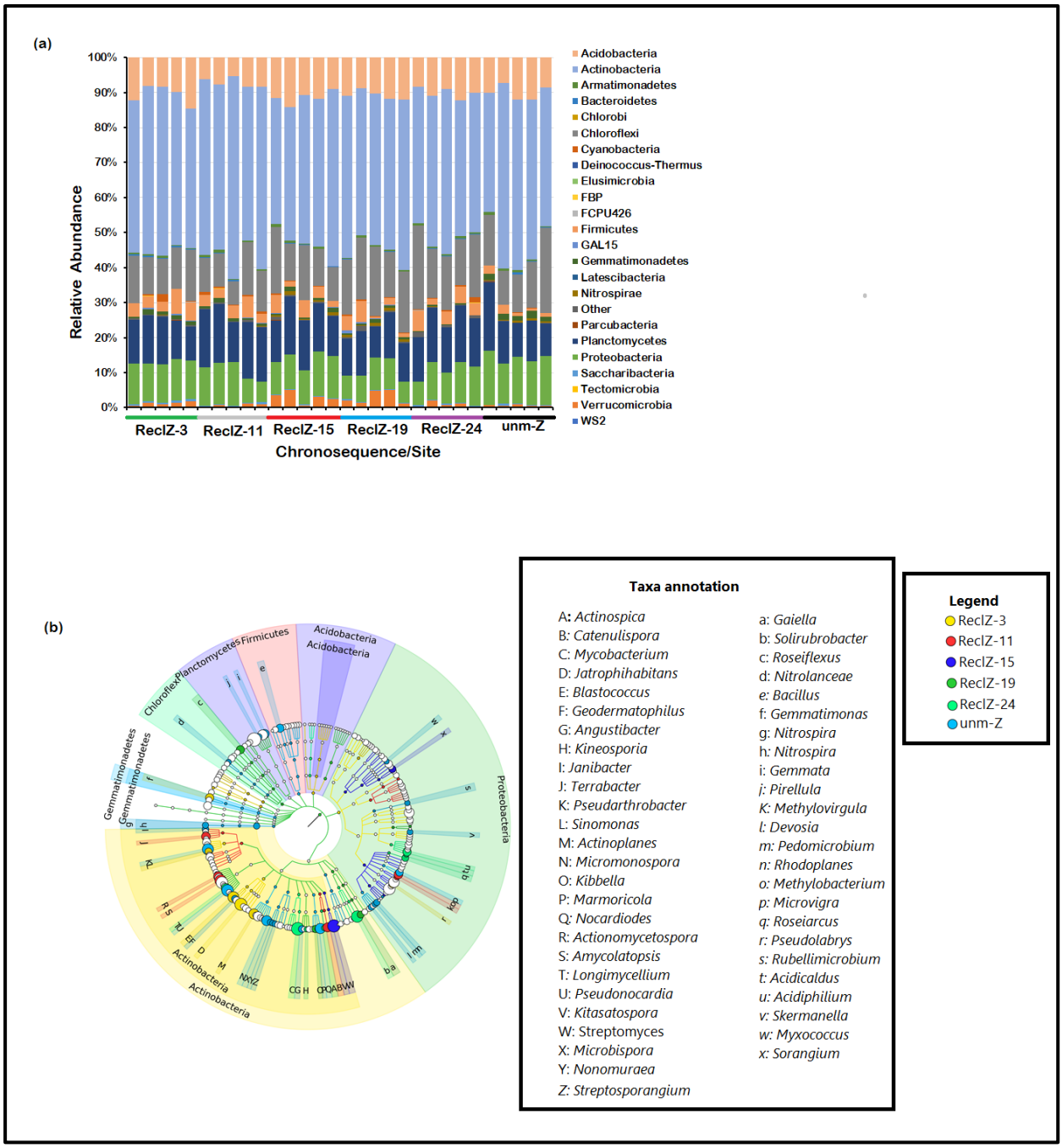

Figure 3.11: Relative abundance of bacterial phylotypes. (a) phylum taxa level (b) Cladistic representation of potential "biomarker" phylotypes across chronosequence and unmined soil. In the cladogram, rings (from inner to outer rings) 1, 2, 3, 4, and 5 represent phylum, class, order, family and genus taxonomic ranks, respectively. Only the top 200 of the 210 discriminative (Kruskal-Wallis $P < 0.05$, LDA score > 2.0) features or clades are shown. Only genus-level classifications (ring 5) are annotated and described in the legend.

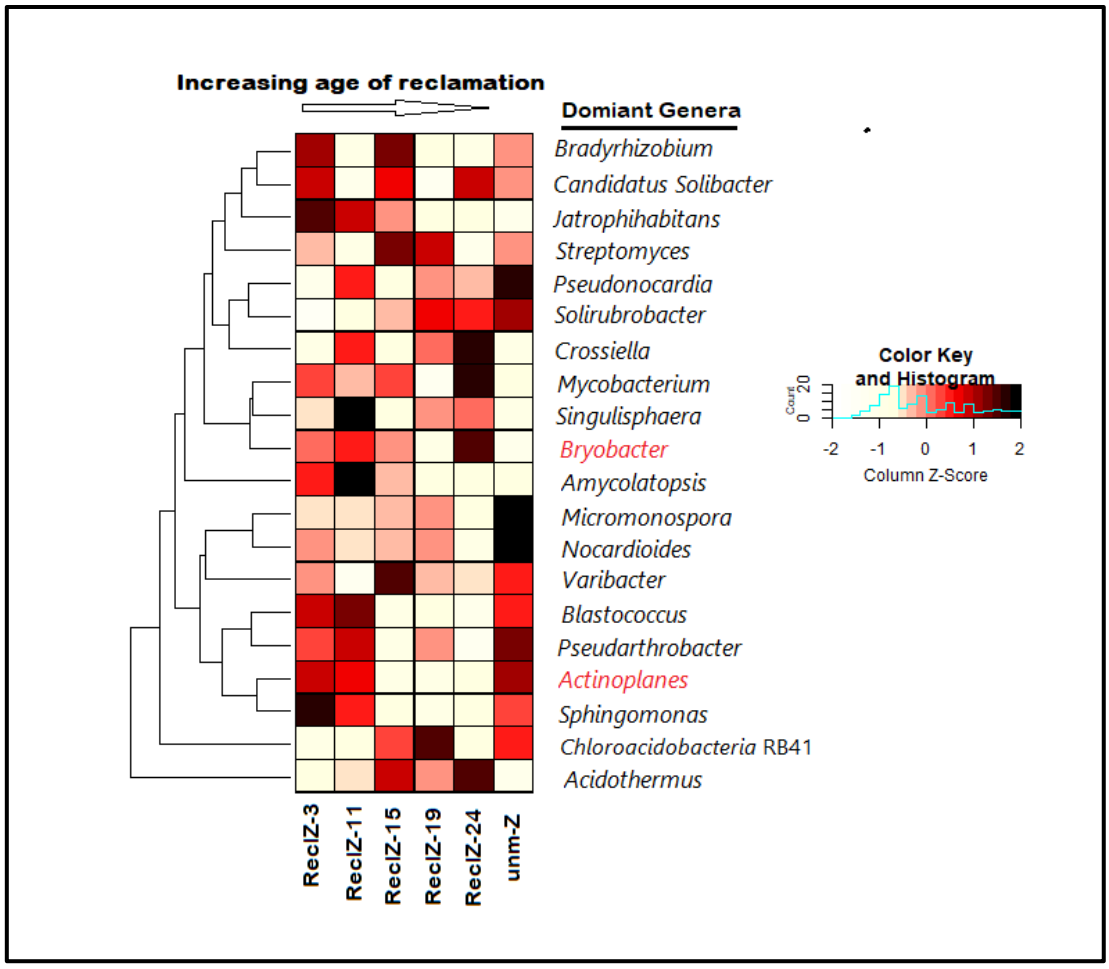

Figure 3.12: Relative abundance of dominant (≥1% average relative abundance) bacterial phylotype at the genus taxonomic rank. Unclassified phylotypes are excluded from the plot. Taxa highlighted in red are significantly (Kruskal-Wallis, $P < 0.05$) different across groups. Hierarchical cluster dendrogram connecting closely associated bacterial genera are based on average distances Colour scaling is across rows (sample groups). See also Table 3.S4.

3.3.7 Diversity, dynamics and the ecological guild of fungal community across post-mining reclamation chronosequence and unmined soil

For the fungal community, Ascomycota was the dominant (approx. 68 – 82 % of sequence) fungal phylum across the chronosequence and unmined soil (Figure 3.13) and accounted for a majority of the potential biomarker phylotypes (Figure 3.13b). Notably, a large number of the fungal ITS2 OTUs were unidentified at the phylum taxonomic rank (Figure 3.13a). Other relatively abundant

phyla (> 1% total relative abundance) included Basidiomycota. Based on differential abundance testing using LefSe, a total of 37 fungal features or clades were differentially abundant (Kruskal-Wallis $P > 0.05$, LDA > 2.0) including 15 genera (Figure 3.13b). Some of these differentially abundant fungal phylotypes in ReclZ-11, ReclZ-15, ReclZ-19, ReclZ-24 and um-Z included *Curvularia*, *Talaromyces*, *Toxicocladosporium*, *Aurebasidum* and *Cercophora*, respectively. Although one bacterial family was differentially abundant (Kruskal-Wallis $P > 0.05$, LDA > 2.0) in ReclZ-3 (see ring 4 in Figure 3.13b), no genus (or classifiable at the genus level) was differentially abundant in ReclZ-3, the youngest reclamation area.

The fungal community dynamics across the chronosequence and unmined soil indicated certain genera were more dominant over years of reclamation (Figure 3.14a). For example, Curvularia was significantly ($P < 0.05$) more abundant in the earlier years of reclamation (ReclZ-3, ReclZ-11 and ReclZ-15) compared to the later years of reclamation (ReclZ-19 and ReclZ-24) and unmined area (Figure 3.14a and Table 3.S5). Whereas, *Cercophora* was significantly more abundant in the unmined area compared to the reclamation areas (Figure 3.14a). Of the fungal genera presented in Figure 3.14a, *Cercophora*, *Coniosporium*, *Curvularia*, *Erchia*, *Endocarpon*, *Neoascochyta*, *Helicoma*, *Rasamsonia* and *Talaromyces* were significantly different across the sampled areas (Table 3.S5).

Across years since reclamation, *Neoascochyta* and *Cladophialophora* were dominant in the early stages (ReclZ-3), *Coniosporium* and *Curvularia* at 11 years (ReclZ-11), *Oidiodendron* and *Talaromyces* at 15 years, *Penicillium*, *Plendomus* and *Endocarpon* at 19 years while *Thielavia* and *Echria* were relatively more dominant at 24 years (Figure 3.14), whereas, *Helicoma* and *Cercophora* dominated in the unmined maize field soil (Figure 3.14a).

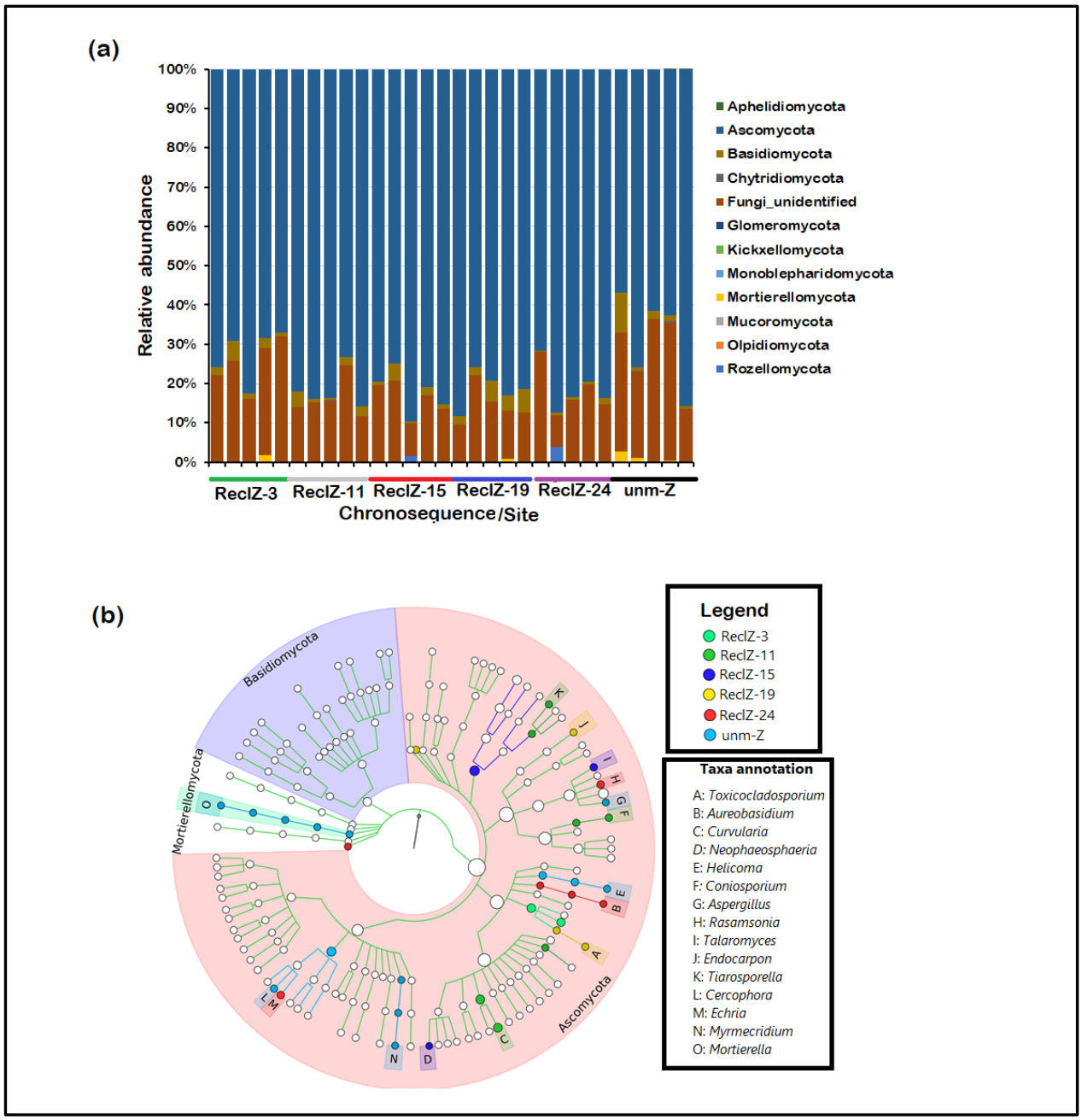

Figure 3.13: **Fungal phylotypes.** (a) Relative abundant phyla (b) Cladistic representation of potential biomarker fungal phylotypes across chronosequence and unmined soil. In the cladogram, rings (from inner to outer rings) 1, 2, 3, 4, and 5 represent phylum, class, order, family and genus taxonomic ranks, respectively. The differentially abundant (Kruskal-Wallis $P < 0.05$, LDA score > 2.0) features or clades are colour-coded by sample group in which they are more abundant. Genus-level classifications (ring 5) are annotated and described in the legend.

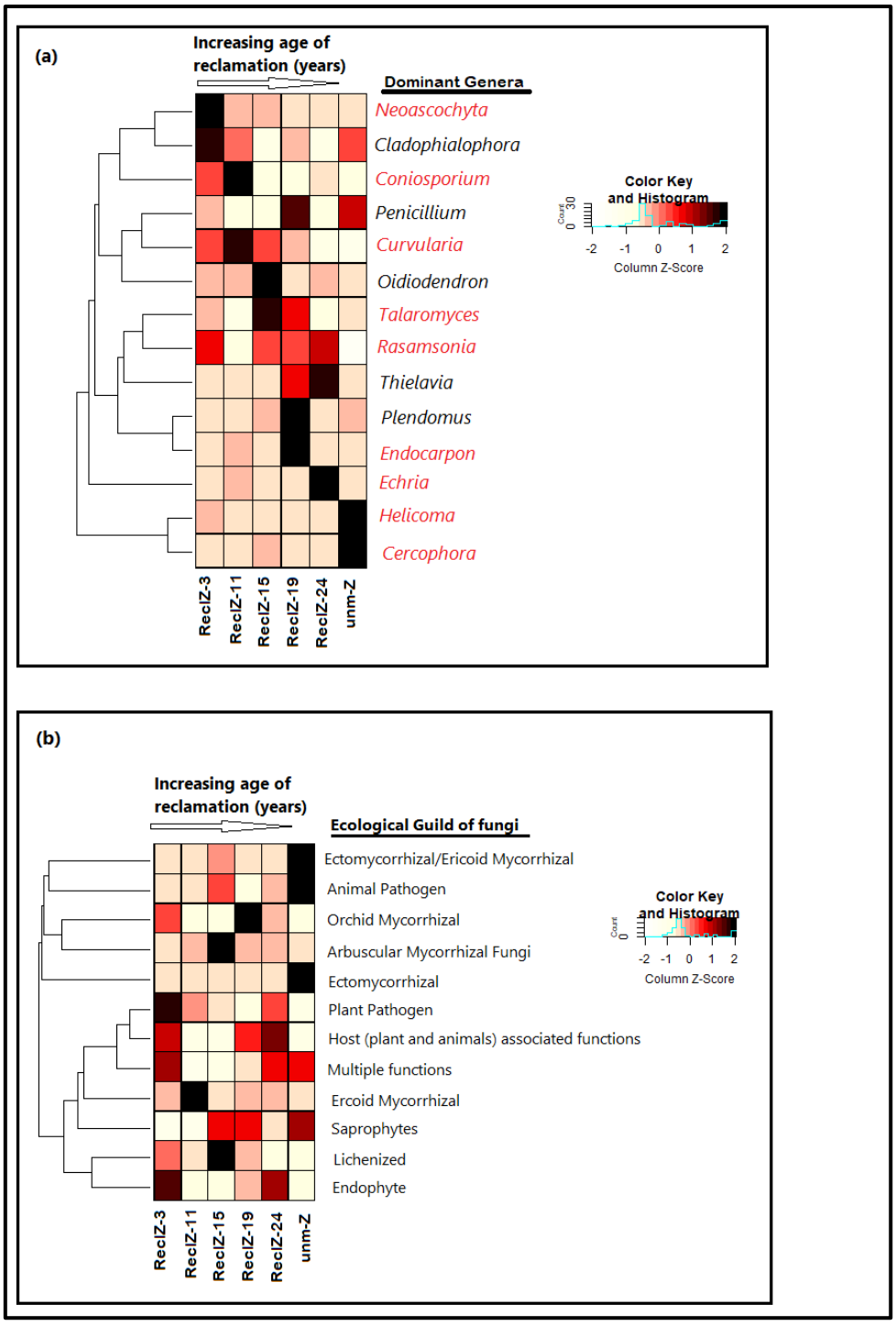

Figure 3.14: Relatively dominant (≥1% average relative abundance in at least one group) fungal genera and ecological guild. (a) Relative abundance (b) Ecological guild. Unclassified phylotypes are excluded from the plot. Genera highlighted in red are significantly (Kruskal-Wallis, $P < 0.05$) different across chronosequence or site (See also Table 3.S3). Ecological guilds assigned as "multiple functions" include fungal phylotypes that are assigned as animal pathogens, endophytes, epiphytes, plant pathogens and undefined Saprophytes.

The ecological guild of the fungal community in the post-mining reclamation soils was largely unknown (data not shown), while the majority of OTUs assigned to ecological guilds were saprotrophs (Figure 3.14b). Summarily, the ecological guild of fungi changed from dominantly plant pathogens and endophytes in the early stages of reclamation to predominantly fungal species with a host (animal and plant) -associated functions by the 24 years of reclamation. In the unmined maize field soil, ectomycorrhizal fungi, saprophytes and animal pathogens were relatively more dominant (Figure 3.14b).

3.3.8 Predicted functional diversity and differentially abundant nutrient-cycling KO terms

The average fraction of OTUs which mapped onto the Kyoto Encyclopaedia for genes and genomes (KEGG) organisms was not significantly different across soils (site and history) (Table 3.S6), suggesting that any comparisons in the diversity of KEGG orthology (KO) terms between soil groups are valid. A total of 6448 KO terms were predicted from all OTUs. The predicted KO diversity (or functional) profile of soil bacterial communities significantly differed between sites (PERMANOVA R^2 = 40.9%, P = 0.001; PERMDISP P = 0.78). However, unlike the bacterial genetic diversity, the predicted community functional diversity was less differentiated (Figure 3. S7) and was not significantly influenced by soil history (PERMANOVA R^2 = 7.91%, P = 0.045; PERMDISP P = 0.016) and interactions between site and history (PERMANOVA R^2 = 9.74%, P = 0.112). From a subset of KO terms/enzyme involved in the metabolism of carbon, phosphorus and amino acid/nitrogen-containing compounds, nine differentially abundant (FDR-adjusted P < 0.1, Indicator value > 0.6) KO terms between reclamation and reference soils were identified (Figure 3.15). Most of the differentially abundant predicted KO terms, including fructan beta-fructosidase [EC:3.2.1.80], dipeptidase [EC:3.4.13], fructose-1,6-biphosphastase III [EC:3.1.3.11] were highest in ReclY. Among reclamation soils, the youngest reclamation area (ReclX) had the least predicted abundance of the differentially abundant KOs (Figure 3.15). However, based on Bray-Curtis distances, close associations (as observed from the hierarchical cluster dendrogram) were observed between ReclX and ReclY compared to ReclZ (Figure 3.15). In addition, the oldest reclamation area (ReclZ) was similar to the adjacent reference area and other reference sites

(Figure 3.15) thereby suggesting that bacterial community functional restoration (similarity to unmined reference) are a function of chronological age since reclamation.

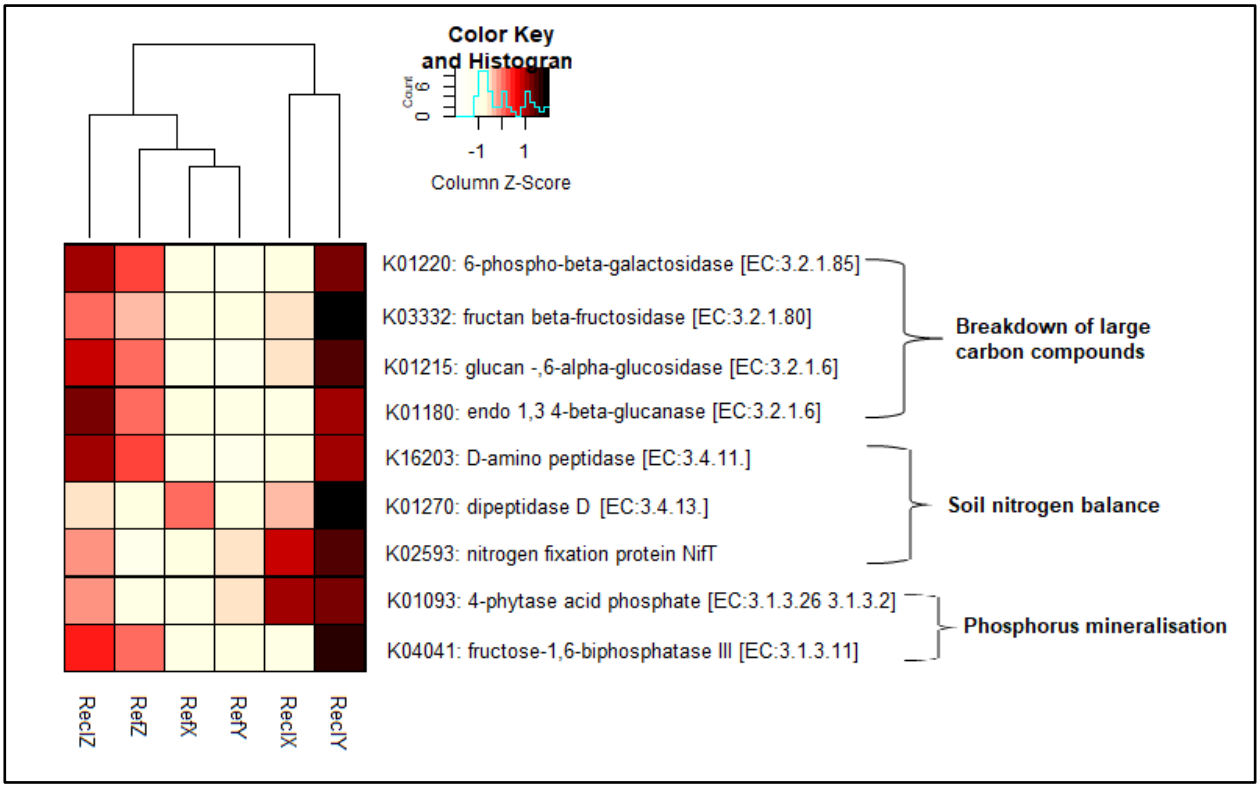

Figure 3.15: Relative abundance of differentially abundant (Mann-Whitney U test FDR-adjusted *P* < 0.1, indicator value > 0.6) KEGG Orthology terms related to carbon, nitrogen and phosphorus. Hierarchical cluster dendrogram are based on the average Bray-Curtis distances between soil groups. Relative abundance (colour key) is scaled across rows. KOs were generated from normalised 16S rRNA copy numbers and a set of pre-computed metabolic reference profiles based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/).

3.3.9 Differentially abundant predicted functions for nutrient cycling across post-coal mining chronosequence and unmined soil

The fraction of OTUs which were assigned to the KEGG orthology (KO) database was not significantly different across the sampled areas (Table 3.S7). Thus, comparisons of specific KOs or predicted functions amongst samples were valid. The predicted function profile of the bacterial

community (based on OTUs that mapped to KEGG database) was not differentiated amongst reclamation ages and/or unmined site in both unweighted and weighted measures (Figure 3.16). PERMANOVA for the sampled area were not significant (unweighted R^2 =21.7%, P = 0.222; weighted PERMANOVA R^2, P = 0.464) confirming observations made in the PCoA plot of Figure 3.16. Of the subset of predicted KO terms/enzymes involved in the catalyses of carbon phosphorus, amino acid/nitrogen and sulphur, only 6-phospho-beta-galactosidase [EC:3.2.1.85] was significantly different among samples (Figure 3.S8).

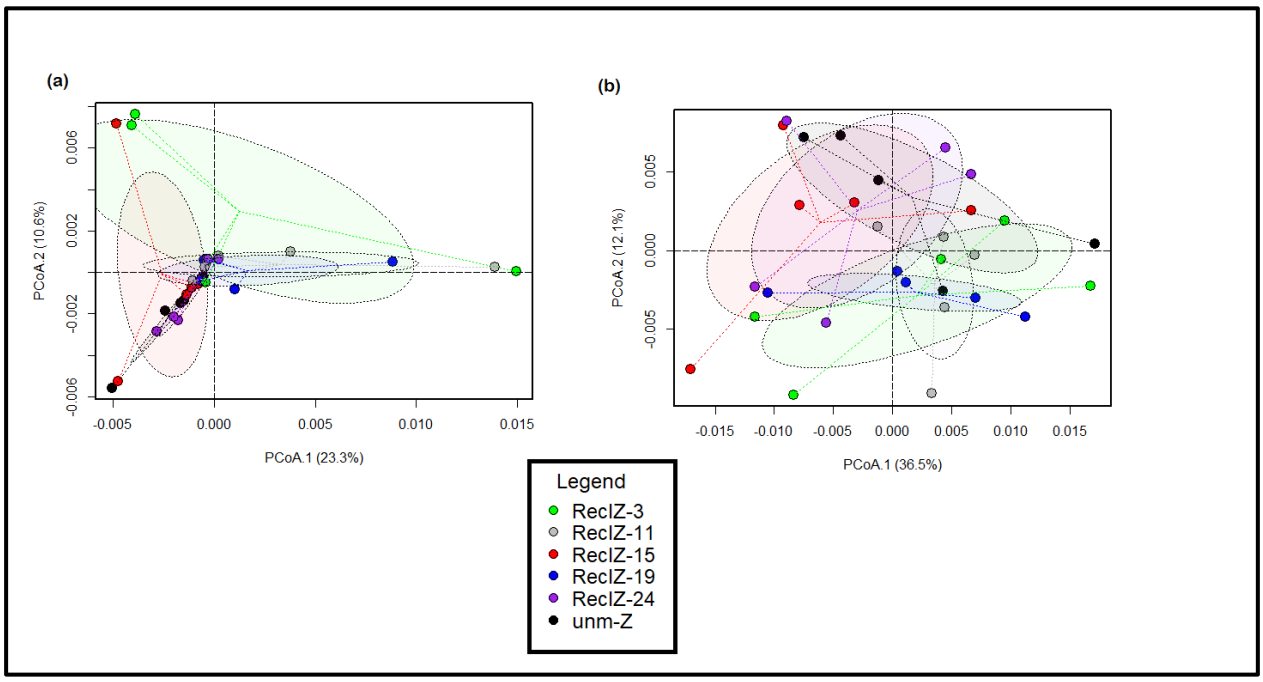

Figure 3.16: Principal coordinate analyses of predicted functional KO profile of bacterial communities. (a) unweighted Bray-Curtis dissimilarity (b) Weighted Bray-Curtis dissimilarity. PCoA were performed on KO profile for KEGG level 3 (enzyme-coding genes). Differences amongst sampled areas for both unweighted and weighted comparisons are not significant (P > 0.05) based on PERMANOVA. Dotted lines (ordispider) in PCoA plots show the distance of every sample to its group centroids in multivariate space, while ellipses show 95% confidence intervals in multivariate space around group centroids for each reclamation area or unmined area.

3.3.10 Influence of soil physicochemical properties on microbial communities

3.3.10.1 Between mining sites and soil history

The CCA model for the triplot depicted in Figure 3.17 is significant (ANOVA F = 1.61, *P* = 0.004). Soil physicochemical properties explain up to 94.3% of the total variation in the bacterial community composition across sites. Site-specific clustering, as opposed to soil history-based clustering, was observed, particularly in site Z (Figure 3.17). Of the soil physicochemical variables included in the CCA model, only silt content, BD, pH, EC, Na and Ca were significant (Table 3.S8). Overall, variations in the microbial community structure in site Y were largely influenced by differences in soil physicochemical properties, while sites X and Z were least influenced.

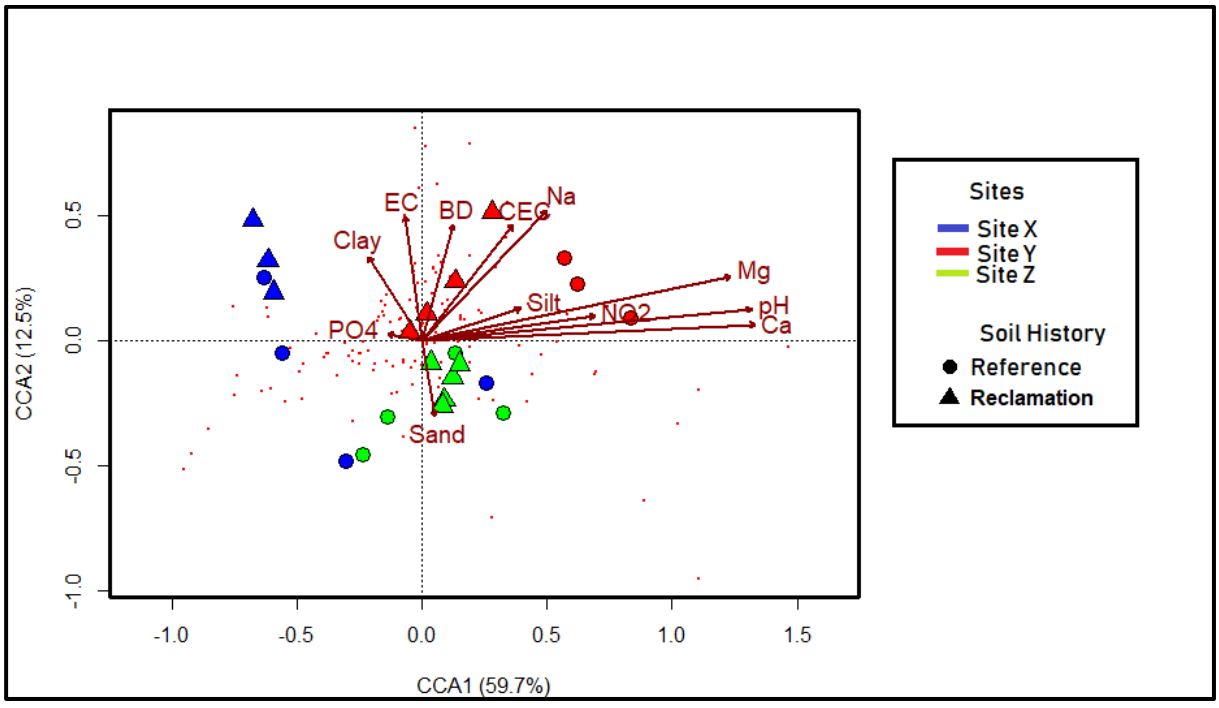

Figure 3.17: CCA triplot depicting the relationship between constraining variables and relative abundance of OTUs (genus taxa rank) across sites and soil history. The first species axis is significant (*P* < 0.05). Tiny red dots indicate genera. Some constraining variables showing collinearity with one or more variables are excluded from the final model plot.

3.3.10.2 Across post-mining reclamation chronosequence and unmined soil

Canonical correspondence analysis revealed that N-forms such as nitrate ($N\text{-}NO_3$) and NH_4 contributed to the bacterial community differentiation between the unmined reference soil and post-mining reclamation soils (Figure 3.18a). The bacterial communities of ReclZ-3 and ReclZ-11 were more influenced by bulk density (BD), whereas clay content and sodium influenced the bacterial communities of ReclZ-15, ReclZ-19 and ReclZ-24 compared to the younger reclamation soils. In contrast, the bacterial communities of all sites were least influenced by C and silt content (Figure 3.18a). Of the soil-physicochemical properties, pH, nitrate ($N\text{-}NO_3$), NH_4-N and clay content were significant in the CCA model depicted by in triplot in Figure 3.18a (see also Table 3.S9).

Similar to the bacterial community, the fungal community was significantly ($P < 0.05$) constrained by pH, nitrate and $N\text{-}NH_4$ (Table 3.S10). Unlike the bacterial community, BD was less associated with the younger (ReclZ-3 and ReclZ-11) reclamation areas (Figure 3.18b) while clay content was not significantly ($P > 0.05$) related to the fungal community of the older (ReclZ-15, ReclZ-19 and ReclZ-24) reclamation areas. Overall, pH, P, nitrate and NH_4 were the soil properties that differentiated reclamation sites from the unmined reference soil (Figure 3.18b).

Across the post-mining reclamation chronosequence (without comparison with the unmined site), the CCA triplot depicting the relationship between soil-physicochemical properties and bacterial communities (Figure 3.S8) suggests that pH, C, BD, Ca, Silt and P are the factors that mostly shape differentiation in the bacterial communities, while pH, C, BD, Na, Silt and P shape fungal communities. BD was more associated with the younger reclamation areas (ReclZ-3 and ReclZ-11) compared to older reclamation areas (Figure 3.S8). Litter, nitrate and ammonium (NH4-N) least influenced the microbial community of the post-coal mining reclamation chronosequence (Figure 3.S8). Overall, based on the CCA model, pH, nitrate and P are significant factors influencing microbial communities along the chronosequence (Table 3.S10).

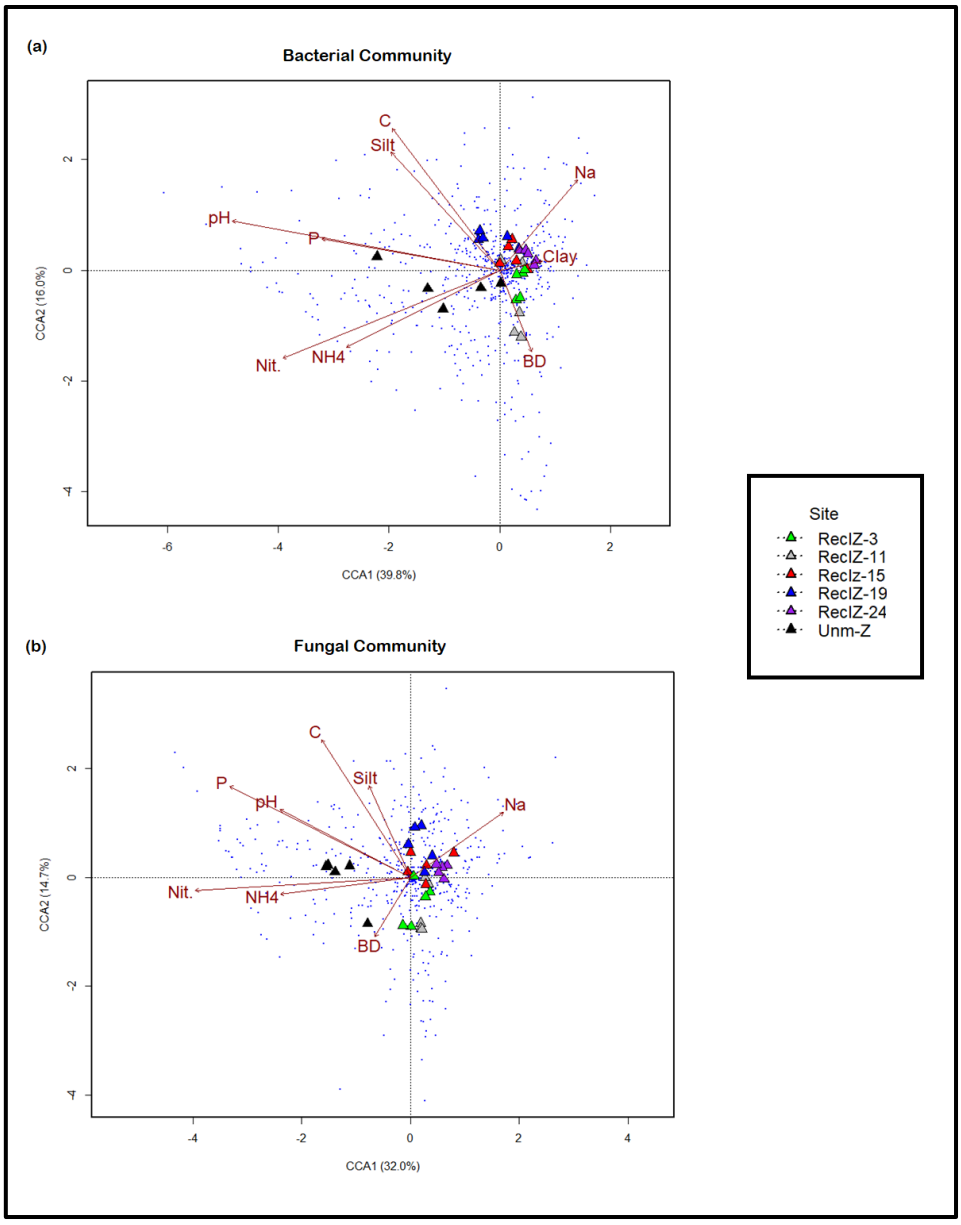

Figure 3.18: CCA triplot depicting the relationship between constraining variables and relative abundance of microbial communities along the chronosequence and unmined soil in site Z. (a) bacterial OTUs (genus taxonomic rank). (b) fungal OTUs. The first two axes are significant ($P < 0.05$). Tiny blue dots indicate genera. Non-significant (Chi-Square, $P > 0.05$) constraining variables with high collinearity (VIF > 10) with one or more variables which are not significant (Chi-Square, $P > 0.05$) were excluded from the final model plot. Nit, Nitrate (NO_3); BD, bulk density.

3.4 Discussion

Soil microbial communities contribute to soil ecosystem processes by regulating the decomposition of organic matter, availability of nutrients and assimilation of soil nutrients by plants amongst other functions. Such functions of soil microorganisms are vital for soil ecosystem functioning and health. The present study investigated the bacterial community structure and potential functions across sites and soil history (reclamation or unmined reference). In addition, the microbial (bacteria and fungi) community differentiation along a post-mining chronosequence was investigated to unravel the microbial diversity and succession over years of reclamation. The study is important for assessing potential restoration across years of reclamation and for identifying potential microbial species which may serve as bioindicator species for assessing soil ecosystem health in mining-impacted soils.

3.4.1 Soil physicochemical properties soil history, site and changes over chronological time

Soil physicochemical properties are often used as indicators of soil quality. However, most physicochemical properties of the soils are often inter-related and thus difficult to interpret (Larson and Pierce, 1991; NRC, 1993). The observation of no significant differences in most chemical properties (e.g. pH, EC, CEC, OM) (Table 3.2) between reclamation sites and adjacent references sites suggest that the chemical properties of reference soils are also influenced by factors other than mining disturbance. Such factors may include extensive leaching of nutrients over many years. Similarly, across the chronosequence, the general increase in specific soil nutrients, especially organic C, suggest increasing organic matter deposition characteristic of ecological development over chronological time (Frouz and Novakova, 2005; Frouz et al., 2013). The observation of no significant differences in selected physicochemical properties between reclamation soils and unmined soils as well amongst reclamation areas of different age is similar to observations made in earlier studies on post-coal mining reclamation sites and unmined adjacent reference soils in South Africa (Claassens et al., 2006; Claassens et al., 2012; Paterson

et al., 2019). Several factors influence the physicochemical compositions of disturbed lands including natural and anthropogenic factors such as erosion and soil use/management, respectively (DePuit and Redente, 1988). For example, intra-site (within mining companies) and inter-site (between different mining companies) variations in soil stockpiling practices (e.g. proper separation of topsoil from subsoils and bedrocks, storage conditions and durations of soils in stockpiles), and reclamation properties (e.g. proper replacement of stockpiled soils in the right order to preserve soil horizons), slopes and erodibility of soil surfaces may predispose differences in soil physicochemical properties between reclamation areas and unmined soils as well as among post-mining reclamation areas across years (Claassens et al., 2008). In addition, the post-mining soil replacements are not always carried out in an organised manner and with high precision and thus may vary from one area to another within and between sites (Tanner and Mohr Swart, 2007; Paterson et al., 2019). Possibly changes in post-mining reclamation practices may have also occurred after years of reclamation (Claassens et al. 2008).

Nevertheless, the generally lower silt fraction and organic matter C content of reclamation soils compared to adjacent reference areas at each mining site sampled in 2016 suggest that reclamation areas were less fertile compared to reference areas. This is because the silt and organic matter content of the soil are vital to nutrient retention and availability as well as for improved soil aeration and structure (McCauley et al., 2017). Furthermore, the higher clay content of reclamation soils across the three sites (ReclX, ReclY and ReclZ) (Table 3.2) and in most of the reclamation areas (of different ages) across the chronosequence in site Z (Table 3.3) may predispose high compaction, poor aeration and poor penetration of plant roots. Reclamation soils generally had higher bulk density compared to adjacent unmined soils (Table 3.2). These observations of higher bulk density in disturbed soils can be attributed to compaction from the use of heavy machinery during the replacement of soils in mined-out areas, and the higher fraction of clayey fractions in reclamation soils and they are similar to observations made in earlier studies on stockpiles and reclamation areas (Ezeokoli et al., 2019a; Ezeokoli et al., 2019b; Paterson et al., 2019). Similarly, these factors including tillage may explain the non-significant difference in

bulk density observed between reclamation areas and the unmined agricultural soil sampled in Site Z during the 2017 sampling. Soil pH was largely (except in RefY) acidic and thus sub-optimal for most crops (Parikh and James, 2012). The low pH (acidity) values are likely due to the leaching of basic ions or oxidation of soil sulphide compounds (Pietrzykowski, 2014; Paterson et al., 2019). From a land-use perspective, soils in both reclamation and reference soils will require liming and fertilisation prior to use in land tillage or pasture (Parikh and James, 2012; McCauley et al., 2017).

3.4.2 Community-level –physiological profiles and enzyme activities as indicators of soil ecosystem health in reclaimed areas

Community-level physiological profiling (CLPP) utilises carbon utilisation patterns to estimate microbial community richness and have been applied to different soil environments including post-mining reclamation soils and agricultural fields (Garland and Mills, 1991; Claassens et al., 2012; Habig et al., 2018). The observations of no differences in the Shannon-Wiener index of diversity and evenness derived from community-level physiological profiling (CLPP) (Table 3.4) suggests that the microbial communities of reclamation soils and reference soils have similar metabolic capabilities with respect to utilising the diverse carbon sources provided in the assay. The evenness values also suggest that the proportion of the physiologically distinct (with respect to carbon utilisation) species in the community are similar across soils. In a study by Markowicz et al. (2015), no differences were observed in the functional diversity (as determined by carbon utilisation profiles) of plant-associated microbial communities in coal mining soil stockpiles of different ages of natural reclamation. Summarily, these observations hint at functional redundancy within the microbial communities with respect to metabolising carbon. However, the observation of higher microbial diversities (H' index) in the reference soil compared to reclaimed soils in most of the sites (Table 3.2) are similar to observations of Lewis et al. (2010) who observed that based on CLPP, higher microbial diversity was observed in unmined soils compared to those in post-bauxite reclamation soils irrespective of reclamation age. The reduction in Shannon-Wiener index with increasing age of reclamation soils may be due to the development of a streamlined

functional community in the older reclamation soils (Krištůfek et al., 2005; Chodak et al., 2009; Frouz et al., 2013).

Soil enzymes are utilised as soil quality indicators for monitoring the response of soil organisms to disturbances in different soil ecosystems (Stenberg, 1999; Acosta-Martinez and Tabatabai, 2000; Tabatabai and Dick, 2002; Turner et al., 2002; Kwak et al., 2015). Most of the enzymes in the soil are extracellular and of microbial origin (Das and Varma, 2010). For the inter-site sampling of 2016, the average β-glucosidase, urease, alkaline- and acid phosphatases activities were lower in most reclamation soils compared to the adjacent reference soils, suggesting potentially lower (compared to reference soils) availability of carbon (e.g. cellobiose), N-mineralization (ammonification) and phosphates in most reclamation soils (Dick et al., 1996; Eivazi and Tabatabai, 1977; Acosta-Martinez and Tabatabai, 2000). Furthermore, the significant interaction effect of fixed factors on these enzyme activities suggests that they are sensitive to differences between sites, and soil disturbances (Stenberg, 1999; Turner et al., 2002; Stege et al., 2010).

Across the post-coal mining soil reclamation chronosequence, the lack of significant difference in phosphatases activities may suggest that both acid- and alkaline phosphatases are not sensitive indicators of soil health. In contrast, the significant differences in urease activity between the unmined maize field and those of the reclamation chronosequence suggest that N-mineralization rates are higher in the maize field as a consequence of nitrogen fertilisation. In comparison to the phosphatases, the pattern of urease activities suggests that the enzyme (urease) is more sensitive to reflect mineral availability or inputs in soils. The levels of the carbon-cycling related beta-glucosidase enzyme and the overall microbial activity indicator dehydrogenase enzyme suggested higher carbon mineralisation and microbial biomass in the unmined maize field compared to the reclamation areas. However, the non-significant differences between the enzyme activity levels in the unmined area and those of most of the reclamation areas (including ReclZ-3) may be due to (statistically) the high within-site variation (standard deviation).

The values obtained for enzyme activities in all reclamation soils are within the range obtained for post-oil mining reclamation soils (Dimitriu et al., 2010) and post-coal mining reclamations soils (Claassens et al., 2011; Claassens et al., 2012). The comparability of the enzyme activity values to those obtained fertilized soils under cultivation (Eivazi et al., 2003; Hojati and Nourbakhsh, 2009) and pasture soils (Turner et al., 2002) suggest similar ecosystem functional capabilities of the reclamation soil microbial communities, and thus, provide indications of the potential suitability of these soils for agriculturally-related uses. The overlaps in the range of enzyme activities values between reclamation soils and adjacent reference soils observed at most sites and within the reclamation chronosequence are similar to the reports of Claassens et al. (2012) and Claassens et al. (2011) on post-coal mining chronosequence. The increase in the activities of β-glucosidase, urease, alkaline- and acid phosphatases with increasing age of reclamation soils for the inter-site sampling of 2016 as well as the general increase in dehydrogenase activity, acid- and alkaline-phosphatase activity across years of post-reclamation (up to the 19th year), suggests the restoration of ecological functions of soil microbial communities over chronological age. The lower activities of most enzymes in the 24 years old reclamation soil compared to the 19-year-old post-reclamation site may be related to the lower nutrient levels in the 24-year-old site (Table 3.3.). As mentioned earlier this may be related to extensive weathering and leaching of nutrients over many years amongst other factors. Indeed, the activities of soil enzymes are usually related to the soil chemical properties, including soil minerals whose cycling they mediate (Das and Varma, 2010; Adetunji et al., 2017). Thus, significant correlations were observed between enzyme activities and selected soil chemical properties (Table 3.5 and Table 3.6).

However, in the 2016 multi-site sample set positive correlations between beta-glucosidase and organic matter were observed although not significant, while the correlations between alkaline- or acid -phosphatases and phosphate content of the soil were very weak. These observations are likely due to the specificity of β-glucosidase to cellobiose, which is only a fraction of the total organic matter, and because underlying effects of site and/or soil histories on soil organic matter and phosphates were not considered in the correlational analysis. As a result, when the site effect

was minimized by sampling along the chronosequence within a single coal mining company in 2017, significant correlations were observed between the assayed enzymes and soil-physicochemical properties. In particular organic carbon was strongly correlated with beta-glucosidase and dehydrogenase activities—enzymes that are mediate c-cycling in the soil (Das and Varma, 2010; Wolińska and Stępniewska, 2012; Adetunji et al., 2017). As a general note, although soil enzyme activities are widely utilised as soil health indicators, the difference between the *in vitro* assay conditions and field conditions may impair their reliability, interpretations and utilisation in predicting soil quality or conditions. In addition, although high enzyme activities may imply high microbial activity, such high enzyme activities may be due to the contribution of microbial species which may be pathogenic to crops when the soil is used for cultivation.

3.4.3 Bacterial species diversity indices and community structure: influence of site and soil history

Microbial community richness and diversity are linked to the plasticity of soil ecosystem functionality (Kennedy and Stubbs, 2006; Maron et al., 2018). The highest number of OTUs shared amongst pairs of reclamation and reference soils were between the oldest reclamation soil (ReclZ) and the adjacent reference suggesting that the older reclamation soils were most similar to the reference soils in terms of bacterial species richness. This information, along with the generally higher species richness and diversity in older reclamation areas (ReclY and ReclZ) compared to the more recently reclaimed area (ReclX), suggests the restoration of bacterial species richness and diversity levels over chronological age and are comparable to those of pre-mining disturbance bacterial species richness and diversity levels. These observations agree with the results obtained for enzyme activity assays discussed earlier. Similarly, high-throughput sequencing-based studies by Li et al. (2014) and Hou et al. (2018) on the microbial diversity and community structure in post-coal mining sites in China indicated lowest bacterial diversity in more recent reclamation soils compared to older reclamation soils. Furthermore, microbial community diversity and structure across post-mining reclamation sites indicated restoration of microbial communities in older reclamation sites when compared to unmined soils (Li et al., 2014; Sun et

al., 2017; Hou et al., 2018). Furthermore, the within- and between- dissimilarity in bacterial communities of the reference sites may be due to other (other than mining) unknown anthropogenic or site-specific influences such as roads and grazing since the mining sites occupy a very large area and are in close proximity to urban areas (Claassens et al., 2012). The significant *P*-values obtained for the fixed-factor PERMANOVA analyses suggest that, indeed, the bacterial community composition and structure are influenced by site differences, as well as soil history/disturbance. This is in agreement with previous findings that bacterial communities are differentiated across landscapes (Hermans et al., 2017), and are sensitive to soil management practices (Dose et al., 2015; Nkuekam et al., 2018).

3.4.4 Trends in microbial species richness, diversity and community structure over years since reclamation

Both bacterial and fungal OTU diversity increased until 19 years and decreased thereafter in the 24-year reclamation area. The observation of increasing species diversity with the age of reclamation is similar to the observation in the earlier mentioned studies (Li et al. 2014; Hou et al., 2018) and suggest the attainment of comparable bacterial species richness and diversity levels as in the unmined maize reference field soil (no significant difference between ReclZ-19 and unm-Z). The observed non-significant ($P > 0.05$) differences in the fungal species richness and diversity (*H'*) in comparison to the significantly different richness and diversity of bacterial species, indicate that bacterial species richness and diversity were more dynamic and more responsive to prevailing ecological factors and/or conditions, as well as soil ecosystem changes over a chronological scale (Frouz et al., 2013). Such an indication is also supported by the homogeneity (closer associations) of the sample group in Figure 3.7 which suggests that fungal communities (Figures 3.7a and 3.7b) are less divergent compared to bacterial communities. In addition, the observed sharp decline in bacterial species richness and diversity in comparison to the rather steady (between ReclZ-19 and ReclZ-24) fungal species richness and diversity after 19 years despite the reduction (from ReclZ-19 to ReclZ-24) in the levels of nutrients in the 24

year-old reclamation site (ReclZ-24) (Table 3.3) further support the hypothesis of bacterial species diversity being more readily perturbed compared to those of fungal species richness and diversity. In the context of potential ecological bioindicator species, and these results indicate the suitability of bacterial species (because they are more sensitive) rather than fungal species (more stable even under harsh conditions) for monitoring soil ecosystem restoration.

Other authors have also observed that bacterial communities are less stable when compared to fungal communities (Sun et al., 2017; de Vries et al., 2018). For example, Sun et al. (2017) during their study on the microbial community patterns during ecosystem restoration of a forest ecosystem, observed that unlike bacterial species, fungal networks did not reflect any age-related trends suggesting that fungal species are more tolerant (less sensitive) to ecological constraints. However, studies elsewhere on responses to non-extreme drought conditions Kaisermann et al., 2015) and heavy metal contamination in soils (Xu et al., 2019) suggest that fungal networks and/or communities are more sensitive compared to bacterial species. The different responses of bacterial and fungal species are influenced by their respective physiological differences, including their ability to utilise diverse carbon substrates that are available in the soil environment (Rinnan et al., 2007; Meidute et al., 2008; Andresen et al., 2014). For example, while fungi may be able to utilise complex organic substrates such as lignocellulose, bacteria are more likely to rely on soluble substrates (Andresen et al., 2014). Furthermore, unlike bacteria, fungi can scavenge for nutrients by extending hyphae into the soil environment, thus ensuring their survival under extreme nutrient limiting conditions compared to bacteria (Andresen et al., 2014).

When considered independently of possible influences of soil physicochemical properties (and perhaps other underlying factors) on the bacterial communities, trends in bacterial and fungal species richness and diversity in the post-mining reclamation soils and in comparison, to the unmined farmland, suggest that species richness and diversity levels are restored between 15- and 19-years following reclamation. Other workers have reported a similar observation of a

potential restoration (in comparison to undisturbed sites) of microbial communities between 15 and 20 years of post-coal mining reclamation on coal mine spoils in China using high-throughput sequencing (Li et al., 2014; Hou et al., 2018). Based on phospholipid fatty acid (PLFA) analysis, Dangi et al. (2012) observed the recovery of microbial biomarkers between 5 to 14 years in reclaimed soils of an opencast coal mining site in Wyoming. The differences in estimated recovery periods in these various studies are most likely related to variations in reclamation methodologies, microbial community analyses methodologies, climatic and site-specific factors amongst others.

Although microbial (bacterial and fungal) species richness and diversity in older reclamation areas were similar to those of the unmined farmland, the community composition and structure revealed differentiations that are most likely due to land types. This observation supports well-documented evidence for the impact of land-use types on soil microbial communities (Dose et al., 2015; Nkuekam et al., 2018). Such differentiation observed points to the sensitivity of microbial communities in reflecting the influence of ecosystem disturbance. Unfortunately, due to the pre-mining land-use practices and other potential anthropogenic contributions around the mining areas, it was practically impossible to obtain an ideal 'reference' soil for comparison and inference on the restoration of community composition and structure over reclamation age. Nevertheless, the differentiation in the microbial community composition and structure amongst reclamation areas of different ages suggests a structured community succession across reclamation years—constant community between 3-11 years, then a shift in community composition that is fairly constant between 15 and 19 years and starts to change after 24 years. This observation suggests that microbial communities during post-mining reclamation ecological development may be successional structured across years.

To conclude this section, the bacterial species richness and diversity indicate recovery between 15 and 19 years after reclamation. While the composition of microbial communities is not similar to those of unmined farmland soils (as a result of different land management practices), their composition and structure show differentiation across reclamation ages. Such differentiations

were observed along the chronosequence between 3-11 years and 15-24 years since reclamation.

3.4.5 Dominance, differentially abundance, succession and potential functions of microbial phylotypes

For the first time, this study provided insight into the soil bacterial community diversity and succession during post-coal mining reclamation in South Africa using high-throughput next-generation sequencing (NGS) technologies. Similar to the study of Li et al. (2014) on post-coal reclamation and unmined soils in China, several dominant and rare bacterial phylotypes, including Proteobacteria, Actinobacteria, Chloroflexi, Acidobacteria, Planctomyces and Verrucomicrobia were observed across reclamation and reference soils. Species of these phyla contribute to the diverse microbial functions and process in the soil ecosystems, including processes that are critical to soil ecosystem sustainability (Markowicz et al., 2015; Youssef et al., 2015; Hermans et al., 2017). The differentially abundant phyla amongst the soil bacterial communities included underexplored phyla divisions such as WPS-2 and FCPU426. Until recent advances in NGS technologies, knowledge of the ubiquity, biogeography and potential roles of WPS-2 and FCPU426 in the soil environment have been limited (Youssef et al., 2015). For example, based on metagenomic analyses, species in the candidate phylum WPS-2 are consistently associated with soil environments globally, and possess capabilities (genes) for anoxygenic photosynthesis which is important towards sequestration of atmospheric carbon and the generation of biomass in the soil ecosystem (Ji et al., 2017; Holland-Moritz et al., 2018). Similarly, the phylum Gemmatimonadetes and Nitrospirae remains underexplored with only a few isolates so far characterized. Some characterised species in the phylum Gemmatimonadetes are reported to be capable of carbon fixation (Zeng et al., 2016), while the phylum Nitrospirae comprise species involved in the biogeochemical cycling of soil sulphur, iron and nitrogen (Lücker et al., 2010; Lin et al., 2014). Nevertheless, the high proportion of unclassified taxa at the genus level clearly indicate that the bacterial community of coal-mining soils are yet underexplored. It also points to the large proportion of the global diversity of bacteria yet uncultivated. Indeed, metagenomics

studies of diverse environments are helping to increase our knowledge of bacterial diversity and to optimize cultivation strategies for bacterial species, which have previously been uncultivated. In the context of bioprospection, the findings of this study point to these soil environments as being rich in underexplored bacterial diversity, including species with economic relevance and potentials for improving soil health.

Several of the dominant genera and differentially abundant genera identified in this study, including *Acidibacter*, *Acidothermus*, *Bacillus*, *Bradyrhizobium*, *Burkholderia-Caballeronia-Paraburkholderia*, *Candidatus Udaeobacter*, *Candidatus Xiphinematobacter*, *Conexibacter*, and *Sphingomonas* have been observed in the bacterial diversity of post-coal mining soils (Frouz et al., 2013; Li et al., 2014; Hou et al., 2018). Overall, species of these genera contribute to soil nutrient cycling, biocontrol of plant diseases, promotion of plant growth, and modulation of plant response to abiotic stress amongst other benefits (Frouz et al., 2013; Nautiyal et al., 2013; Tiwari et al., 2017). Specifically, species of *Microvirga* and *Bradyrhizobium* are plant-growth-promoting rhizobacteria which contribute to nitrogen fixation in the rhizosphere (Frouz et al., 2013; Msaddak et al., 2017), while *Bacillus* species are well known for their plant-growth-promoting ability, biocontrol of plant pest and pathogens, and the modulation of plant-hormone expression and adaptation to abiotic stress (Nautiyal et al., 2013; Tiwari et al., 2017). Similarly, the genera *Sphingomonas* comprise species with diverse functions in the soil ecosystem, including the degradation of polycyclic aromatic compounds (Nautiyal et al., 2013). *Candidatus Udaeobacter*, which was observed in all soils at relatively high abundance, is an oligotroph that can thrive in nutrient-poor conditions, thus suggesting, its potential use as an indicator species for reflecting poor soil nutrient state.

Within the context of ecological relevance and potential application of differentially abundant species in soil monitoring, both reclamation soils and reference soils harbour species (based on available information on characterised species) which have potentials for Biocontrol e.g. *Lysobacter*, *Micomonospora*, *Dactylosporangium*, *Actinoplanes*, *Pseudonorcadia*, *Haliangium*

and *Streptomyces* (Kundim et al., 2003; de Lima Procópio et al., 2012; Lee et al., 2013); Nutrient mineralization e.g. *Dyella*, *Pseudaminobacter*, *Labrys*, *Pedomicrobium*, *Gemmatimonas*, *Sinomonas*, *Terrabacter*, *Mucilaginibacter*, *Conexibacter*, *Bryobacter* and *Candidatus Koribacter* (Ridge et al., 2007); Plant-growth-promotion e.g. *Mesorhizobium*, *Microvirga*, *Bradyrhizobium* and *Solirubrobacter* and *Norcadioides* (Frouz et al., 2013; Msaddak et al., 2017); Supporting soil ecological food web by being primary producers e.g. *Rhodoplanes* (Srinivas et al., 2014); and pathogenicity e.g. *Pajaroellobacter* (Brooks et al., 2016) (Table 3.7). These plant-growth-promoting bacterial species could be isolated and employed as inoculum (biofertilizers) during post-mining reclamation—a practice which is not currently implemented in post-mining reclamation practices within South Africa. Thus, as a recommendation towards best practices for mediating restoration success, the inclusion of a microbial consortium in the current local reclamation practice may facilitate restoration success. Ideally, such microbial consortium should comprise beneficial (e.g. plant-growth promoters, primary producers and nutrient-mobilizers) bacterial species and may be obtained from undisturbed or virgin sites using conventional methods for cultivating indigenous microorganisms (Park and DuPonte, 2008; Kumar and Gopal, 2015). In addition, based on observations from previous studies on stockpile (Paterson et al., 2018; Ezeokoli et al., 2019a; Ezeokoli et al. 2019b), the facilitation of microbial species proliferation and diversity during storage of topsoil in stockpiles (heaps) as well as the minimization of compaction during soil replacement may aid soil health and consequently overall restoration success during post-mining reclamation.

Additionally, based on the growth requirement or adaptation of some characterized species to given environmental conditions, some differentially abundant genera may be further explored for potential utilisation as bioindicators for reflecting soil conditions (Table 3.7), including salinity (e.g. *Haliangium* (Kundim et al., 2003)) and acidity (e.g. *Bryobacter*, *Acidothermus* and *Singulisphaera* (Li, et al., 2018)). Summarily, both reclamation and reference these soils are rich in microbial diversity and may serve as a suitable source for the bioprospection of novel species as well as species with potential industrial importance such as the production of secondary metabolites.

Most of the fungal species were of the phylum Ascomycota. This is similar to the observations made in stockpiles in Chapter 2 (Mashigo, 2018) and previous microbial ecology studies of post-coal mining reclamation soils (Sun et al., 2017). This observation suggests that species of Ascomycota play major ecological roles such as the accumulation of organic matter and the degradation of plant litter inputs throughout the development and restoration of ecosystem functions in the reclamation soils (Frouz et al., 2013; Tedersoo et al., 2014; Sun et al., 2017). A majority of the fungal species detected across both reclamation and unmined reference soils are saprophytes as has been also observed in Fens (Wang et al., 2019b) and in most soil environments (Ingham, 2000; Jenkins, 2005). As saprophytes, these fungal species degrade plant litter and various other complex macromolecules including lignocellulose materials present in the soil environment, thus contributing to the soil food chain by serving as decomposers (Ingham, 2000; Andresen et al., 2014; Tedersoo et al., 2014). The potential ecological guild of the dominant and differentially abundant fungal genera is summarised in Table 3.8.

The succession in bacterial and fungal species communities are driven by a complex interaction between soil physicochemical properties, plant community and other stochastic processes (Dumbrell et al., 2010a; Davison et al., 2012; Frouz et al., 2013; Hou et al., 2018). Thus, the changes in soil-physicochemical properties observed over years of reclamation may be driven changes in the soil microbial communities (along with their metabolic activities) and vice versa. This complex interaction underscores the complexity of defining what constitutes soil health and what parameters are most critical in the context of soil health (Stenberg, 1991; Arias et al., 2005; Cardoso et al., 2013). At best, the succession in the bacterial and fungal species observed over chronological time only provides insights into the community dynamics in light of changing soil conditions (Frouz et al. 2013). Such insights can be exploited in further bioprospection- and anthropogenic impact-assessment-based studies that are focused on target species. Furthermore, the inability of the sequencing methodology applied in this study to delineate microbial species to species taxonomic level limits inference on potential functions of the microbial species (genera taxonomic rank) at specific time points during post-mining reclamation.

Within the soil environment analysed, soil pH, phosphorus and nitrate were the most critical (based on statistical significance) factors influencing both fungal and bacterial communities. pH is one of the most important soil properties which affect microbial activity, organic matter mineralisation, nutrient mobilisation and overall soil ecosystem dynamics (Smith and Doran, 1996; Miller, 2016). For example, the mobilisation of phosphorus and N in the soil are affected by pH (Jensen et al., 2010; Miller et al., 2016). The availability of these nutrients may influence microbial communities directly or indirectly through the activities of plants (nutrient uptake, root exudates etc) in response to changes in the nutrient availability levels (Hawkins and Crawford, 2018). Furthermore, in the observation that specific soil-physicochemical properties influenced bacterial and fungal communities differently (e.g. clay content, electrical conductivity, Na and Ca on bacterial communities, while ammonium-N solely influenced fungal communities) may be related to differences in adaptative traits, and diversification of metabolic capabilities of bacterial and fungal species (Rinnan et al., 2007; Meidute et al., 2008; Rousk et al., 2009; Andresen et al., 2014; Kaisermann et al., 2015).

Table 3.7: Potential economic importance and function of some differentially abundant bacterial taxa in the soil ecosystem

Economic importance	*Genus (notes)	[1]References
Biocontrol/ Antibiotics	-*Lysobacter* -*Micomonospora* -*Dactylosporangium* -*Actinoplanes* (produce pharmaceutically important compounds including ramoplanin, teicoplanin and valienamine) -*Pseudonorcadia* -*Haliangium* (Antifungal compounds; Haliangicins) -*Streptomyces* (bioactive metabolites, antifungals)	Kundim et al. (2003); Chater (2006); de Lima Procópio et al. (2012); Seipke et al. (2012); Yamamura et al. (2012); Lee et al. (2013).
Biomineralization/ Nutrient cycling/ Bioremediation	-*Dyella* (Phosphate solubilising) -*Pseudaminobacter* (Degradation of aromatic hydrocarbon) -*Labrys* (Biodegradation of fluorobenzene) -*Pedomicrobium* (Manganese) -*Gemmatimonas* (polyphosphate accumulation) -*Sinomonas* (use in metallurgy) -*Terrabacter* (Biomineralization of nickel, copper, lead, cobalt, zinc, cadmium and calcium. Reduction of nitrates to nitrites) -*Mucilaginibacter* (chemoorganotrophic) -*Conexibacter* (Reduce nitrate nitrite (Nitrification), Biodegradation) -*Bryobacter* (decomposers, chemoorganotrophic) -*Candidatus Koribacter* (degradation of complex polymers and CO oxidation (Carbon cycle))	Ridge et al. (2007); Kampfer et al. (2014).
Endosymbiont/Plant growth-promoter	-*Mesorhizobium* (Nitrogen fixation) -*Microvirga* (Endosymbiont nodulating *Lupinus luteaus*) -*Bradyrhizobium* (Nitrogen fixation)	Frouz et al. (2013); Msaddak et al. (2017).

Economic importance	*Genus (notes)	[1]References
	-*Solirubrobacter* (root endophyte) -*Norcadioides* (leaf endophyte)	
Autotrophs-biomass, food chain	-*Rhodoplanes*	Srinivas et al. (2014)
Zoonotic/Parasitic	-*Pajaroellobacter* (epizootic bovine abortion) -*Crossiella* (causing nocardioform placentitis)	Brooks et al. (2016); Erol et al. (2012).
Potential soil quality Indicator organisms		
Salinity	-*Altererythrobacter* -*Pirellula* -*Halingium*	Glöckner et al. (2003); Kundim et al. (2003).
Acidity	-*Bryobacter* (Acidity) -*Acidothermus* (thermophilic, acidophilic) -*Singulisphaera* (degrading several biopolymers under acidic conditions)	Kishimoto et al. (1991); Li et al. (2018).

*See Figures 3.8, 3.9, 3.11 and 3.12. See also Table 3.S2 for distribution of these genera. [1]References are not exhaustive.

Table 3.8: Ecological guild of dominant and/or differentially abundant fungal genera

Trophic mode/Guild	Genus (*Distribution)	†References
Saprophyte	-*Cercophora* (ReclZ-11) -*Cladophialophora* (ReclZ-11; ReclZ-3) -*Echria* (ReclZ-24) -*Penicillium* (All soils) -*Rasamsonia* (ReclZ-24) -*Talaromyces* (ReclZ-15) -*Thielavia* (All sites) -*Aspergillus* (unm-Z)	Bell (1983); James et al. (2006); Cannon and Kirk (2007); Bills et al. (2013); Tedersoo et al. (2014); Sterkenburg et al. (2015).
Multiple potential functions (Animal Pathogen-Endophyte-Epiphyte-Plant pathogen-undefined Saprophyte).	-*Aureobasidium* (ReclZ-24)	Tedersoo et al. (2014); Wachowska and Glowacka (2014); Irinyi et al. (2016).
Pathotroph-Saprotroph	-*Coniosporium* (ReclZ-11) -*Helicoma* (unm-Z) -*Neoascochyta* (All sites)	Cannon and Kirk (2007); Li et al (2008); Tedersoo et al. (2014).
Plant pathogen	-*Curvularia* (ReclZ-11)	Tedersoo et al. (2014)
Symbiotroph: Lichen	-*Endocarpon* (ReclZ-19)	Esslinger (2014)
Ericoid Mycorrhizal	-*Oidiodendron* (All sites)	Newsham (2011)
Unknown	-*Neophaeosphaeria* (ReclZ-15) -*Mortierella* (unm-Z)	-
Endophyte	-*Tiarosporella* (ReclZ-11)	Carroll and Carroll (1978)

*Soil sample or site in this study in which the genus is dominantly or differentially present. See Figures 3.13 and 3.14, and also Table 3.S3. †Based on entries in the output from FunGuild (Nguyen et al., 2016).

Unlike bacterial genetic diversity, the predicted functional diversity was not significantly influenced by soil history. This observation suggests redundancy in the functional capabilities of soil bacterial communities and agrees with results obtained in the phenotypic-based assays and the microbial community of fens (Wang et al., 2019b). Such redundancy in microbial community functional capabilities is linked to the resilience of soil bacterial community to environmental constraints and to the role of soil microbial communities in ensuring resilience in the soil ecosystem function (Allison and Martiny, 2008; Maron et al., 2018). The significant differences observed for specific

predicted functions suggest that some bacterial communities are functionally more capable of regulating specific soil processes than others. Because the functional profiling performed in this study are based on predictions using marker genes, discussions on the succession of potential functions are not discussed. Further studies are however needed to evaluate the expression of functional genes in these soils because the presence of genes does not necessarily translate to phenotypic expressions.

3.5 Conclusion

Although bacteria species richness was impaired in stockpiles compared to the reference soils in stockpiles (chapter 2), the results from the present chapter showed that over chronological time, the microbial community richness approaches levels similar to those of unmined areas. Also, the hypothesis that the microbial community structure and function are site-specific was affirmatively confirmed. The results further provided evidence to support the hypothesis that indeed, the microbial communities of post-coal mining soils are differentiated along a temporal scale of years since reclamation and between unmined areas.

Of all microbial community profiling methods utilised in the study, community-level physiological profiling failed to detect differences in microbial community richness and diversity across all soil histories (reclamation and unmined) and between sites. Although the functional capabilities of bacterial communities were redundant across reclamation and unmined soils, microbial community differences between soils suggest that certain functions of a majority of the soil microorganisms remain largely unknown. Thus, these soils may serve as a bioresource for the exploration of novel bacterial species and ecosystem functionalities.

More importantly, while the structural differentiation in the microbial communities amongst reclamation soils and between reclamation soils and unmined reference show that microbial communities are sensitive ecological bioindicators, these differences are also driven by soil physicochemical properties. Therefore, the utilisation of microbial communities as soil ecosystem

health indicators for monitoring the restoration of post-mining reclamation areas, as well as the adequacy of current reclamation protocols in the South African coal mining industry must be integrated with soil physicochemical properties.

The restoration of bacterial community richness and diversity levels in post-coal mining soils in comparisons to unmined areas is a function of time, and occur between 15 and 19 years after reclamation. Furthermore, the relative stability in fungal community diversity over years of reclamation compared to bacterial community diversity suggests that bacterial communities, not fungal communities, are more likely to serve as indicators of ecosystem restoration. However, it is possible that certain groups of fungi such as the obligate plant-symbionts, arbuscular mycorrhizal fungi, may reflect trends consistent with the age of post reclamation areas. Further studies are needed to investigate this possibility.

CHAPTER 4:

ARE THE ARBUSCULAR MYCORRHIZAL FUNGAL COMMUNITIES ALONG A POST-COAL MINING RECLAMATION CHRONOSEQUENCE DIFFERENTIATED?

4.1 Introduction

Because one of the goals of the study was to identify potential ecological indicator species, the observation in Chapter 3 that fungal community richness and diversity (without taking into cognizance specific fungal guilds) show stability over reclamation years generated an additional question on whether the richness and diversity of a specific fungal group are less stable and potentially reflect a trend proportional to age of reclamation. Arbuscular mycorrhizal (AM) fungi were chosen because they have been reported to play roles in ecological succession during ecosystem development (Johnson et al., 1991; Koske and Gemma, 1997; Husband et al., 2002; Kikvidze et al., 2010) and because of the availability of a specialised database for Arbuscular mycorrhizal fungal (AMF) taxonomic delineation. Furthermore, Lekberg et al. (2018) showed that although universal fungal primers such as those targeting the Internally-transcribed spacer 2 (ITS-2) utilised in Chapter 3, may detect identical ecological responses like Glomeromycotan-specific primers, differences in the phylogenetic structure and family level abundances of AMF species may occur. Such differences may predispose the underestimation of true AMF species richness in a given community, as well as mask structural differentiation patterns (Lekberg et al. (2018).

Previously (Chapter 2), it was observed that AMF spore density in soil stockpiles (stored for later use in post-mining reclamation) was not different from unmined soils, although AMF spore viability may reduce with respect to stockpile height. In the present chapter, investigations were carried out to ascertain whether differences exist in the AMF richness and diversity in reclamation soil areas and/or along years since reclamation. Based on the preceding justifications, studies on the structural differentiation along the post-mining reclamation chronosequence in site Z were studied.

In soil ecosystems, the interactions between above-ground and below-ground biotic communities are vital to ecosystem processes and health (Dickie et al., 2013; de Leon et al., 2016). Such interactions include the symbiotic association between most vascular plants and their below-ground mycorrhizal symbionts (Smith and Read, 2010). AM fungi are obligate symbionts of a majority of vascular plants (Barea et al., 2002). The symbiosis between AM fungi and plant roots appear to be cosmopolitan (Davison et al., 2015). In the symbiotic association, AM fungi facilitate the assimilation and mobilisation of soil nutrients in exchange for plant sugars (Barea et al., 2002; Smith and Read, 2010). Additionally, AM fungi play roles in plant fitness and growth (Utkhede, 2006; Porras-Soriano et al., 2009), control of plant pathogens (Barea et al., 2002; Utkhede, 2006), soil structure improvement (soil aggregation) through the production of glomalin (Rillig et al., 2002; Rillig et al., 2010) and plant community succession (Kikvidze et al., 2010).

Within a landscape, differentiation in AMF assemblages is driven by niche partitioning and stochastic processes (Dumbrell et al., 2010a; Dumbrell et al., 2010b; Moebius-Clune et al., 2013a). Differentiation in AMF species due to niche differentiation are mediated by the prevailing environmental factors, including soil pH, salinity, particle size and nutrients, as well as plant vegetation type (Zaller et al., 2011; Tahat and Sijam, 2012; Moebius-Clune et al., 2013b; Marín et al., 2017). Whereas, stochastic processes which predispose the dispersal of AM fungi within and between geographical barriers are mediated by the transportation of AMF propagules (spores and hyphae) through abiotic factors such as wind and water runoff (Warner et al., 1987) as well as by biotic factors, including humans, rodents and birds (Mangan and Adler, 2002; Correia et al., 2019).

In natural and/or managed soil ecosystems, shifts in diversity and community structure of AMF assemblages over chronological space and spatial boundaries are due to plant community succession, anthropogenic activities and changes in environmental conditions (Johnson et al., 1991; Davison et al., 2012; Cui et al., 2016; de León et al., 2016; Krüger et al., 2017; Roy et al., 2017). Such successional shifts in community structure have also been observed in the

community of AM fungi (Johnson et al. 1991; Koske and Gemma, 1997) other soil microbial groups (Sun et al., 2017), and are often associated (or correlated) with variations in soil ecosystem properties, including soil organic matter development, pH changes, plant succession, and nutrient availability amongst others (Urbanová et al., 2011; Frouz et al., 2013; Li et al., 2014; Hou et al., 2018).

The relationship between plant, soil and mycorrhizal fungi are important to the restoration of ecological functions and processes in disturbed soil ecosystems such as post-mining sites (Krüger et al., 2017). Thus, differentiation in AMF communities may provide useful insights into the impacts of anthropogenic and natural activities on soil health (Stenberg, 1999). Thus, in the present chapter, the AMF community along the post-coal mining reclamation chronosequence described in Chapter 3 was investigated using Glomeromycotan-specific primers to: (1) explore the diversity and succession of AMF species along the reclamation chronosequence studied in Chapter 3. (2) Determine factors which shape AMF assemblages, including vegetation cover and influence of a single dominant vegetation (through host-colonization) and (3), gain insights into the suitability of utilising AMF community differentiation as part of a minimum dataset for monitoring ecosystem restoration following post-mining reclamation in the South African coal mining industry. The hypothesis that the AMF assemblages within a post-coal reclamation soil chronosequence are differentiated across years since reclamation, and differ from those of unmined soils was tested. Furthermore, the hypothesis that AMF species in soil co-occur with those colonising the roots of the dominant vegetation was tested. Lastly, utilising soil physicochemical and plant cover estimation data, ordination analyses were performed in order to obtain insights into environmental factors that shape AMF assemblages within the post-coal mining chronosequence across both soil and rhizoplane of the dominant vegetation.

4.2 Materials and Methods

4.2.1 Study site

The chronosequence identified in mine Z (Figure 3.2) was used for the study. This site was used because unlike the reclamation areas in other mining sites, the reclamation areas were intimately associated (in proximity to one another). The proximity of the reclamation areas provided a unique opportunity to study arbuscular community succession following post-mining reclamation while minimising the number of other confounding variables that may be directly proportional to spatial distances between reclamation areas.

4.2.2 Sampling and plant cover estimation

Sampling was done concurrently with the sampling described in Chapter 3. Before sampling, the reclamation areas were initially surveyed to identify the dominant vegetation type across reclamation sites. Soil sampling was conducted as described in Chapter 3—sampling along five 40 m quadrant spaced 100 m apart in random orientations. The origin and orientation of the first transect were purposefully selected to obtain a representative sample of each reclamation or reference site and to block out as much as possible, slope gradients effects which are typical of reclamation areas, respectively. Samples of rhizosphere soil were aseptically collected at 10 m intervals along each transect and composited to form one bulk sample. A total of five transects were used per reclamation area shown in Figure 3.2 (see also Table 3.1).

Root samples were also collected from the dominant vegetation, *Eragrostis tef* (annual grass), across the reclamation areas. Root samples were not collected from the maize plants in the reference field for two reasons. Firstly, comparisons of maize root samples in the managed farmland with those of the dominant vegetation may be inconclusive due to the well-known phenomenon of host-specificity for AMF species (Smith and Read, 2010; Torrecillas et al., 2012). Secondly, the time of sampling was during post-harvest and hence the plants were already harvested. The analysis of the AMF assemblages colonising roots was important in order to gain

insights into whether the arbuscular mycorrhizal fungal community diversity in soils differ from those colonising the roots of the dominant vegetation and may further elucidate differentiation in AMF communities in relation to a specific (or single) host. Samples were transported on ice and stored at -70°C until processing.

4.2.3 DNA extraction from soil and roots

DNA extractions from soils were as described in Chapter 3. For DNA extraction from root samples, roots were gently washed repeatedly in running tap water to eliminate soils. Thereafter, at least 30 tender root hairs per sample were excised, freeze-dried in liquid nitrogen and ground using tungsten beads in a homogenizer (SpeedMill Plus, Analytik Jena AG, Jena, Germany). Thereafter DNA was extracted from 0.1 g of ground root tissues by using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA extraction was performed in duplicates for both soil and roots samples in order to obtain sufficient DNA yields (> 10 ng) as determined by a Nano-drop spectrophotometer (ND-1000, Nano Drop Technologies LLC, Wilmington, DE, USA).

4.2.4 Preparation of partial Glomeromycotan ribosomal SSU library

The Glomeromycotan ribosomal small subunit (SSU) was amplified using a nested-PCR approach for both soil and root DNA samples. A nested approach was used because a single PCR step did not yield sufficient amplicons for subsequent downstream library preparation and sequencing. The primer pairs AML1 (5´-ATCAACTTTCGATGGTAGGATAGA-3´) and AML2 (5´-GAACCCAAACACTTTGGTTTCC-3´) (Lee et al., 2008) was used for the first PCR (yielding approx.800 bp amplicons) while primer pairs AMV4.5NF (5´-AAGCTCGTAGTTGAATTTCG-3´) and AMDGR (5´-CCCAACTATCCCTATTAATCAT-3´) (Sato et al., 2005) were used for the second PCR (approx. 260 bp amplicons). Primers AMV4.5NF and AMDGR contained forward and reverse Illumina overhang adapters, respectively (Illumina Inc., San Diego, CA, USA). Components for the first PCR included 20 ng DNA template, 5 µL of 5 X reaction buffer (New England BioLabs, Ipswich, MA, USA), 0.02 U/µl high-fidelity DNA polymerase (New England

BioLabs), 0.2 µM dNTPs, 0.4 µM of each primer and PCR-grade water in a total reaction volume of 25 µL. The PCR condition was an initial denaturation of 98°C for 3 min, 35 cycles of 98°C for 45 s, 51°C for 40 s and 72°C for 1 min and a final extension of 72°C for 10 min. The components for the second PCR were similar to the first PCR except that 1µl of a 1:10 dilution of the amplicon from the first PCR was used as the template and the primer set AMV4.5NF/AMDGR was used. The conditions for the second PCR was an initial denaturation of 98°C for 5 min, 35 cycles of 98°C for 40 s, 58°C for 1 min and 72°C for 1 min and a final extension of 72°C for 10 min. All PCRs were performed in an Eppendorf 2231 thermal cycler (Eppendorf AG, Hamburg, Germany). All PCR products were verified on 1.5% agarose. Subsequently, amplicon libraries were prepared as described by Illumina (Illumina, CA, USA) for paired-end (2 x 300 bp) sequencing on an Illumina MiSeq sequencer with the MiSeq v3 chemistry (Illumina Inc., USA). Sequencing was performed at the Genomics Platform of the Institute of Integrative and Systems Biology, Universite Laval, Quebec, Canada.

4.2.5 Bioinformatic analyses

Demultiplexed paired-end reads obtained from the sequencing facility were quality-checked using FastQC software version 0.11.5 (Babraham Institute, United Kingdom). Thereafter, Trimmomatic software (version 0.38) (Bolger et al., 2014) was used to quality-trim paired-reads, including clipping off any Illumina barcodes and eliminating reads with an average quality score (Phred Q score) lower than 20. Quality-filtered paired-reads were then analysed in the Quantitative Insight into Microbial Ecology (version 2) (QIIME2) software (Bolyen et al., 2018) by using the DADA2 denoiser (Callahan et al., 2016) to obtain amplicon sequence variants (ASVs) (so-called 100% unique sequences). ASVs were further binned into operational taxonomic units (OTUs) at 97% similarity by using the “open reference" approach which involved aligning ASVs against the AMF virtual taxa (VT) (containing 344 AMF VT sequences) from the MaarjAM database (Öpik et al., 2010), and further de novo clustering of ASVs that failed to hit the AMF VT reference. OTU clustering was performed using VSEARCH (Rognes et al., 2016). Thereafter, the sequences for soil and roots were separated and the respective OTU count tables (for soils and roots) depleted

of singletons and OTUs that occurred in less than three samples. Taxonomic assignment of 97% OTUs were performed against the MaarjAM AMF VT reference taxonomy (Öpik et al., 2010) using the Scikit-learn feature classifier with the default confidence threshold of 0.7 (Pedregosa et al., 2011; Bokulich et al., 2018). The OTU count tables were further sub-sampled (rarefied) to even depths of 19 700 sequences and 10 300 sequences for soils and root samples, respectively, prior to computing alpha- and beta- diversities (in QIIME2) and statistical analyses.

4.2.6 Statistical and AMF community structure analyses

Unless otherwise stated, all statistical analyses were performed in R software version 3.5.3 (R Core Team, 2017). For statistical analyses of AMF community between sample groups (beta-diversity), relative count data were log10-transformed [$\log_{10}$ (x) +1, where x > 0] (Anderson et al., 2006) using the "decostand ()" function in the vegan package (version 2.5-5) of R software (Oksanen et al., 2019). Comparisons between sample groups were based on both unweighted (presence or absence) and weighted (relative abundance) Bray-Curtis dissimilarity matrices. The dissimilarity matrices were further subjected to principal coordinate analyses. Differences in multivariate space were tested using the permutational multivariate analysis of variance (PERMANOVA) in the vegan package (version 2.5-5). To complement the PERMANOVA test, permutational multivariate analysis of dispersion (PERMDISP) was performed to test the homogeneity of multivariate dispersions within groups. Post hoc test for significant PERMANOVA ($P < 0.05$) were further performed using the "pairwiseAdonis ()" function (Martinez, 2019) in the vegan package. A Venn diagram was constructed to depict the number of shared OTUs among sites using the online interactive tool (Heberle et al., 2015).

The influence of environmental factors such as soil physicochemical properties and vegetation cover on the AMF community diversity and structure in both soil and roots were further investigated by Spearman rank correlation and by canonical correspondence analysis (CCA), respectively. CCA was performed on the log-transformed environmental and AMF community data by using an automatic forward and backward stepwise model ("ordistep ()" function) in the

vegan package. Test for significance of the constraining variables were based on a permutation test. Multicollinear environmental (constraining) variables (Variance inflation factor > 10) were excluded from the final CCA plot. The contribution of soil properties and vegetation cover estimate to the AMF community abundance was further elucidated by performing a variance partitioning (chi-square- based partitioning) in the vegan package of R software.

4.2.7 Data availability

Paired-end sequence reads for the partial Glomeromycotan SSU generated from this study have been deposited in the sequence read archives of the national centre for Biotechnological Information under the BioProject ID PRJNA547880 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA547880/).

4.3 Results

4.3.1 AMF OTU diversity in soils and roots

A total of 973 468 and 439 599 high-quality partial Glomeromycotan 18S rRNA sequences from soil and roots, respectively, were assigned into OTUs. Rarefaction at an even depth of 19 700 (155 total OTUs) and 10 300 (80 total OTUs) sequences for soil and roots, respectively, were sufficient to describe the OTU diversity (See rarefaction curve in Figure 4.S1). The total number of OTUs in the unmined soil (113 OTUs) was higher than observed in soils of the reclamation areas (Figure 4.1). The number of shared (‘core’) OTUs (32 OTUs, 5.9 %) amongst all the six soil “treatment” groups (one unmined and five reclamation ages) were greater than those shared amongst five or less soil ‘treatment’ groups. Amongst reclamation soils, the total number of OTUs increased directly with reclamation age up to the nineteenth year (i.e. 75 OTUs in ReclZ-3 < 80 OTUs in ReclZ-11 < 95 OTUs in ReclZ-15 < 103 OTUs in ReclZ-19) but reduced by the twenty-fourth year (77 OTUs in ReclZ-24) (Figure 4.1a). No OTUs were unique to ReclZ-3 and ReclZ-24. Lesser OTU richness was observed in roots compared to the soil for each reclamation age (Figure 4.2 and Figure 4.S2). Similar to the soil OTU/species richness (SR), the number of OTUs

(26 OTUs) observed in roots from all five reclamation soil ages was higher than those shared between any four or fewer treatment groups (Figure 4.1b). Likewise, the lowest total number of OTUs were observed in ReclZ-3 (Figure 4.1b).

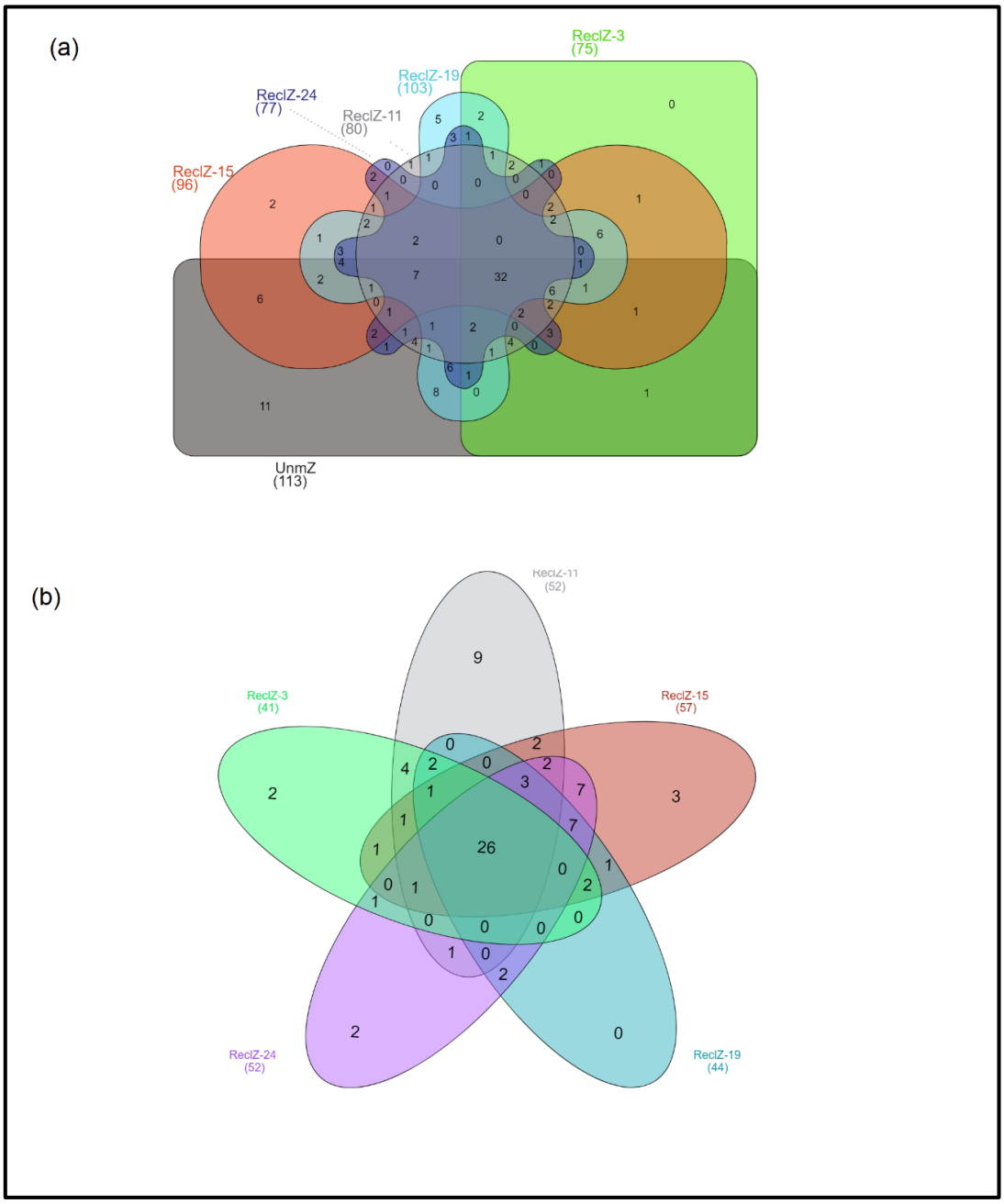

Figure 4.1: **Shared AMF OTUs.** (a) Soil (b) Root. OTU count table for soil and root are rarefied to a depth of 19700 and 10300 sequences, respectively. The values in the bracket after each site name is the total number of OTUs in the group. Venn diagram was constructed by using the online interactive tool (Heberle et al., 2015).

Table 4.1: Mean alpha diversity indices for AMF communities across soils and roots

Diversity indices	Sample type	Reclamation chronosequence					Unm-Z
		ReclZ-3	ReclZ-11	ReclZ-15	ReclZ-19	ReclZ-24	
OTU richness	Soil	36.00±7.00[a]	34.00±6.00[a]	43.00±6.00[a]	45±13.00[a]	35±10.00[a]	47.00±29.00[a]
	Root	18.00±6.00[a]	24.00±9.00[a]	30.00±4.00[a]	24±6.00[a]	28.00±6.00[a]	-
Shannon-Wiener (*H´*)	Soil	3.36±0.34[a]	3.19±0.37[a]	3.71±0.63[a]	4.03±0.28[a]	3.59±0.25[a]	2.94±1.51[a]
	Root	2.45±0.17[a]	2.94±0.56[a]	3.01±0.21[a]	2.97±0.18[a]	2.60±0.44[a]	-
Dominance	Soil	0.65±0.06[a]	0.63±0.05[a]	0.68±0.10[a]	0.72±0.06[a]	0.71±0.07[a]	0.53±0.19[a]
	Root	0.32±0.03[a]	0.23±0.08[bc]	0.22±0.02[c]	0.21±0.03[c]	0.30±0.08[ab]	-
Pieolu's evenness	Soil	0.18±0.07[a]	0.18±0.05[a]	0.15±0.09[a]	0.12±0.06[a]	0.15±0.04[a]	0.32±0.23[a]
	Root	0.60±0.04[a]	0.65±0.06[a]	0.62±0.02[a]	0.65±0.05[a]	0.54±0.07[a]	-

Values (mean ± SD) on the same row with different superscript letters are significantly different ($P < 0.05$) based on Fisher's least significant difference. The average OTU richness is rounded off to the nearest whole number. -, not determined.

The average SR, Shannon-Wiener index of diversity (*H´*), dominance (D) and Pieolu's evenness (*J'*) (a measure of species proportionality within a community) for soil AMF OTUs were not significantly different (Table 4.1). In the root AMF community, D was significantly higher ($P < 0.05$) in ReclZ-3 compared to ReclZ-15, ReclZ-11 and ReclZ-19, whereas SR, *H´* and *J'* were not significantly different ($P > 0.05$) (Table 4.1). Within each reclamation age, SR was significantly different ($P < 0.05$) between soil and roots in ReclZ-3 and ReclZ-19 (Figure 4.S3). Whereas, *H'* and D were significantly different ($P < 0.05$) between soil and roots in each of ReclZ-3, ReclZ-19 and ReclZ-24 (Figure 4.S3). In contrast, *J´* was not significantly different ($P > 0.05$) between soil and roots in all reclamation soils (Figure 4.S3).

4.3.2 Differentiation of AMF communities in soil and roots

Bray-Curtis dissimilarity between soil AMF community composition (unweighted measure, absence or presence) and structure (weighted measure, relative abundance) revealed large differentiation between reclamation and unmined soils (Figure 4.2a and 4.2b). Close associations were observed in multivariate space between ReclZ-3 and ReclZ-11, and between ReclZ-19 and ReclZ-24 in both the unweighted and weighted PCoA plots of the Bray-Curtis dissimilarities (Figure 4.2a and 4.2b). The differentiation observed in multivariate space for soil AMF community are significant (Unweighted PERMANOVA $R^2 = 36.2\%$, $P = 0.001$, PERMDISP $P = 0.26$; Weighted PERMANOVA $R^2 = 35.9\%$, $P = 0.001$, PERMANOVA $P = 0.124$). These observations are also similar to those obtained for the Unifrac (phylogenetic) distances (Figure 4.S4a and 4.S4b). Pair-wise post hoc comparisons for PERMANOVA revealed that AMF community composition and structure are significantly different (False discovery rate (FDR)-adjusted $P < 0.05$) between the unmined soil and each of the post-mining reclamation soil ages (Table 4-S1), as well as between some pairs of post-mining soil ages (Table 4.S1). In particular, AMF community composition and structure in ReclZ-15 significantly differed (FDR-adjusted $P < 0.05$) from those of all other ages (Table 4-S1). Phylogenetically (unifrac distances), differentiation in AMF community composition within the reclamation soil

chronosequence appear stochastic over chronological time—the pattern of significant differences between ages did not indicate a specific trend (Table 4-S1).

AMF community composition and structure colonising roots in older reclamation areas (ReclZ-15, ReclZ-19 and ReclZ-24) are similar (Figure 4.2c and 4.2d) but heterogeneous within and between younger reclamation areas (ReclZ-3 and ReclZ-11) (Figure 4-2c and 4.2d). Differentiations in the AMF community composition (unweighted) colonizing *Eragrostis tef* roots are generally significant (PERMANOVA R^2 = 24.8%, *P* = 0.044; PERMDISP *P* = 0.220), whereas, differentiation in community structure (weighted) are not significant (PERMANOVA R^2 = 24.8%, *P* = 0.05; PERMDISP *P* = 0.238). However, the FDR-adjusted *P*-values for differences in multivariate space for AMF community composition and structure between any two reclamation ages are not significant for both Bray-Curtis dissimilarities and unifrac distances (Table 4.S2).

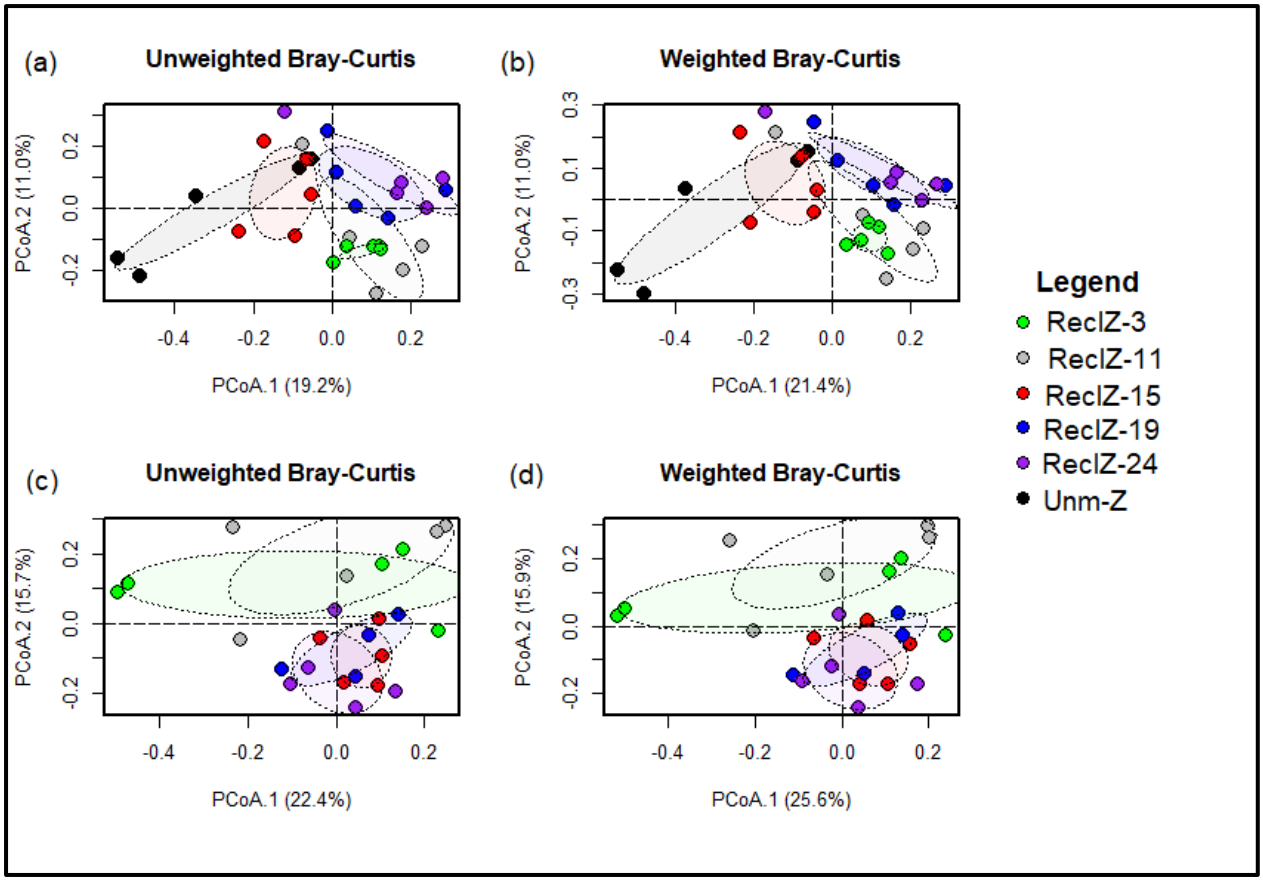

Figure 4.2: Principal coordinate analysis (PCoA) of the Bray-Curtis dissimilarity between AMF communities. (a-b) soil (c-d) root. The ellipses show 95% confidence interval in multivariate space around each group's centroid (median). Pair-wise posthoc comparisons for soil and root communities are presented in Table 4-S2 and Table 4-S3, respectively.

4.3.3 AMF species composition and differentiation across chronological time-space

The genera *Archaeospora*, *Acaulospora*, *Diversispora*, *Scutellospora*, *Claroideoglomus*, *Glomus* and *Paraglomus* were detected in soil and roots (Figure 4.3a). *Glomus* dominated (relatively) the AMF communities of both soil and root, while *Diversispora* was least dominant and less associated with roots (Figure 4.3a). *Acaulospora* was more relatively more abundant in root than in soils. On average, most unclassified sequences at the genus taxonomic level are from the soil (Table 4-S3 and Figure 4.3a). Furthermore, *Diversispora* was only associated with soils in ReclZ-15, ReclZ-19 and the unmined soil. Whereas in roots, *Diversispora* was

only associated with ReclZ-11 (Table 4.S3). The average relative abundances of *Archeospora*, *Claroideglomus*, *Paraglomus* and *Scutellospora* were higher in all reclamation soils compared to soil of the unmined maize field (Table 4.S3). In contrast, *Glomus* was relatively more abundant in the unmined soil compared to reclamation soils (Table 4.S3). Across reclamation areas, the average relative abundance of *Glomus* increased from 66.93±12.19% in ReclZ-3 (the third year after reclamation) to 76.85±19.00 % in ReclZ-24 (the twenty-fourth year after reclamation), whereas *Acaulospora* generally reduced over reclamation years (from an average of 4.51% in ReclZ-3 to 0.95% in ReclZ-24). Significantly different (FDR-adjusted $P < 0.05$) genera amongst soils included *Archaeospora*, *Claroideoglomus*, *Diversispora* and *Paraglomus* (Figures. 4.4a-d).

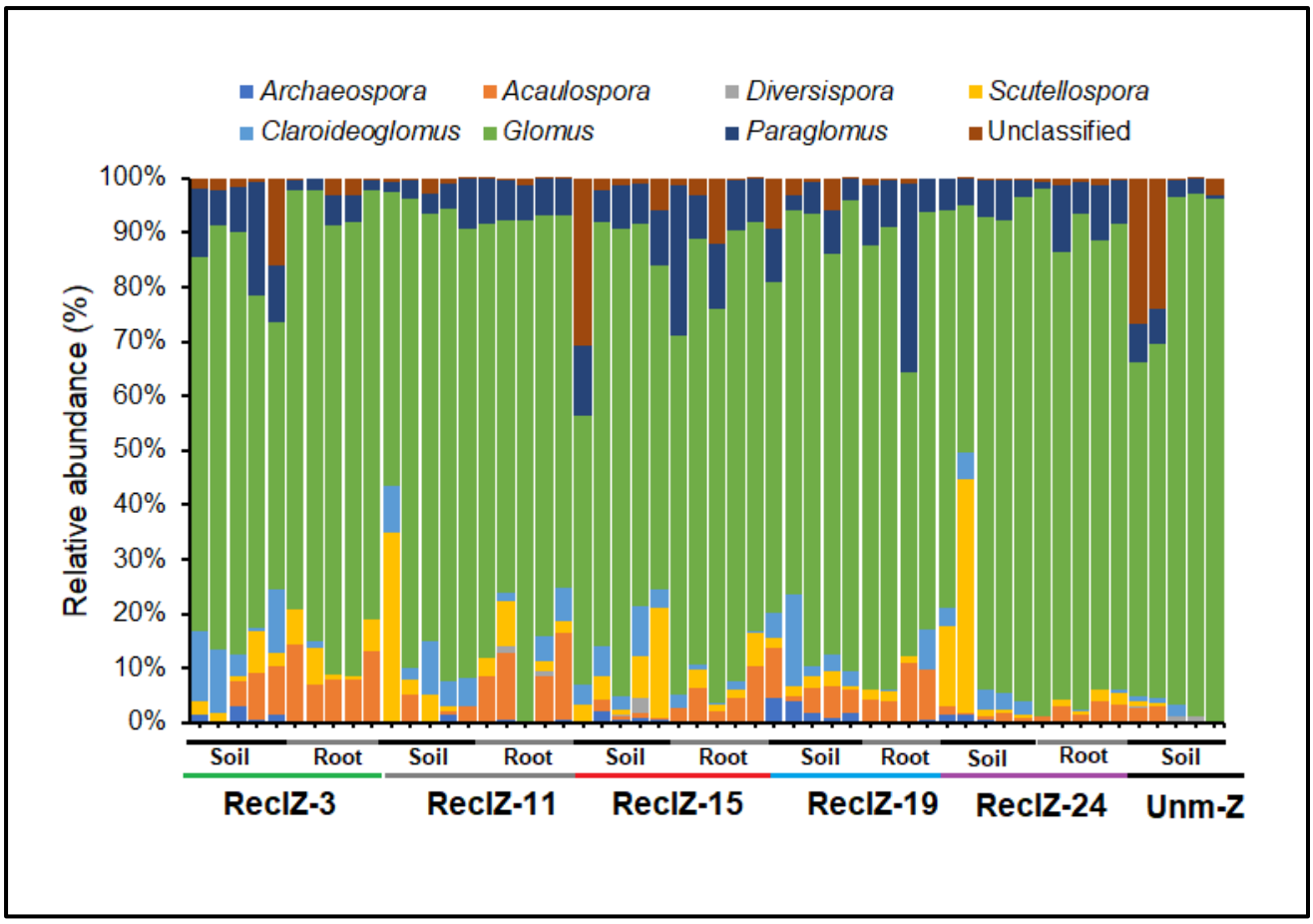

Figure 4.3: **Relative abundance of AMF genera in soil and roots.** OTU count table for soil and roots were both rarefied to a depth of 10300 sequences for the comparison.

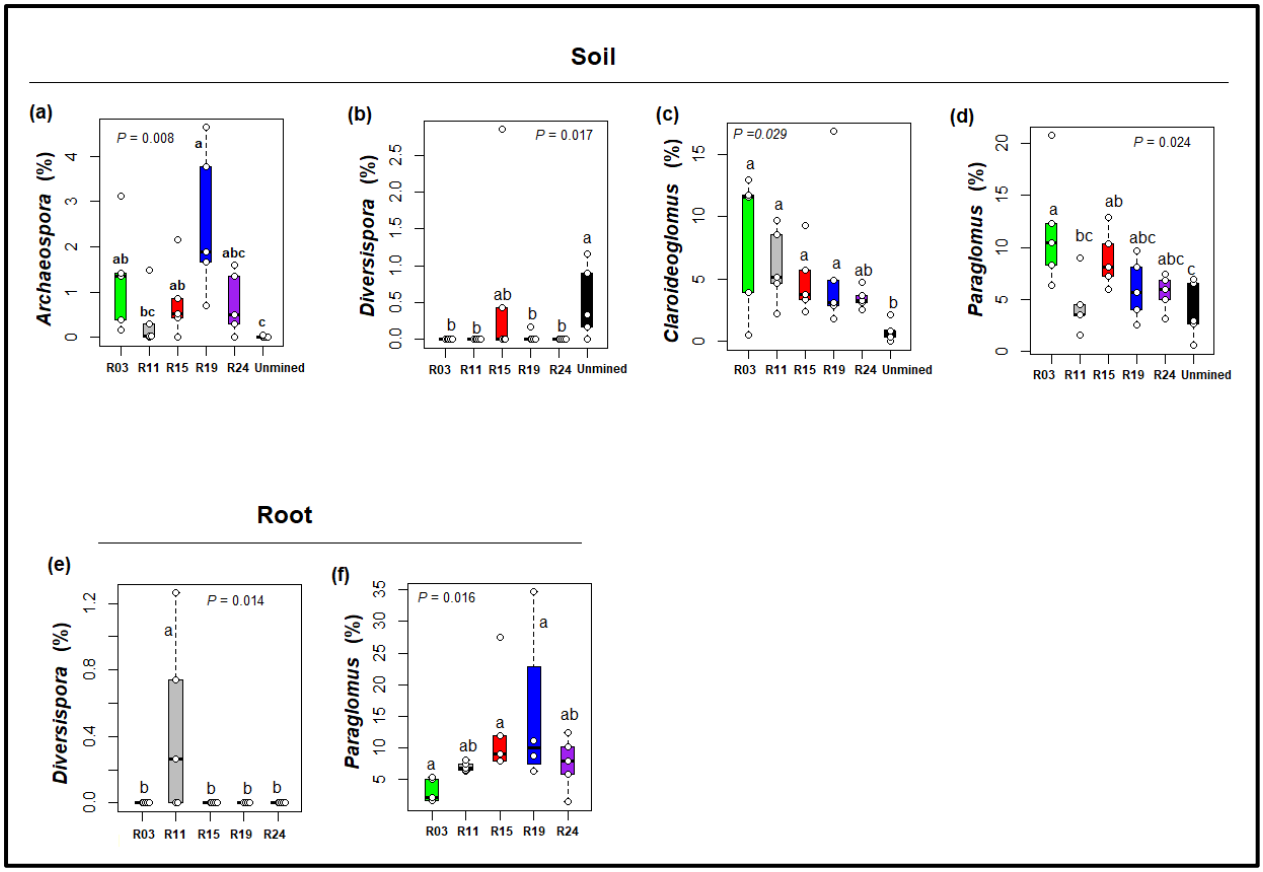

Figure 4.4: **Significantly differentially abundant AMF taxa.** (a-d) Soil (e-f) Roots. Significantly difference is based on Kruskal-Wallis $P < 0.05$. Boxplots with different alphabet letters are significantly different based on Fisher's LSD (false-discovery rate-adjusted $P < 0.05$). R03, ReclZ-3; R11, ReclZ-11, R15, ReclZ-15; R19, ReclZ-19; R24, ReclZ-24; Unmined, Unm-Z.

Similar to patterns in soil, the average relative abundance of the dominant *Glomus* species colonising roots increased over reclamation years (Table 4.S3). However, unlike in soil, the average relative abundance of *Glomus* species initially reduced somewhat steadily until the nineteenth year of reclamation (73.78% in ReclZ-19) and subsequently increased to an average of 87.75% by the twenty-fourth year of reclamation (ReclZ-24) (Table 4.S3). The average relative abundance of *Paraglomus* increased steadily over reclamation years and peaked in the nineteenth year of reclamation (Table 4.S3). In contrast, *Acaulospora* generally reduced across reclamation years in the root (Table 4.S3). Significantly different (FDR-adjusted $P < 0.05$) genera amongst roots included *Paraglomus* and *Diversispora* (Figures 4.4b

and 4.4c). On the other hand, significantly different genera between soil and roots for each reclamation age/area included *Glomus*, *Acaulospora*, *Paraglomus* and *Claroideoglomus* in ReclZ-3 (Figure 4-S5a-d), *Acaulospora* and *Claroideoglomus* in ReclZ-15 (Figure 4.S5e-f), *Archaeospora* in ReclZ-19 (Figure 4.S5g) and *Claroideoglomus* in ReclZ-24 (Figure 4.S5h).

4.3.4 Influence of environmental factors on AMF community and species diversity

Correlation test between alpha diversity indices of AMF OTUs in soil and soil physico-chemical properties revealed that *H´* was significantly correlated with N-NH_4^+ (Spearman rank correlation r = -0.43, *P* = 0.03), silt (r = -0.44, *P* = 0.03) and litter (r = -0.54, *P* = 0.01), while *J'* was significantly correlated with nitrate (r = 0.51, *P* = 0.04) and amount of litter (r = -0.47, *P* = 0.02) (Table 4.S4). In contrast, D was positively correlated with nitrate (r = 0.41, *P* = 0.04). In roots, *H´* was negatively correlated with foliar (r = -0.57, *P* < 0.00) and basal (r = -0.45, *P* = 0.03) covers, while D was negatively correlated with pH (r = -0.42, *P* = 0.04) but positively correlated with foliar cover (r = 0.55, *P* = 0.01) and basal cover (r = 0.49, *P* = 0.02).

The step-wise model for the CCA triplot in Figure 4.5 is significant (*P* = 0.028) for AMF community in soil (Figure 4.5a), but not significant the root AMF community (*P* = 0.511) (Figure 4.5b). Precisely 64.8% and 74.2% of the variation in AMF community distribution in soil and root, respectively, were explained by environmental variables. However, with plant cover estimates included in the CCA model for only chronosequence soils, up to 84% variation in the AMF community was explained by environmental variables (data not shown). Significance of environmental terms fitted into the step-wise CCA model revealed that nitrates (NO_3^-), clay content and BD significantly influenced (*P* < 0.05) the AMF community in soil (Table 4.S5). Whereas, the influence of environmental variables on AMF species colonising the root was not significant (*P* > 0.05) (Table 4.S5). Nevertheless, the distribution of *Archaeospora* and *Diversispora* in roots appear to be associated with Na and nitrogen forms (NH_4^+ and NO_3^-), while *Paraglomus*, *Glomus*, *Acaulospora* and *Claroideoglomus* were least influenced by

environmental variables (Figure 4.5b). Based on variance partitioning (Figure 4.6), soil physical properties (textural fractions and bulk density) solely explained 26% of the variation in AMF community structure along the post-mining reclamation chronosequence, while soil nutrients and pH explained a combined 5%. However, a higher proportion of the variation in soil (58%) and roots (approx.100%) could not be explained by the environmental variables (Figure 4-6), thereby suggesting the role of other underlying factors in the shaping AMF structure in soil and roots of the dominant vegetation along the chronosequence.

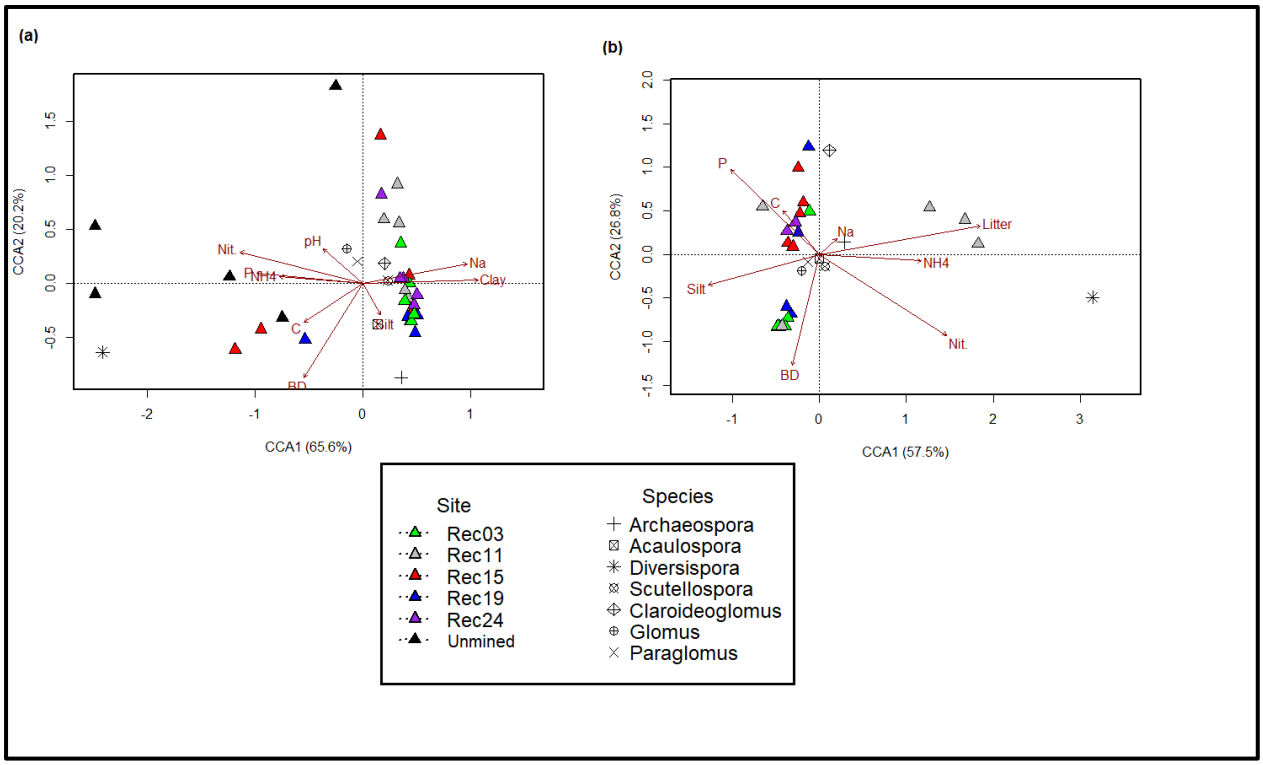

Figure 4.5: Canonical correspondence analysis (CCA) showing the relationship between soil Physico-chemical properties and AMF community. (a) soil (b) roots. CCA model for soil is significant ("anova.cca ()", $P < 0.05$) whereas model for root is not significant ($P > 0.05$). The first canonical axis (CCA1) of the CCA plot for soil is significant ($P = 0.044$).

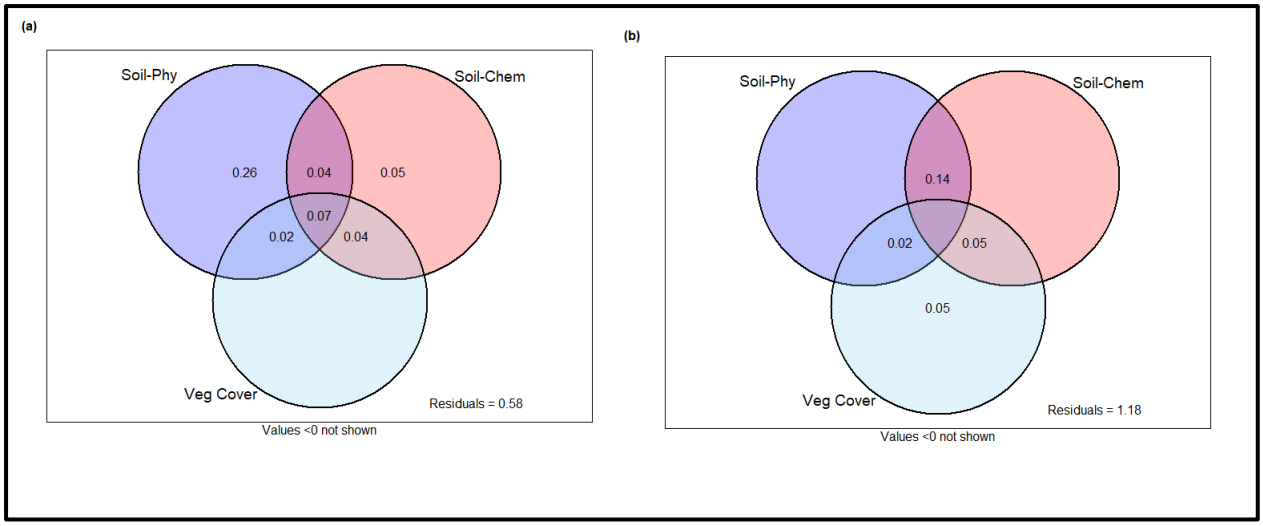

Figure 4.6: Variation in the AMF community structure explained by environmental variables across chronosequence. (a) Soil (b) root. Soil-Phy: textural fractions and bulk density; Soil-Chem, soil nutrients and pH; Vegetation: plant cover, litter and basal covers. Values are partitioned chi-square adjusted values.

Analysis of associations between soil physico-chemical properties and relative proportions (%) of AMF genera in soil revealed significant correlations ($P < 0.05$) between pH and *Diversispora* (r = -0.51, $P < 0.01$), *Paraglomus* (r = -0.51, $P < 0.01$) and *Scutellospora* (r = -0.58, $P < 0.01$) (Table 4-S6). Similarly, nitrates were correlated with *Diversispora* (r = -0.61, $P < 0.01$) and *Scutellospora* (r = -0.47, $P < 0.01$), while phosphorus was correlated with *Glomus* (r = 0.42, $P = 0.02$), *Diversispora* (r = -0.59, $P < 0.01$) and *Scutellospora* (r = -0.40, $P = 0.03$). Most of the cations (K, Mg, Ca and Mg) were significantly correlated ($P < 0.05$) with *Scutellospora* in the soil (Table 4.S6). *Claroideoglomus* correlated with Na (r = -0.39, $P = 0.03$) and clay content (r = -0.42, $P = 0.02$), while the association between BD and *Acaulospora* was significant (r = 0.38, $P = 0.04$) (Table 4.S6). On the other hand, *Glomus* in root was significantly ($P < 0.05$) correlated with pH and Ca (Table 4.S7), while *Paraglomus* was significantly correlated with pH, nitrates, phosphorus, Na, foliar cover and basal cover (Table 4.S7).

4.4 Discussion

AMF assemblages within a landscape are shaped by niche differentiation although random processes such as those mediated by anthropogenic activities may also play roles in shaping AMF assemblages (Dumbrell et al., 2010a; Dumbrell et al., 2010b; Moebius-Clune et al., 2013b). The present study utilised high-throughput sequencing of the Glomeromycotan small subunit of the ribosomal gene to investigate the diversity and structure of AMF assemblages across a post-coal mining reclamation soil chronosequence. In addition, insights into environmental variables which shape differentiation in AMF assemblages in soil and root of the dominant vegetation across the chronosequence were explored.

4.4.1 OTU richness and diversity across the chronosequence

Core OTUs (number of OTUs shared across all soil or root samples) were larger than the number of OTUs common to any other sub-groups indicating a high similarity of common species across the landscape. This core OTUs are likely to be influenced by the dominant singular vegetation species across the reclamation landscape rather than soil physicochemical properties, which varied across the chronosequence. In a study by Krüger et al. (2017), AMF structure along a post-coal mining soil heap in Czechia was predominantly shaped by plant communities in comparison to abiotic factors. The influence of plant communities on AMF diversity over chronological time may be due to changes in plant-specific nutrient requirement and host-specificity due to plant community succession during post-mining ecosystem development (Martínez-García et al., 2015; de León et al., 2016; Krüger et al., 2017). Similar to the observations made by Krüger et al. (2017) and Johnson et al. (1991), the average species richness and diversity (*H'*) in soil and root AMF community did not significantly differ over chronological time. The observation is also similar to those obtained with universal fungal ITS2 primers in Chapter 3, and thus negates one of the underlying assumptions for the specific analyses of AMF diversity within the post-coal mining reclamation

soil. Because a single grass species was dominant across the post-mining reclamation, the non-significant differences across the landscape may be due to a strong association between AMF and the dominant host plants across the chronosequence. In their work, Martínez-García et al. (2015) reported that of all factors influencing a chronosequence, the host plant type appeared to be the dominant drivers of AMF diversity. Being strict endosymbionts, AMF are tightly coupled to a specific host (Martinez-Garcia et al., 2015). However, Cui et al. (2016) report that AMF diversity significantly reduced over time in a reclaimed coastal alkaline-salt soil revegetated with different plant species, while Koske and Gemma (1997) observed increase in AMF species richness over chronological time in sand dunes of large-scale plantings of *Ammophila breviligulata* (beachgrass) near Provincetown, Massachusetts, USA. Progressive and retrogressive stages of long-term ecosystem development are characterised by alternating levels of soil nutrients and plant diversity at a given time point (Dickie et al., 2013). Such alternating levels of specific soil nutrients may foster or negate plant (host)-dependency on mycorrhizal interactions for nutrient scavenging at any given time point. The preceding phenomenon was probably the case with the observed alternating trends in OTU richness and diversity with reclamation age. For example, the fertilisation of post-mining lands as part of the reclamation process may increase available soil P, thereby limiting the need for mycorrhizal symbiosis between plants and AM fungi. However, because these sites are never fertilized over many years since reclamation, levels of available phosphorus may decline thereby necessitating plants' need for mycorrhizal symbiosis.

Conventional agriculture and ecological disturbances have been reported to influence soil AMF richness (Roy et al., 2017; Stover et al., 2018). However, the non-significant differences in OTU richness and diversity between the reclamation areas and the unmined agricultural soil suggest otherwise. This observation is similar to those obtained for the fungal community analyses utilising universal primers in Chapter 3. It is also plausible that the contrasting observation may be attributable to the commonly reported likelihood of high-throughput

sequence count rarefication to distort the 'true' species richness of the studied environment (McMurdie and Holmes, 2014; Hart et al., 2015). Nevertheless, the significantly higher species dominance in the root of the youngest reclamation site indicates possible host selection preference for specific species, which may contribute to the adaptation of *Eragrostis tef* (or other vegetation species) in the early years since reclamation (or revegetation). Some studies have shown that colonisation of plants by specific AMF species are required for the plant establishment during early stages of ecological succession (Johnson et al., 1991; Koske and Gemma, 1997; Husband et al., 2002). For example, Johnson et al. (1991) identified certain AMF species that are clearly early successional and late-successional in field and forest sites in Minnesota, USA. Similarly, Kiers et al. (2000) reported that seedlings of pioneer plants were more dependent on AMF inoculations for initial growth and survival, while Husband et al. (2002), observed that certain species which were rare in seedlings were more dominant after a year of growth in a tropical forest and replaced species which were earlier present in the seedlings. Such succession in AMF species colonising plant during ecological successional stages may be due to AMF functionalities. For example, late-successional AMF species have been found to require more plant carbon (high carbon drain) than early-successional counterparts and hence were less beneficial during initial seedling development in a tropical forest in Mexico (Allen et al., 2003).

4.4.2 Community structure and composition

Like for the fungal community analysed using fungal ITS2 primers (in Chapter 3), the community composition and structure of AMF assemblages were differentiated in multivariate space, particularly between reclamation- and unmined agricultural soils. However, unlike in Chapter 3, the differentiation in community composition and structure were not very distinct (overlaps in confidence intervals in multivariate space was observed in multivariate space for each reclamation age and unmined farmland soil community) suggesting that AMF community composition and structure are not sensitive when considered solely of other fungal groups.

Nevertheless, the somewhat differentiation suggests the influence of host-plant communities between the managed agricultural ecosystem and reclamation sites, as well as the potential influence of a complex interaction between soil-physicochemical properties (Dumbrell et al., 2010a; Moebius-Clune et al., 2013a; Moebius-Clune et al., 2013b). The close associations observed in the AMF community composition and structure in soil between the 3-year-old and 11-year-old site and between the 19-year-old site and 24-year-old site suggest gradual shifts in AMF community composition over chronological time, which may be related to plant community succession during ecosystem development (Johnson et al., 1991; Husband et al., 2002; Dickie et al., 2013; de León et al., 2016). Furthermore, the non-significant difference observed in the community structure of AM fungi colonising the roots of the single dominant host (*Eragrostis tef*) across the chronosequence indicates host-association effects. However, the observed similarity in the composition and structure of mycorrhizal communities colonizing roots in older reclamation areas (ReclZ-15, ReclZ-19 and ReclZ-24) but heterogeneity within and between younger reclamation soil suggest differentiation into late and early successional stages observed during ecological development (Johnson et al., 1991; Koske and Gemma, 1997), as well as the development of a climax community (stability in structure in later years) with respect to chronological time.

4.4.3 Dominance and potential roles of AMF species

Similar to other studies (Daniell et al., 2001; Cui et al., 2016; Zhao et al., 2017), the genus *Glomus* was the most dominant in both soil and roots across the reclamation and unmined. Similarly, the persistence of a dominant AMF species has been observed in soils across a successional gradient—from open sand prairie to a closed oak-hickory forest (Benjamin et al., 1989). The dominance of the *Glomus* species in the soils and roots may be due to host-specificity preference, functional relevance, adaptation and ease of propagation in the soil ecosystem (Daniell et al., 2001; Hart and Reader, 2002; Cui et al., 2016; Wang et al., 2019a). For example, *Glomus* species were reported to preferentially colonise herbaceous plants

(Torrecillas et al., 2012) while Spruyt et al. (2014) observed higher colonisation preference for *Tamarix usneoides* (wild tamarisk) in comparison to other plants used in post-mining mine waste rehabilitation. The differential abundance patterns (increase or decrease) observed for specific genera over reclamation years are most likely due to the factors which influence species richness and diversity discussed earlier.

Although mechanisms driving the functional diversity of AMF species are still unclear, evidence suggests that functional contribution of AMF species are most likely related to plant-specific nutrient requirements against the backdrop of the prevailing soil physicochemical conditions (Feddermann et al., 2010; Lee et al., 2013). The differentially abundant *Archaeospora*, *Diversispora*, *Claroideoglomus* and *Paraglomus* at different reclamation years may indicate that these species are more functionally relevant to ecological processes and less demanding (sustainable partners for plants) on plant resources at specific successional stages (Johnson et al., 1991; Allen et al., 2003). In the present study, patterns in the average relative abundance of species colonising roots of *Eragrostis tef* suggest that *Paraglomus* species are likely more functionally active in later stages of post-mining ecosystem development, while *Acaulospora*, *Diversispora* and *Scutellospora* and are more functionally involved in the earlier stages. The observation of *Diversispora* in roots of *Eragrostis tef* in only reclamation site of 11 years may be linked to the lower average phosphorus levels in the site compared to other sites. In available P-limiting soils, the establishment of mycorrhizal symbiosis is beneficial to plant for scavenging and mobilising phosphorus (Barea et al., 2002; Smith and Read, 2010). Additionally, the detection of *Diversispora* in soils (of the 15- and 19-years sites) suggest the completion of their life cycle from colonising roots (of the 11-year site) to sporulation in the soil—the spores persist and are detected in later ages (15-yr and 19 yr. sites). The differentiation in the relative abundance of these species over chronological time may be adapted for use as soil ecosystem development indicators and can also inform decision on the selective application of AMF species as biofertilizer inoculum at different time points of reclamation—at the onset of reclamation and over several years. Due to the use of

short-read fragments of the SSU, the identification of the AMF diversity could not be reliably identified up to species level. Indeed, taxonomic delineation to finer taxonomic ranks (e.g. species level) will further enhance the accuracy of such potential bioindicator applications. The development and utilisation of high-resolution taxonomic markers, the sequencing of longer DNA fragments using latest sequencing technologies such as the single molecule real-time sequencing, as well as the availability of an expanded Glomeromycotan database will likely help identify AMF species to such finer taxonomic levels (Schlaeppi et al., 2016; Bruns et al., 2018).

4.4.4 AMF community diversity and structure are influenced by environmental parameters

The influence of soil-physicochemical properties on AMF community diversity is predominantly due to the dynamic relationship between plant-dependency on mycorrhizal associations and nutrient availability—nutrient-rich or limiting conditions (Raverkar and Bhattacharya, 2014). Although AMF-plant symbiosis is generally fostered by nutrient-limiting conditions, AMF communities in the soil ecosystem may be influenced by a complex interaction between abiotic and biotic factors (Dumbrell et al., 2010b). The significant inverse associations between soil AMF species diversity (*H'*) and NH_4-N, silt and litter content suggest that AMF species are less diverse and probably less functionally active in the presence of higher amounts of ammonium compounds and nutrient-rich silt fraction and plant litter. A similar inverse relationship between *H'* and organic carbon, NH_4^+ and NO_3^- have been reported by Cui et al. (2016) on reclaimed coastal saline-alkaline lands in China. On a community scale, the differentiation in soil AMF communities were significantly explained by soil nitrate, clay fraction and bulk density. The influence of nitrogen (Avio et al., 2013; Xu et al., 2017), soil texture (Moebius-Clune et al., 2013b) and bulk density (Yang et al., 2018) on AMF communities have also been reported. On a population level, the influences of environmental variables, including pH, nitrate, phosphorus, cations (K, Mg, Ca and Mg, Na) and plant cover may be species-specific as

observed in the present study and a study by Wang et al. (2019a). However, because most soil properties are often inter-related, the influence of individual environmental properties on AMF communities may be consequent on one or more other co-dependent factors, and may thus vary for different ecosystems. Nevertheless, for a given soil ecosystem, a combination of soil physiochemical properties which directly or indirectly influence nutrient availability and mobilisation is most likely to contribute to AMF community differentiation in soil and plant root.

4.5 Conclusion

Compared to Universal fungal primers used in Chapter 3, more AMF species were identified showing that Glomeromycotan-specific primers are more suitable for studying AMF richness and diversity. However, unlike observations made in Chapter 3, the structure and composition of AMF communities in soils are less differentiated across the ecosystem (post-reclamation) age or between reclamation and the unmined farmland. Thus, for the present study sites, AMF assemblages appear to be less suitable bioindicators of ecosystem development compared to the total fungal assemblages of the soil as determined by the analyses of the ITS2 region sequences.

Furthermore, seven Glomeromycotan genera, as well as dominant and differentially abundant genera across stages of ecosystem development post-mining reclamation, were unravelled. The differentiation in the relative abundance of these species over chronological time may be adapted for the selective application of AMF species as biofertilizer inoculum at different time points of reclamation—at the onset of reclamation and over several years. Species richness and diversity in both soil and root were influenced by several environmental factors, including ammonium, nitrate, silt content, litter, pH, foliar and/or basal covers. On a community level, nitrate, clay and bulk density significantly influenced the AMF communities.

Based on the co-occurrence of similar AMF species in soil and roots of the dominant vegetation, the study points to the influence of host-specificity in shaping AMF assemblages

in soils along the post-mining reclamation chronosequence. Because the obligate symbiotic relationship between AM fungi and the plant hosts also depend on a complex interaction in the levels and dynamics of soil nutrients, the utilisation of AMF species richness, diversity and community structure for monitoring ecosystem restoration in post-mining reclamation areas is not as straightforward compared to utilising non-obligate soil microflora. Rather, the comparable diversity and richness of the AMF observed across the reclamation soil chronosequence and unmined farmland when taken together with the results of AMF spore density across stockpiles reported in Chapter 2, may suggest an equal likelihood of root colonization and nutrient mobilization by AMF across the topsoil horizon in mining-impacted (stockpile and reclamation) soils.

In the overall context of the study, the present chapter and preceding experimental chapters (2 and 3) show the potential of soil microbial communities as ecological indicators of ecosystem restoration as well as suggest restoration of microbial community richness and diversity (especially for bacterial species) over time. Because, the soil ecosystem supports numerous interactions such as those between soil microflora and fauna, further studies utilizing other soil bioindicator fauna may provide a holistic picture regarding the potential of post-mining reclamation soils to support such interactions (biocoenosis). Such an assessment may be required in order to establish support function of coal-mining impacted soils and the potential restoration of such support functions over years since reclamation.

CHAPTER 5:

UTILISING EARTHWORM (*EISENIA ANDREI*) BIOASSAYS IN ASSESSING ECOSYSTEM SUPPORT FUNCTION OF STOCKPILES AND POST-MINING RECLAMATION SOILS

5.1 Introduction

The soil is a habitat for diverse micro flora and fauna. Within the soil environment, a number of interrelationships exist, including trophic relationships such as food chains or webs. Within the soil biological communities, ecological niches such as those of decomposers, and ecological engineers such as earthworms are vital to the ecological balance of the soil ecosystem.

Earthworms are important soil fauna that are essential to several soil health processes including decomposition and stability of organic matter, nutrient cycling and soil structure (Aira et al., 2006; Thakuira et al., 2010). They are 'key species' in the soil food, occupying an important niche as soil ecosystem engineers (Thakuira et al., 2010; Pulleman et al., 2012). Their disappearance can have strong impacts on other levels of organisation of ecosystem biological hierarchy (Pulleman et al., 2012). Hence, they are important bioindicators of soil ecosystem health (Paoletti, 1999; Pulleman et al., 2012). The role of the earthworm in organic matter decomposition and nutrient cycling is dependent on earthworm-gut microbiota interactions (Aira et al., 2006; Thakuira et al., 2010; Zhao et al., 2010). The earthworm gut is an anaerobic cavity which supports a number of ecological functional microbes, including nitrogen-fixing, methanogenic, nitrate-reducing and fermentative bacteria (Thakuira et al., 2010; Pass et al., 2014). These bacteria transform the chemistry of the soil as it passes via the gut (Aira et al., 2006; Pass et al., 2014), thereby transforming the soil ecosystem overall. Also, the microbiota of the surrounding soil and substrate the earthworm feeds on contributes to the earthworms' gut-associated microbiome. However, the microbial profile of the earthworm gut is not a coincidental composite of the microbiota of the surrounding soil and

ingested substrate—some of the microorganisms pass through the gut and are shed off in the cast, while some may colonize the gut (Thakuira et al., 2010). At different taxonomic levels, gut host-associated microbiota may be linked to certain hosts' phenotypic expressions. For example, the different earthworm ecological groups (anecic, endogeic, epigeic) have been found to possess ecological-group specific microbiota which reflects their digestion and assimilation patterns (Thakuira et al., 2010). Hence, disturbances in the soil ecosystem which alter the physical-chemical and biological properties of the soil can have impacts (of large ecological proportions) on the gut microbiome and plasticity of the earthworm.

Current post-coal mining reclamation practices do not take into cognisance the restoration of soil support function as suitable habitat, especially for soil biocoenosis. The ability of the soil to support the growth, fitness, reproduction, ecological interactions and functions of soil biological communities is an indication of the soil's suitability and health. Because the South African climate is semi-arid and field-soils (non-agricultural) generally dry and compact, field assessments of earthworm abundance and fitness are usually not as realistic as they are for moister and less compact agricultural soils (Person Com. Prof M.S. Maboeta). Hence, bioassays which ascertain the fitness and reproduction of soil mesofauna when exposed to test soils can be utilised as indirect measures for assessing the habitat function of soil (ISO, 2008; Otomo et al., 2013; Maboeta et al., 2018).

Such bioassays utilising earthworms as representatives of the soil biocoenosis have been standardized and performed on soils obtained from rehabilitated and non-rehabilitated gold mine tailings (van Coller-Myburgh et al., 2015; Maboeta et al., 2018), platinum mine tailings (Maboeta et al., 2008) and chromium mine waste (van Coller-Myburgh, 2014) in South Africa, as well as to test the potential effect of agrochemicals on the soil biota (Fouché et al., 2016; Oladipo et al., 2019). However, there is a paucity of such assessments for coal mining-impacted soils, including coal mines heap (soil stockpiles) or post-coal mining reclamation soils in South Africa. Currently, it is unknown what impacts coal-mining activities may have on the ecosystem support functions of soils around coal mines, especially soils in areas that have

been deemed “reclaimed”. Therefore, this study aimed to utilise an ecotoxicological approach in assessing the support and habitat function of coal mining-impacted soils (stockpiles and reclamation soils) by utilising earthworm bioassays. By taking into cognizance the ages of the reclamation areas, inference on the potential restoration of ecosystem support functions over the years since reclamation were drawn. If significantly different sub-lethal effects were observed amongst soil treatments, further analysis of possible effects on the earthworm gut would be investigated. Thus, the outcome of the study also provided a justification for further microbial ecotoxicology studies on post-coal mining reclamation soils.

5.2 Materials and methods

5.2.1 Test and OECD control soil

For the earthworm bioassays, bulks soil samples (test soils) were collected in 2018 from coal mining site Z (described in Chapter 3) and an additional mine M (Figure 5.1). In each of these mining sites soil stockpiles (heaps), post-mining reclamation soils (of different ages) and unmined soils were sampled. Soils from unmined areas and stockpiles were sampled at random points, while samples from reclamation soils were sampled in a similar manner as described in Chapter 3—along a 50 m transect at intervals of 10 m, with all six points on a transect composited. In mine M, five reclamation areas of different ages (or years since reclamation), an unmined area and a recent soil stockpile (3 months-old, designated Stock-M) were sampled (Figure 5.1). The reclamation areas sampled in mine M included an area reclaimed in 2017 (1-year-old, designated ReclM-2Y), two areas reclaimed in 2015 (each 3 years old; designated ReclM-3YA and ReclM-3YB), an area reclaimed in 2013 (5 years old, designated ReclM-5Y) and an area reclaimed in 2008 (10 years old, designated ReclM-10Y). Information on the soils used for this study is summarised in Table 5.1. All soil samples were collected at the 0-15 cm using a sterile auger along five 50 m transects at 10 m intervals. The orientation of transects was such that representative samples Prior to the assay, soil samples were homogenized and sieved through 2-mm sieves.

For the earthworm bioassay, bulk artificial soils (control soils) were prepared. The artificial soils contained 70% silica sand, 20% kaolinite clay and 10% sphagnum peat on a dry weight basis (OECD, 2016). The mean pH in (KCl) of the artificial soil was 5.75. Prior to the assays, the pH and maximum water holding capacity of the test and control soil was determined as described by the ISO (1994) and ISO (1996), respectively. Each representative quintuplicate sample collected from each site served as replicates for all the bioassays.

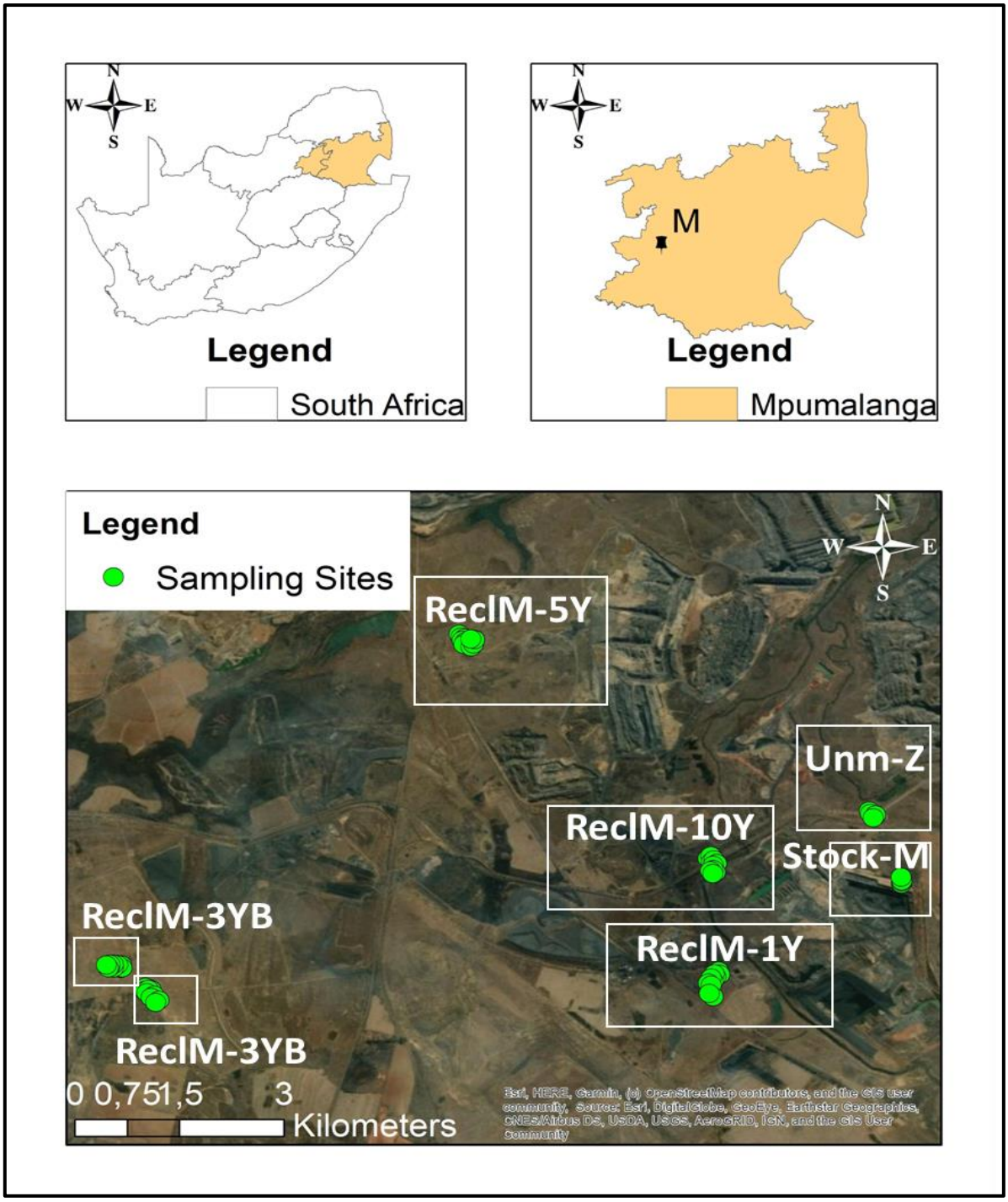

Figure 5.1: Map of sampling area in mine M sampled in 2018 (See Table 5.1 for more information).

Table 5.1: Description of study sites

[λ]Site/Code	Site Description	Year of seeding/stockpiling ([†]age in years)	[†]No. of bulk samples	Veg. cover estimate (Mean ± SD %)	Notes
Mine M					
ReclM-1Y	Reclamation area	2017 (1)	5	53.6±22.2	No activity since reclamation/revegetation
ReclM-3YA	Reclamation area	2015 (3)	5	23.0±12.1	Same notes as ReclM-1Y
ReclM-3YB	Reclamation area	2015 (3)	5	50.4±14.6	Same notes as ReclM-1Y
ReclM-5Y	Reclamation area	2013 (5)	5	ND	Burnt shortly before sampling.
ReclM-10Y	Reclamation area	2008 (10)	5	40.0±10.0	Same notes as ReclM-1Y
Unm-M	Unmined area	-	5	85.0±10.0	Fallow for 6 years. Previously used for maize farming
Stock-M	Soil stockpile heap	2018 (0.25)	5	~0	Recently stockpiled. No vegetation growth.
Mine Z					
ReclZ-3	Reclamation area	2014 (4)	5	84.0±13.9	No activity since reclamation/revegetation
ReclZ-11	Reclamation area	2006 (12)	5	96.0±4.0	Same notes as ReclZ-3
ReclZ-15	Reclamation area	2002 (16)	5	67.5±19.4	Same notes as ReclZ-3
ReclZ-19	Reclamation area	1998 (20)	5	53.8±4.80	Same notes as ReclZ-3
ReclZ-24	Reclamation area	1993 (25)	5	ND	Burnt shortly before sampling
Unm-Z	Unmined area, agricultural field	-	5	ND	Post-harvest sunflower field at the time of sampling. In the preceding year, maize was planted.
Stock-Z	Soil stockpile heap	Unknown (≥ 5)	5	22.0±8.0	Sparse vegetation cover. A lot of bare patches/areas.

[†]Age at the time of sampling in 2018. ND, not determined. The sampled areas in mine Z are the same as those sampled in 2017 (Chapter 3, See Figure 3.1). [†]Each bulk sample is a composite of five soil cores collected at 10 m intervals along a 40 m transect. A total of five transects were used per site. [λ]Although age of sampled areas in mine Z as at the time of sampling in 2018 changed (age in 2017 + 1), site codes and/or coding convention used in Chapters three and four are retained in order to minimize any ambiguities. -, undated. See Figure 5.1 for the map of the sampling area in mine M.

5.2.2 Physico-chemical analyses of test and control soil

For the determination of pH and maximum water holding capacity of test and OECD control soil prior to the earthworm bioassays, the methods described by ISO (1994) and ISO (1996), respectively, were followed. Analyses of other soil physicochemical properties were performed by the standard methods referenced in Chapter 3 (Section 3.2.2).

5.2.3 Earthworm bioassay

5.2.3.1 Test organisms

Clitellate *Eisenia andrei* worms (>250 mg wet weight) were utilised for the tests. The worms were selected from a breeding stock available at the Ecotoxicology laboratory of the Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa. The test species population were cultured on the artificial OECD soils and fed pasteurized air-dried horse manure in a climate-controlled chamber maintained at 20±2°C.

5.2.3.2 Avoidance behaviour test

Avoidance behaviour, the tendency of earthworms to avoid test (mining-impacted) soils in preference to control soils, is recognized as a valuable endpoint in measuring the habitat function of soils. Compared to other ecotoxicological tests, an avoidance behavioural test is a quick approach to determining the presence of contaminants and bioavailability of chemicals in the soil (ISO, 2008; Clasen and Lisboa, 2019). The avoidance behaviour test was performed according to the standardized guidelines of the International Organisation for Standardisation (ISO) (ISO, 2008). An avoidance behaviour or response values > 80% (equal to less than 20% of total worms in test soils after the test) indicate that a soil habitat function is limited (ISO, 2008). Avoidance response was performed in a two-chamber vessel. The chamber was a rectangular plastic container of a total capacity of 1.2-litre and a cross-sectional area of approximately 215 cm^2. The lid of the test chamber was transparent and perforated to allow gaseous exchange without allowing the escape of the test worms. Control and test soil that has been moistened to 60% of

their maximum water holding capacity were then added to each equally divided halves of the two-chamber vessel to a height of 5 cm. Ten adult-worms which had been pre-acclimatized to the control soil for at least 24 hours were placed at the centre of the dividing section and incubated in the dark for 48 hours at 20±2°C. Thereafter, the number of worms in each section was then recorded. Avoidance response (or net response) was calculated as follows (ISO, 2008):

$$AR = \frac{N_C - N_T}{N} X\ 100 \qquad \text{(Equation 5.1)}$$

Were AR= Avoidance response

N_c = the number of worms observed in the control soil,

N_T = the number of worms in the test soil, and

N = total number of worms in both control and test soil.

Mean AR values for all replicates were reported. Positive AR values indicated that test species avoided the test soil while zero or negative AR values suggested otherwise (preference).

5.2.3.3 Mortality, growth and reproduction test

Precisely 500 g of test soil or control soil was transferred into a 1-litre cylindrical plastic vessel. The soils were then moistened to 60% of their maximum water holding capacity. Thereafter, ten adult clitellate earthworms (*Eisenia andrei*) were individually weighed and introduced into the vessel. Earthworms were fed 5 g of moistened horse manure on the second day of the test and subsequently every week (OECD, 2016). Earthworm mortality and biomass was recorded at seven days intervals for a total of 28 days. Soil moisture was maintained throughout the duration of the test by the addition of distilled water weekly or when required.

At the end of 28 days, the adult worms were removed from the text vessels and individually weighed. Because it was not possible to utilise earthworms with the same mass, the relative growth rate was calculated. The relative growth rate of the worms was then calculated as follows:

$$RGR = \frac{(W_t - W_0)}{W_0}$$ (Equation 5.2)

Where W_0 is the average weight of earthworms at the start of the experiment, and

W_t is the average weight of earthworms on days 7,14, 21 and 28 (Oladipo et al., 2019).

The number of cocoons in the soils at the end of the 28 days was counted. Thereafter, the soil (and cocoons) was replaced into the containers and incubated for a further 28 days (OECD, 2016) during which feeding with 5 g moistened horse manure was maintained. At the end of the second 28 days (day 56 days of the assay), fecundity (test endpoint), the number of juveniles hatched from cocoons, was enumerated by hand sorting of worms (OECD, 2016).

5.2.4 Statistical analyses

Except otherwise stated, statistical tests were performed in R software version 3.5. (R Core Team, 2017). All data, including soil physicochemical properties, avoidance response, biomass, relative growth rate, number of cocoons and number of juveniles, were compared across soil samples collected within each mining site (Z or M) and to those of OECD control soil. Prior to statistical analysis, appropriate data transformations, including log-10 transformations, were applied where necessary in order to meet normality criteria for the parametric one-way analysis of variance (ANOVA) test. Where normality could not be achieved through transformations, the non-parametric Kruskal-Wallis one-way analysis of variance was used. The Tukey HSD posthoc test was used to separate means where the parametric one-way ANOVA was significant, while the Fisher least significant difference was used for the non-parametric tests. Test for significance was set at probability (P) < 0.05.

Lastly, Spearman Rank correlation was performed to test the relationship between physicochemical properties and bioassay endpoints. The correlations were performed for each mining site separately.

5.3 Results and discussion

5.3.1 Physico-chemical properties

The textural classification of soils largely varied within sites, with sandy-clay-loam the prominent soil textural classification (Table 5.2). In mine M, particle size fractions of clay, silt and sand were significantly ($P < 0.05$), different among sampled areas. Whereas in mine Z, only differences in sand and silt fractions were significant across sampled areas. The unmined reference and stockpile soils in both mines were classified as sandy loam and sandy clay loam soils, respectively, while the textural classification of soils within the post-mining reclamation areas varied largely between sandy clay loam and sandy loam soils. The differences in soil texture among areas sampled within sites somewhat indicate high intra-site variations in soil properties and may suggest that stockpiling, soil preservation and reclamation practices within mines vary widely. With respect to the bioassay, soil texture may influence inference drawn on the soils ability to support biocoenosis compared to the control soil (ISO, 2008; Chauhan, 2014). This is because soil texture is linked to permeability, water and nutrient retention, as well as compaction (Agrawal, 1991; Crouse, 2018). In most cases, highly drained or highly permeable soils contain low moisture and low nutrient levels and are therefore not suitable for the growth and survival of soil biota. Similarly, high clay content may lead to compaction which affects the ability of earthworms to borrow through the soil.

With the exception of soil K in mine Z, all the chemical properties analysed were significantly different ($P < 0.05$) among sampled areas within each coal mine (Table 5.3). Overall, the pH of the soils was generally acidic (3.37 – 6.70) (Table 5.3), while Na content of soils was undetectable in post-mining reclamation soils of mine Z (Table 5.3). The Ca, Mg, Na and C contents of the OECD control soil largely differed significantly ($P < 0.05$) from the test soils but the ammonium (NH_4-N content of the control soil was less than those of some test soils from both coal mining sites. Comparisons within mining-impacted soils revealed that soil from stockpiles generally had lower pH, Ca, and Mg content compared to reclamation areas.

Table 5.2: Particle size distribution of soil

	Sites	Particle size distribution			Textural Class
		Sand (%)	Silt (%)	Clay (%)	
Mine M	OECD Ctrl.	70.00±0^{c}	10.00±0^{a}	20.00±0^{bc}	Sandy Clay Loam
	unm-M	82.60±1.67^{a}	4.00±0.71^{c}	13.40±1.95^{e}	Sandy Loam
	Stock-M	69.80±1.30^{c}	2.80±0.45^{d}	27.40±1.14^{a}	Sandy Clay Loam
	ReclM-1Y	76.40±1.14^{b}	6.00±0.71^{b}	17.60±1.52^{d}	Sandy Loam
	ReclM-3YA	62.60±1.14^{d}	7.20±1.10^{b}	30.20±0.84^{a}	Sandy Clay Loam
	ReclM-3YB	70.00±2.55^{c}	8.00±2.00^{ab}	22.00±0.71^{b}	Sandy Clay Loam
	ReclM-5Y	78.00±3.74^{b}	4.00±1.22^{c}	18.00±2.55^{cd}	Sandy Loam
	ReclM-10Y	84.00±3.46^{a}	2.80±0.84^{d}	13.20±2.95^{e}	Loamy Fine Sand
Mine Z	OECD Ctrl.	70.00±0^{b}	10.00±0^{a}	20.00±0^{a}	Sandy Clay Loam
	unm-Z	81.50±1.00^{a}	2.75±0.50^{c}	15.75±1.23^{a}	Sandy loam
	Stock-Z	75.67±5.03^{ab}	4.33.00±0.58^{ab}	20.00±4.58^{a}	Sandy Clay Loam
	ReclZ-3	73.00±3.74^{ab}	3.80±1.30^{bc}	23.20±2.95^{a}	Sandy Clay Loam
	ReclZ-11	80.60±4.77^{a}	3.00±1.00^{c}	16.40±4.39^{a}	Sandy Loam
	ReclZ-15	73.40±2.04^{ab}	3.60±0.55^{bc}	23.00±1.73^{a}	Sandy Clay Loam
	ReclZ-19	77.40±8.62^{a}	3.80±0.84^{bc}	18.80±8.35^{a}	Sandy Loam
	ReclZ-24	76.40±2.61^{ab}	3.60±0.55^{bc}	20.00±2.55^{a}	Sandy Clay Loam

Values are mean ± SD (N = 5). Values with different superscript letters are significantly different (P < 0.05) based on Tukey's HSD posthoc test for parametric one-way ANOVA or the non-parametric Fisher's least significant difference.

Table 5.3: Chemical composition of soil

Site code	pH (KCl)	NO_3-N (mg/kg)	NH_4-N (mg/kg)	P (Bray1) (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	Na (mg/kg)	%C
Mine M									
*OECD Ctrl.	5.75±0.01^{bc}	8.86±0.21^{a}	0.53±0.04^{d}	37.50±0.71^{ab}	77.50±3.54^{d}	1330.00±169.71^{a}	734.00±65.05^{a}	56.50±16.26^{a}	4.51±0.07^{a}
unm-M	6.14±0.36^{ab}	1.18±0.47^{ab}	4.34±0.55^{ab}	49.00±13.82^{a}	177.20±26.36^{a}	541.80±104.38^{bc}	124.80±27.23^{bcd}	0±0^{c}	0.99±0.28^{b}
Stock-M	4.148±0.02^{d}	1.57±0.40^{ab}	2.29±1.07^{bc}	1.60±0.55^{e}	86.40±16.21^{cd}	56.80±14.41^{e}	39.00±7.42^{e}	5.20±1.79^{ab}	0.27±0.06^{e}
ReclM-1Y	6.70±0.49^{a}	5.50±2.11^{ab}	8.04±1.87^{a}	21.20±4.27^{abc}	137.40±22.95^{ab}	923.60±391.42^{ab}	159.40±23.82^{bc}	0.60±1.34^{c}	1.20±0.16^{b}
ReclM-3YA	5.17±0.31^{c}	0.26±0.24^{b}	2.11±0.44^{bc}	2.00±0.71^{e}	118.80±31.76^{abcd}	361.80±33.84^{cd}	178.80±28.70^{b}	0.60±1.34^{c}	0.48±0.08^{d}
ReclM-3YB	5.66±0.16^{bc}	0.33±0.26^{b}	1.18±0.28^{cd}	4.80±3.11^{de}	97.80±25.61^{bcd}	395.00±18.371^{cd}	154.80±24.01^{bc}	4.80±2.05^{b}	0.38±0.09^{de}
ReclM-5Y	5.41±0.97^{bc}	0.97±1.43^{ab}	3.50±1.40^{b}	14.40±13.15^{cd}	120.20±17.63^{abc}	280.80±131.43^{d}	91.80±24.04^{d}	1.20±1.64^{c}	0.65±0.16^{c}
ReclM-10Y	4.90±0.11^{cd}	5.92±7.69^{ab}	2.42±1.36^{bc}	17.20±9.55^{bc}	100.60±20.84^{bcd}	369.40±101.44^{cd}	117.20±46.07^{cd}	0.60±1.34^{c}	1.05±0.20^{b}
Mine Z									
*OECD Ctrl.	5.745±0.01^{a}	8.86±0.21^{a}	0.53±0.04^{c}	37.50±0.71^{a}	77.50±3.54^{a}	1330.00±169.71^{a}	734.00±65.05^{a}	56.50±16.26^{a}	4.51±0.07^{a}
unm-Z	4.85±0.47^{ab}	2.98±2.30^{ab}	6.15±1.89^{a}	35.00±8.83^{a}	90.25±20.11^{a}	322.75±113.13^{ab}	69.00±10.55^{ab}	0.75±1.5^{b}	0.83±0.05^{b}
Stock-Z	3.37±1.83^{de}	0.60±0.89^{bc}	3.14±2.56^{ab}	11.75±10.42^{b}	80.27±46.23^{a}	142.35±26.84^{cd}	26.60±7.70^{d}	0±0^{c}	0.99±0.56^{a}
ReclZ-3	4.48±0.54^{cd}	0.56±0.68^{c}	2.40±0.80^{bc}	7.80±2.77^{bc}	96.00±19.24^{a}	181.80±92.99^{cd}	48.20±11.48^{bc}	0±0^{c}	0.56±0.14^{c}
ReclZ-11	4.42±0.15^{bc}	0.48±0.42^{bc}	4.29±2.02^{ab}	8.80±4.71^{b}	85.20±28.82^{a}	156.20±46.75^{abc}	47.20±16.19^{bc}	0±0^{c}	0.80±0.23^{b}
ReclZ-15	4.68±0.17^{ab}	2.29±4.78^{bc}	2.26±1.40^{bc}	13.00±3.54^{bc}	108.00±12.55^{a}	245.00±13.51^{cd}	72.80±10.03^{a}	0±0^{c}	0.79±0.22^{b}
ReclZ-19	4.35±0.13^{cd}	0.93±1.79^{c}	2.97±1.59^{b}	12.20±9.60^{bc}	80.20±49.40^{a}	196.20±106.28^{bcd}	46.20±23.93^{cd}	0±0^{c}	0.68±0.28^{bc}
ReclZ-24	4.11±0.10^{e}	0.01±0.00^{d}	2.45±0.41^{bc}	6.40±3.36^{c}	64.60±18.73^{a}	134.80±110.94^{d}	38.80±18.20^{cd}	0±0^{c}	0.73±0.15^{bc}

*Included (duplicated) for comparison. Values for test soils are mean ± SD of quintuplicate samples. Values within each coal mine (M or Z) with the same superscript letters are not significantly different ($P > 0.05$) based on the parametric Tukey's HSD or the non-parametric Fisher's least significant difference.

The soil pH, soil texture and organic matter are crucial parameters that may affect earthworm survival (ISO, 2008; Brami et al., 2017). Soil pH determines the availability of nutrients in the soil, while organic matter and texture may influence the permeability and conditioning of the soil as a suitable habitat for the earthworms (Chauhan, 2014; Brami et al., 2017; Hallett and Caird, 2017). The pH of the stockpile soils and ReclM-10Y in Mine M, as well as the soils from sampled areas in mine Z, were all below the optimum range (5.5 to 6.5) recommended for earthworms (*E. andrei* and *E. fetida*) (OECD, 2016). A previous study by Ohno (2001) showed that acidity reduces the survival of the Japanese earthworms (*Allolobophora japonica*). Similarly, Bengtsson et al. (1986) observed that acidity reduced the cocoon number and hatching success of the earthworm *Dendrobaena rubida*. Thus, the largely acidic pH of the stockpile soils and most of the soils in mine Z may not be ideal for the fitness of earthworms and consequently, their (earthworms') soil ecosystem functions.

5.3.2 Avoidance behaviour

All validity criteria for the avoidance test, including the homogenous distribution of worms in a test chamber (i.e. both sections of the two-chamber vessel) filled with the same control soil and the number of dead or missing worms ≤10% were satisfied (ISO, 2008). Analysis of the net avoidance response (AR) of soils indicated significant differences (Kruskal-Wallis, $P < 0.05$) among "treatments" (Figure 5.2). However, based on the > 80% avoidance response cut-off, the soil habitat function of one of the two three-year old reclamation areas in mine M (ReclM-3YA) is limited (Figure 5.2). This observed 'limited habitat function' may be due to the relatively higher clay content in ReclM-3YA (see Table 5.2). This is because high clay content increases the likelihood of soil compaction. Compaction of the soil may lead to relative difficulty in penetration (and lower preference) of the soil by earthworms (Yvan et al., 2012; ABC, 2019). A similar effect of soil compaction on the richness and activity of other soil biota (e.g. microorganisms) have been observed (Frac et al., 2009; Ishak et al., 2016).

Natal-da-Luz et al. (2008) observed that earthworms exhibited a strong likelihood to avoid soils with a high clay content (finer texture).

The net avoidance response of earthworms when exposed to unmined soils from mines M (Figure 5.2a) and Z (Figure 5.2b) was significantly different ($P < 0.05$) (i.e. more preference for unmined soils) from those in the all-OECD control soil vessels and stockpiles soils. Although the significant difference in AR may suggest that the artificial OECD control and stockpile soils are significantly less preferred (or functionally limited) compared to the unmined soils (unm-Z and unm-M), these differences are most likely related to the differences in selected physicochemical properties of the soil (ISO, 2008). This assertion is supported by the significant correlations between AR and selected soil physicochemical properties (Table 5.3). Furthermore, the influence of soil physicochemical properties on the avoidance response validates the sensitivity of the standardized tests in reflecting soil conditions (ISO, 2008; Natal-da-Luz, 2008). However, Hund-Rinke and Wiechering (2001) demonstrated that avoidance behaviour is largely determined by pollutants in the soil relative to chemical-physical soil properties. Studies have also shown that the avoidance test is sensitive in determining a wide range of soil contaminants, including heavy metals, petroleum hydrocarbons and polycyclic hydrocarbon (Schaefer, 2001; Reinecke et al., 2002; ISO, 2008). Thus, the low avoidance response observed in the present study suggests that the coal mining associated soil environments are not contaminated by such pollutants.

The trends in the habitat functions of mining-impacted soils (based on the mean avoidance response) were generally in the reducing order of unm-M > ReclM-1Y > ReclM-5Y > ReclM-10Y > Stock-M > ReclM-3YB > ReclM-3YA in mine M and in the decreasing order unm-Z > ReclZ-15 > ReclZ-11 > ReclZ-19 > Stock-Z > ReclZ-3 > ReclZ-24 in mine Z (Figure 5.2). For both coal mining sites, these trends suggest that the habitat function of mining-impacted soils (stockpiles and reclaimed areas) are limited compared to the unmined areas and that

these functions are not proportional to age of reclamation. Rather, the ecosystem functions of these soils as revealed by the AR is more likely a function of the soil physicochemical conditions which varied largely across reclamation areas of different ages. The significant positive correlation observed between pH and AR in mine Z (Table 5.4) suggest that earthworms are sensitive to an increase in soil pH (towards alkalinity).

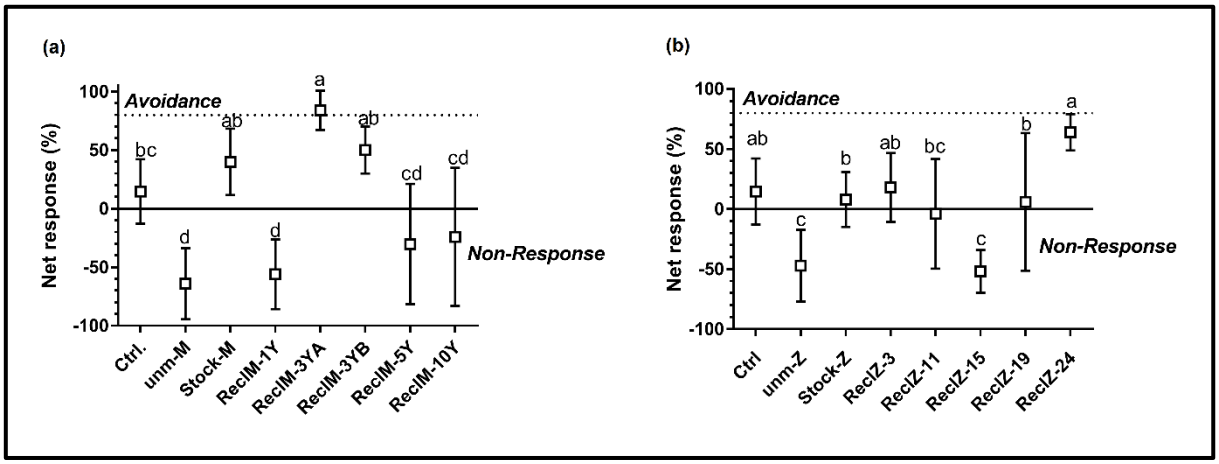

Figure 5.2: Avoidance behaviour. (a) Mine M (b) Mine Z. Boxes are mean values ± SD of five replicates. Values with different superscript letters are significantly different ($P < 0.05$) based on Fisher's least significant difference. "Ctrl", the OECD control soil. In the control test, both sections of the test chambers were filled with the OECD control soil.

Table 5.4: Spearman correlation coefficient for the relationship between soil physicochemical properties and earthworm bioassay endpoints

	Mine M				Mine Z			
Soil Properties	**RGR@ WK4**	**AR**	**No. of cocoons**	**No. of juveniles**	**RGR@ WK4**	**AR**	**No. of cocoons**	**No. of juveniles**
pH	-0.05	0.21	0.10	0.08	0.01	0.36*	-0.09	-0.14
NNO3	0.11	0.15	0.26	0.09	0.17	0.11	0.01	-0.06
NNH4	-0.35	0.39	0.04	0.10	0.12	-0.38*	0.06	0.10
P	0.09	0.18	0.36*	0.22	0.25	0.19	0.01	-0.03
K	-0.33	0.31*	0.06	0.18	0.19	-0.24	-0.22	-0.21
Ca	0.13	0.09	0.16	0.15	0.05	0.34*	-0.04	-0.12
Mg	0.06	-0.05	-0.04	0.13	0.09	0.39*	-0.01	-0.17
Na	0.37*	-0.50***	-0.07	-0.21	0.01	0.50*	-0.03	-0.12
%C	0.21	0.32*	0.15	0.10	0.44	-0.03	0.10	-0.09
%Sand	0.27	0.21	0.42**	0.20	0.00	-0.29	0.18	0.15
%Silt	-0.02	-0.11	-0.26	-0.08	0.21	0.28	0.09	0.11
%Clay	-0.32	-0.14	-0.33	-0.14	-0.07	0.05	-0.24	-0.17

*Significant at probability level of 0.05; **Significant at probability level of 0.01; ***Significant at probability level of 0.001. AR, avoidance response; RGR, relative growth rate.

5.3.3 Biomass, mortality, relative growth rate and reproduction success

The mortality criteria for the validity of the assay was met (the mortality did not exceed 10% in any of the control or test soils). The mean biomass of worms at the onset of the reproduction assay ranged from 0.43 to 0.49 g (Figure 5.4). Except for Stock-M, the mean biomass of worms was highest after the first week of the test in both control and test soils (Figure 5.4). In Stock-M, the highest average biomass was observed after the second week (Figure 5.4a). The general decline in biomass towards the end of the assay coincided with the period in which high cocoon production was observed in the test chambers (data not shown). A similar decline in earthworm biomass towards the end of the test period has been reported in other ecotoxicology studies on mining soils (van Coller-Myburgh et al., 2015; Maboeta et al., 2018).

As expected from the biomass pattern, the relative growth rate (RGR) of worms over the 28 days reduced from initial (week 1) values. The RGR was significantly different (Kruskal-Wallis, $P < 0.05$) throughout the test period in mine M, with the relative growth rate lowest in either ReclM-3YA or ReclM-3YB at the end of each week. However, the RGR of worms in these test soils (ReclM-3YA or ReclM-3YB) did not differ significantly from those of the control soil (Table 5.5). Whereas, in mine Z, the RGR was not significantly different (Kruskal-Wallis, $P > 0.05$) during the first three weeks of the test. However, at week 4, the RGR of worms in the control soil significantly ($P <0.05$) differed from those of the oldest reclamation area (ReclZ-24) in mine Z (Table 5.5). Overall, and compared to the control soil, the relative growth rate did not suggest any obvious effects on earthworms. The differences observed in growth rates might be due to inherent genetic or metabolic differences amongst the worm population used in the test (despite the use of the same population breed).

Compared to the avoidance test, the biomass and RGR did not appear to reflect differences in soil properties. Thus, compared to the avoidance behaviour test, these endpoints (biomass and RGR) are not very sensitive for assessing the capability of these soils to support biocoenosis. Similarly, van Coller-Myburgh et al. (2014) also concluded that biomass was not a sensitive endpoint during their assessment of chromium mine wastes in South Africa using *E*. *andrei*.

The validity criterium for the reproduction test was met in that over 40 juveniles were observed in the OECD control soil (OECD, 2016). Although the number of cocoons produced after 28 days of the test was significantly (Kruskal-Wallis, $P < 0.05$) different in mine Z, the number of hatched juveniles did not significantly (Kruskal-Wallis, $P > 0.05$) differ amongst test soils in both mining sites, thereby indicating that the test soils do not impair earthworm fecundity in reference to the control soil. Thus, the reproduction test corroborated the basic conclusion drawn from the avoidance test: that these soils potentially do not contain contaminants that may impede the fitness of earthworms. It has been suggested that the avoidance test is equally sensitive to the reproduction test (ISO, 2008).

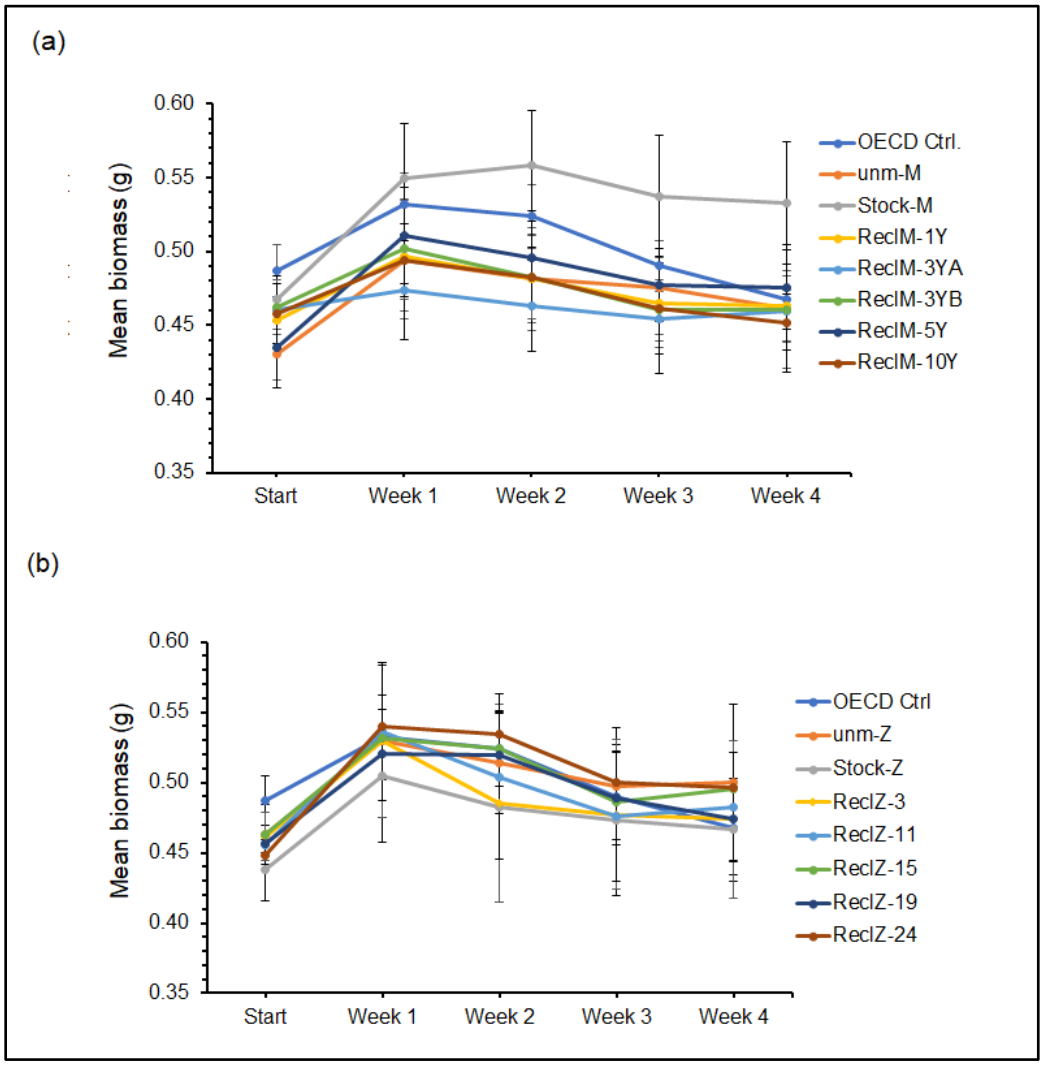

Figure 5.3: Mean biomass of adult *Eisenia andrei* over 28 days in test and control soils (see Table 5.S1 for the statistical test of differences)

Table 5.5: Relative growth rate (RGR) and reproduction success

	RGR (%)				No. of cocoons	No. of juveniles
	Week 1	Week 2	Week 3	Week 4		
Mine M						
OECD Ctrl.	9.41±7.29^{ab}	7.72±7.21^{bcd}	0.81±6.26^{bc}	-3.84±4.13^{d}	61.4±8.23^{a}	362.6±34.78^{a}
unm-M	14.71±6.3^{a}	11.96±6.15^{abc}	10.54±7.25^{ab}	7.53±12.3^{abc}	65.0±15.23^{a}	266.4±117.19^{a}
Stock-M	17.52±8.77^{a}	19.36±7.97^{a}	15.03±9.76^{a}	13.96±9.15^{a}	77.8±14.38^{a}	340.4±87.56^{a}
ReclM-1Y	10.83±7.28^{ab}	7.20±7.69^{bcd}	4.66±6.96^{abc}	3.61±5.65^{abc}	65.0±10.82^{a}	273.4±65.81^{a}
ReclM-3YA	2.86±4.58^{b}	0.64±6.57^{d}	-1.37±6.99^{c}	-0.18±7.2^{bcd}	65.4±7.92^{a}	310.4±47.15^{a}
ReclM-3YB	8.55±6.42^{ab}	4.65±8.29^{cd}	-0.24±7.42^{c}	-0.06±8.05^{bcd}	66.2±9.12^{a}	293.6±85.11^{a}
ReclM-5Y	18.01±11.65^{a}	14.38±10.6^{ab}	9.99±7.44^{ab}	9.68±8.25^{ab}	66.75±10.55^{a}	292.5±111.07^{a}
ReclM-10Y	8.03±2.01^{ab}	5.52±3.10^{bcd}	0.80±3.77^{c}	-1.19±1.58^{cd}	76.8±7.92^{a}	375±32.83^{a}
Mine Z						
OECD Ctrl.	9.41±7.29^{a}	7.72±7.21^{a}	0.81±6.26^{a}	-3.84±4.13^{b}	61.4±8.23^{b}	362.60±34.78^{a}
unm-Z	14.3±14.3^{a}	10.97±10.97^{a}	7.38±7.38^{a}	8.04±8.04^{ab}	71.2±11.12^{ab}	322.2±71.18^{a}
Stock-Z	15.47±15.47^{a}	10.62±10.62^{a}	8.5±8.5^{a}	6.98±6.98^{ab}	67±4.30^{ab}	280.2±24.24^{a}
ReclZ-3	15.58±15.58^{a}	6.15±6.15^{a}	4.27±4.27^{a}	3.49±3.49^{ab}	78.8±9.96^{ab}	338.8±79.41^{a}
ReclZ-11	18.65±18.65^{a}	11.38±11.38^{a}	5.5±5.5^{a}	6.62±6.62^{ab}	83.2±7.05^{a}	366.2±70.32^{a}
ReclZ-15	15.18±15.18^{a}	13.62±13.62^{a}	5.35±5.35^{a}	7.6±7.6^{ab}	72.2±15.14^{ab}	339.8±66.38^{a}
ReclZ-19	14.22±14.22^{a}	14.11±14.11^{a}	7.32±7.32^{a}	4.21±4.21^{ab}	73.2±15.37^{ab}	331.8±68.53^{a}
ReclZ-24	20.72±20.72^{a}	19.5±19.5^{a}	11.57±11.57^{a}	10.84±10.84^{a}	73.4±13.24^{ab}	334.6±66.71^{a}

RGR, Relative growth rate. Values are mean values ± SD of five replicates. Values with different superscript letters on the same column within each site (mine M or Z) are significantly different ($P < 0.05$) based on Fisher's least significant difference.

5.4 Conclusion

The work in this chapter involved the utilisation of avoidance behaviour responses, reproduction, mortality and biomass as endpoints in assessing the habitat support function and presence of contaminants in coal-mining soil environments. Although the avoidance behaviour of earthworms in one of the reclamations sites indicated an avoidance response, the general trends observed for all soils did not suggest that the soil habitat functions were limited. Similarly, other endpoints such as biomass, fecundity and mortality did not suggest that the soil environment contained substances which may be harmful to earthworm survival and fitness. Of all endpoints measured, the avoidance test was the most sensitive and generally reflected the differences in soil physicochemical properties amongst test soils. Furthermore, the avoidance test indicated a preference (by the earthworms) for the unmined soils compared to the mining soil stockpiles and reclamation soils in both coal mining sites.

CHAPTER 6:

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Soil is a key resource that is fundamental to the existence of life forms. There has been an increasing loss of soil ecosystem function and services globally as a result of intensified anthropogenic activities. Importantly, the impacts of anthropogenic activities on soil health as well as the adequacy of current post-disturbance reclamation approaches need to be re-evaluated. Due to the intricate nature of soil and the complexity of the ecological interactions and/or processes in the soil ecosystem, a vast number of parameters are needed for such evaluations. In practice, it may not be economically feasible to evaluate all these different parameters. Thus, the identification of a minimum dataset which best describes the soil ecosystem health is paramount. Such a minimum dataset should ideally integrate the different soil components—physical, chemical and biological. There are no such comprehensive integrated soil health monitoring indices for coal mining soil environments in South Africa.

This study formed part of a multi-disciplinary research collaboration (Mushia et al., 2016; Paterson et al., 2016; Mashigo, 2018; Mushia, 2018; Paterson et al., 2019), which aimed at obtaining a comprehensive insight (from the different soil components) into the quality of soils in stockpiles and reclamation areas of coal mining companies in South Africa. Specifically, the collaboration sought to evaluate the appropriateness of current practices, ascertain potential recovery of reclaimed areas, put forward recommendations on best practices and develop a minimum dataset and/or tools for soil quality monitoring in the South African coal mining industry. Amongst the different components of the soil ecosystem, the soil biological communities are crucial to a number of ecological processes. Such processes

are fundamental to the health of the soil environment and consequently ecosystem services. In particular, microorganisms play critical roles that are vital to soil ecosystem sustainability (Hayatsu et al., 2008; Smith and Read, 2010; Steffan et al., 2015; Adeleke et al., 2017). Therefore, this study sought to investigate the richness and community structure of soil microbial populations in coal-mining soil environments (stockpile and reclamation areas). The data obtained in this study were analysed based on two assumptions: (1) Increase in species richness and diversity of microbial species over a time gradient indicate potentials for ecosystem recovery and (2) Non-significant differences in microbial species richness and diversity between soils of unmined areas and coal mining-disturbed soils (stockpiles and reclamation soils) indicate restoration and/or stability of microbial species richness and diversity.

The study was undertaken in three stages. Firstly, an initial analysis of soil stockpiles was conducted in order to understand the potential contribution of current stockpiling practices to post-coal mining reclamation soil health. Secondly, by utilising the richness and structure of microbial communities as bioindicators, potential ecosystem restoration in post-coal mining reclamation areas were inferred. Lastly, to gain insight into the potential capability of soils to support biocoenosis, an ecotoxicological approach was adopted by using earthworms as bioindicators. The subsequent sections provide key conclusions from these studies.

6.1.1 The potential contribution of soil stockpiles to post-coal mining reclamation soil health

Soil stockpiles which were preserved for post-mining replacement were sampled at two depths (0-20 cm and >20 cm) across three coal mining sites. The abundance and viability of AMF spores, microbial community profile and richness as well as enzyme activities and

soil physicochemical properties were determined. There was no evidence to suggest that soil stockpile had lower AMF spore abundance and viability compared to the unmined reference soil. Rather, differences were largely influenced by sampling depth. Moreover, bulk density, cation exchange capacity, clay content and organic C were factors which were significantly correlated with AMF spore abundance in the topsoil depth.

Based on PCR-DGGE profiles, the microbial community structure of unmined soils was not similar to those of soil stockpiles. Similarly, microbial richness and Shannon-Wiener index of diversity in soil stockpiles were impaired compared to the unmined soil. In contrast, the enzyme assay indicated higher beta-glucosidase activities in stockpile soils compared to the reference soil. This suggests a higher biological activity in response to carbon availability observed in soil stockpiles. Enzyme analyses suggest that beta-glucosidase activity is a more sensitive indicator for assessing soil stockpile health compared to urease activity. Soil-physicochemical properties which influenced microbial communities were pH, cation exchange capacity, phosphorus and total nitrogen content.

In summary, the impaired microbial community structure and diversity observed in stockpiles suggest potentially negative implications for soil biological processes driven by microbes, especially those that are critical for nutrient cycling and ecosystem sustainability. Consequently, post-coal mining land use capability may potentially be affected. Based on the trends observed for AMF spore density, stockpiling heights may affect the abundance and viability of AMF spores. Furthermore, the significant relationship between bulk density and AMF spore density in soil stockpiles suggest that alteration in soil structure and compaction in soil stockpiles influence AMF spore density and viability. As a result, the capability of the soil to support plant growth (via infectivity of roots by mycorrhizae) during post-mining land use may be negatively affected.

6.1.2 Structural and functional differentiation of microbial communities in post-coal mining reclamation soils

In this part of the study, the richness, diversity, structure and function of microbial communities were assessed across reclamation soils from three coal mines initially, and then along a post-coal mining chronosequence in a single coal mining company. The composition and structure of microbial communities in reclamation soils revealed site-specific differences. The restoration of pre-disturbance bacterial richness levels is estimated to occur between 15 and 19 years after reclamation. Furthermore, bacterial communities were more differentiated and potentially more responsive to ecological factors than fungal communities. In multi-variate space, the composition and structure of microbial communities in the reclamation areas differed from those of unmined areas, suggesting that land use type influences soil microbial communities. Furthermore, this observation suggests that although species richness and diversity levels may be restored over years after reclamation, the composition and structure of the microbial communities may never be restored to pre-disturbance states. However, the foregoing conclusion may be influenced by the lack of a true “reference” site or soil for comparison.

The beta-glucosidase activity was the most discriminatory of all the enzyme activities assayed in reclamation areas. Beta-glucosidase activity generally increased with reclamation age. Based on the 16S-marker gene predictions, the potential functional capabilities of bacterial communities did not significantly differ amongst reclamation soils and compared to unmined areas, suggesting functional redundancy in bacterial communities. However, such observed functional redundancy could be due to the functional capabilities of a large proportion of the microbial species are yet unknown.

Bacterial species and/or communities may serve as bioindicators of ecosystem recovery. However, their responsiveness to changes in soil physicochemical properties suggests that their utilisation to infer soil ecosystem health and restoration must be integrated with selected soil physicochemical properties. Based on data generated in this study, such soil properties include pH, particle distribution (or texture), bulk density (potential for compaction) and selected nutrients (e.g. nitrogen and phosphorus).

6.1.3 The potential of utilising arbuscular mycorrhizal fungi as bioindicators of ecosystem restoration in post-mining reclamation areas

Investigations into the richness, diversity and community composition of arbuscular mycorrhizal fungi towards ascertaining their suitability to serve as bioindicators revealed that (1) AMF richness and diversity in soils are not significantly different across reclamation soils and unmined areas, (2) community differentiation of AMF along a post-coal mining reclamation chronosequence is not as clear-cut as those of total fungal communities, utilising ITS2 markers, (3) the dominance of a single AMF species throughout the post-coal reclamation provides little resolution for detecting changes in soil conditions and for estimating ecosystem recovery and, (4) the influence of revegetation species, the emergence of a dominant vegetation across reclamation areas, and the existence of host-specificity among AMF species make their interpretation and integration into a soil health monitoring scheme relatively difficult compared to other microbial groups. Further studies on the viability or infectivity of AMF propagules in reclamation soils may elucidate the potential of the soils to support plant growth during post-reclamation land use, especially for cropping.

6.1.4 Habitat support function of coal-mining associated soils as determined by earthworm bioassays

An ecotoxicological approach was adopted in assessing the habitat function of stockpile and reclamation soils. Earthworm (*E*. *andrei*) fitness in the test soils was used as a representative of the soil to support biocoenosis. Standardized tests that included avoidance behaviour and reproduction tests were used. Data on biomass, relative growth rate, mortality avoidance behaviour and reproduction success did not provide strong evidence for limited habitat functions of stockpile and reclamation soils. Although test worms showed a higher preference for unmined soils, the response to stockpile and reclamation soils were mostly below the recommended limit for adjudging the soils as having limited habitat function. The result also showed that the coal mining soil environments are not contaminated by common soil pollutants which could limit biocoenosis. Thus, from an ecotoxicological perspective, coal mining soil stockpile and reclamation soils in the different coal mines could potentially provide habitat support for soil biota and the complex inter-relationships among different biological species.

6.2 Recommendations

6.2.1 Future research directions

- Future longer-term studies are required to investigate microbial community patterns within reclamation areas. Such studies will help confirm the reliability of the current observations under different climatic conditions. It may be necessary to include replicate reclamation areas of similar age in the design of such studies in order to obtain a statistically robust inference. However, due to the difficulty in obtaining replicate sites in

nature, such long-term studies may be performed under a simulated reclamation scenario.

- Although high-throughput sequence analysis method employed in this study is desirable due to its relative ease and rapidness, the relative abundance of specific taxa may be misleading due to the variations in the rRNA copy number and PCR biases that form part of the limitations of the method. Hence, future studies should focus on how adjustments in rRNA copy can be incorporated into taxa abundance estimation. While the 16S rRNA gene copy number variability still affects microbiome abundance estimation, the study by Guo et al. (2019) on root microbiome show great promise and can be adopted for studying the microbiome of soil and other environments.

- Shotgun metagenomics of the soil microbial communities may also provide better insights into functional capabilities of the microbial communities. Furthermore, with recent developments in long-read sequencing technologies such as the Single-molecule real time (SMRT) technology (Pacific Biosciences, CA, USA) it may become possible to identify microbial species to species taxonomic rank with greater confidence. However, the current relative high cost may limit its utilisation for meta sample analysis in the interim.

- Future studies should attempt the absolute quantification and isolation of the differentially abundant and potential soil health indicator species detected in this study. Following isolation, *in vitro* and field studies may be designed to evaluate their physiological traits and ecosystem functionalities. Furthermore, whole genome sequencing of these isolates may be performed to gain insights into their potential metabolic repertoire.

- The development of microbial cocktail or inocula from adjacent unmined areas and application of the same during post mining reclamation may speed up ecosystem restoration by ensuring that pre-disturbance microbial diversity is restored. Because the microbial inoculum is indigenous to the mining geographical space, the likelihood of their survival and persistent in the reclamation soil environment may be higher compared to commercially available microbial inoculants (Kumar and Gopal, 2015). The development of such microbial cocktail of inocula may assume well-established traditional methods used for cultivating indigenous soil microbes (Park and DuPonte, 2008). Briefly, such methods include passing clean water through virgin soil trapped in sieves and the filtrate collected in a rich microbial nutrient medium (e.g. molasses, glucose syrup). Subsequently, the filtrate-medium mixture is allowed to brew for a few days. The “brew” can then be used as inoculants for soil amendments.

- Further studies may be performed to investigate the role of specific reclamation vegetation species in shaping microbial communities of post-mining reclamation soils. This should be achieved through multidisciplinary research collaborations.

6.2.2 Best practices for stockpiling and post-mining reclamation: the mine environmental officer’s guide

- **Ensure adequate soil stockpiling practices**. Such adequate practices include:

    ii. Careful separation and storage of the different soil horizons;

    iii. Minimising topsoil storage durations in stockpiles;

    iv. Minimising the use of heavy machinery on stockpiles in order to prevent soil compaction;

v. Minimising the height of soil stockpiles because deeper portions of the heap become anaerobic and consequently affect soil biological activities;

vi. Considering soil stockpiles as an active soil ecosystem rather than a “static” one. The seeding of stockpiles with cover vegetation may minimize soil erosion and leaching of nutrients through water runoff during stockpile storage;

vii. Avoiding subsequent heaping of new topsoil on previous soil heaps. This will help with maintaining storage duration records; and

viii. Keeping adequate records, including date of stockpiling, horizon information, and coordinate information.

- **Ensure best reclamation practices.** Such best practices should include:

i. Ensuring soils are replaced in a manner that pre-mining soil horizons are recreated as best as possible. The pre-mining soil survey should be consulted during reclamation;

ii. Prioritising high-end land use capability such as arable use by ensuring adequate topsoil layers (> 30 cm) are applied. The homogenous re-distribution of topsoil across the entire reclamation areas should be prioritize. Treat the soil as a precious scarce resource;

iii. Minimising steeps and slopes during reclamation. Adequate grading of the reclamation area must be ensured. This is important in order to minimize erodibility;

iv. Taking necessary steps to minimise soil compaction in reclamation areas. Such steps include minimising the use of heavy equipment on reclamation areas as well as replacing adequate topsoil layers;

v. Inclusion of microbial inocula during reclamation. Aim for soil biological species restoration and activity rather than solely focusing on vegetation growth. The application of organic soil amelioration such as vermicompost, vermicompost tea etc. should be considered. Because chemical fertilizers may impede microbial community richness and activity, their application rate should be checked;

vi. Avoiding re-vegetation species which outcompetes other species. Current indications are that *Eragrostis tef* produces metabolites in its rhizosphere which antagonizes other plant species. Careful selection of a mixture of plant species which can co-exist should be used;

vii. Keeping adequate and detailed record of all activities performed on reclamation areas. Information to be kept include seeding date, soil amendment applied and rate of application, topsoil depth applied and topography details; and

viii. Continuous maintenance of reclamation areas with nutrients should be done until it becomes a self-sustaining soil ecosystem.

ix. Long term monitoring of post-mining soils is necessary in order to ascertain if reclamation efforts are sufficient and if “restoration” of pre-mining biological species levels are obtainable.

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SUPPLEMENTARY MATERIALS

Supplementary Tables

Table 2.S1: Summary of aligned rank transform non-parametric factorial ANOVA

Parameter	P-value		
	Site	Depth	Interaction (Site x Depth)
pH	5.35e-06*	0.85	0.60
% Sand	6.47e-07*	0.41	0.02
% Silt	0.03*	0.71	0.01*
% Clay	4.17e-05*	0.01	0.02*
Na (cmol kg-1)	1.64 e-07*	0.43	0.04*
K (cmol kg-1)	2.78e-05*	1.39e-04*	8.69e-03*
Ca (cmol kg-1)	3.31e-04*	0.82	0.79
Mg (cmol kg-1)	2.03e-09*	0.26	0.82
S-V unit (cmol kg-1)	3.03e-06*	0.68	0.72
Cation exchange capacity	6.33e-04*	0.39	0.15
Bulk density (g cm-3)	7.55e-03*	ND	ND
Organic carbon (%)	0.10	6.5e-03*	0.12

ND, not determined. *Significant p-values (α=0.05)

Table 2.S2: Taxonomic affiliations of AMF operational taxonomic units

OTU No.	No. of sequences	*Site (Depth)	Closest Defined AM fungi Relative in the GenBank (Accession number)	Max Identity (%)
1	21	X(TS), C (SS), B(TS), A(TS)	*Paraglomus occultum* (DQ273827)	97.9
2	3	B(TS), A(TS)	*Paraglomus occultum* (DQ273827)	97.2
3	1	X(TS), C(TS)	*Paraglomus occultum* (DQ273827)	97.8
4	1	B(TS)	*Paraglomus occultum* (DQ273827)	94.0
5	1	A(TS)	*Paraglomus occultum* (DQ273827)	92.0

OTU, operational taxonomic unit; *Site: X, Control site; A, Mine A; B, Mine B; C, mine C; Depth: TS, Topsoil; SS, Subsoil.

Table 2.S3: Diversity indices of Microbial communities soils

	Bacteria						Fungi					
	Topsoil			Subsoil			Topsoil			Subsoil		
Season/Site	**†Obs.**	***H'***	***J'***	**Obs.**	***H'***	***J´***	**Obs.**	***H'***	***J'***	**Obs.**	***H'***	***J'***
Summer												
Reference	14	2.38	0.90	18	2.60	0.90	27	2.02	61	15	2.42	0.90
Mine A	8	1.90	0.91	13	2.38	0.91	15	2.64	0.97	10	2.04	0.87
Mine B	5	0.43	0.27	7	0.83	0.43	16	2.22	0.80	7	1.79	0.92
Mine C	8	1.88	0.91	12	2.24	0.90	6	1.67	0.93	7	1.75	0.90
Winter												
Reference	15	2.36	0.87	16	2.55	0.92	25	2.15	0.67	15	2.46	0.91
Mine A	5	0.39	0.24	12	2.26	0.91	2	0.60	0.86	6	1.50	0.89
Mine B	5	0.43	0.27	12	1.34	0.54	19	2.34	0.80	6	1.51	0.84
Mine C	15	2.55	0.94	18	2.69	0.93	11	1.94	0.81	6	1.68	0.94
Spring												
Reference	15	2.36	0.87	16	2.33	0.84	19	1.97	0.67	16	2.43	0.88
Mine A	4	0.32	0.23	9	2.11	0.96	11	2.24	0.93	8	1.56	0.75
Mine B	6	0.76	0.42	5	0.80	0.50	4	1.29	0.93	8	1.65	0.79
Mine C	11	2.12	0.88	18	2.59	90	15	2.34	0.87	7	1.74	0.89

†Obs., Observed species, indicates different bands (vertically) in PCR-DGGE images of Figure 2.4.; *H'*, Shannon-Wiener index, *J'*, Evenness. Obs., *H'* and *J'* were computed based on the general assumption that different species (sequence) migrated to different positions in the DGGE gel. *H´* and *J´* were computed in the vegan package of R software.

Table 2.S4: *P*-values (permutation test) for the significance of the contribution of Physico-chemical properties to the RDA model of Figure 2.5.

Soil properties	Topsoil	Subsoil
S-V	0.837	0.070
CEC	0.597	0.268
pH	0.534	0.019*
Organic C	0.510	0.036*
Total N	0.659	0.023*
C: N	0.410	0.078
Phosphorus	0.621	0.025*
Sand	0.863	0.327
Silt	0.329	0.322

*Significant at the probability level of 0.05.

Table S3.1: Permutational tests for microbial community structure between reclamation and reference soils per site based on Bray-Curtis distances

Factors	Unweighted Bray (composition)			Weighted Bray (Structure)		
	PERMANOVA		PERMDISP	PERMANOVA		PERMDISP
	R^2 (%)	*P*	*P*	R^2 (%)	*P*	*P*
Pair-wise site comparison						
Site X	29.20	0.018	0.001	26.95	0.090	0.001
Site Y	53.09	0.018	0.120	51.50	0.026	0.119
Site Z	13.58	0.280	0.035	12.66	0.406	0.058
Sample-wide analyses						
Site	27.29	0.001	0.22	28.16	0.001	0.263
Soil History	6.70	0.007	0.04	8.13	0.004	0.032
Site x Soil History	13.65	0.001	ND	14.80	0.001	ND

ND, Not determined. PERMANOVA tests were performed by using the “adonis ()” of the vegan package of R software and are based on 999 iterations.

Table 3.S2: Posthoc PERMANOVA (Pair-wise comparison) for microbial communities of chronosequence and unmined area in site Z

	Bacterial community				Fungal community			
	Unweighted Bray-Curtis		Weighted Bray-Curtis		Unweighted Bray-Curtis		Weighted Bray-Curtis	
pairs	R^2	*P* (fdr-adjusted)	R^2	*P* (fdr-adjusted)	R^2	*P* (fdr-adjusted)	R^2	*P* (fdr-adjusted)
unm-Z vs ReclZ-11	0.36	0.02*	0.36	0.02*	0.46	0.01*	0.46	0.01*
unm-Z vs ReclZ-15	0.346	0.02*	0.35	0.02*	0.46	0.01*	0.45	0.01*
unm-Z vs ReclZ-19	0.27	0.02*	0.27	0.02*	0.46	0.01*	0.45	0.01*
unm-Z vs ReclZ-3	0.43	0.02*	0.43	0.02*	0.48	0.01*	0.47	0.01*
unm-Z vs ReclZ-24	0.47	0.02*	0.48	0.02*	0.64	0.01*	0.59	0.01*
ReclZ-11 vs ReclZ-15	0.23	0.02*	0.25	0.02*	0.20	0.01*	0.21	0.01*
ReclZ-11 vs ReclZ-19	0.29	0.02*	0.28	0.02*	0.25	0.01*	0.26	0.01*
ReclZ-11 vs ReclZ-3	0.14	0.10	0.15	0.06	0.13	0.18	0.14	0.16
ReclZ-11 vs ReclZ-24	0.21	0.02*	0.25	0.02*	0.33	0.02*	0.31	0.01*
ReclZ-15 vs ReclZ-19	0.20	0.02*	0.19	0.02*	0.18	0.02*	0.18	0.01*
ReclZ-15 vs ReclZ-3	0.23	0.02*	0.25	0.02*	0.24	0.01*	0.24	0.01*
ReclZ-15 vs ReclZ-24	0.22	0.02*	0.24	0.03*	0.29	0.01*	0.28	0.01*
ReclZ-19 vs ReclZ-3	0.34	0.02*	0.33	0.02*	0.27	0.01*	0.27	0.01*
ReclZ-19 vs ReclZ-24	0.34	0.02	0.35	0.02*	0.37	0.01*	0.35	0.01*
ReclZ-3 vs ReclZ-24	0.21	0.02	0.27	0.02*	0.36	0.01*	0.32	0.01*

*False discovery rate-adjusted $P < 0.05$.

Table S3.3: Statistical test for discriminative genus-level features between reclamation and reference soil. See Figure 4.

	P values	FDR-adjusted P-values	Class	LDA score
Massilia	0.003	0.337	Reclamation	4.65
Sporosarcina	0.004	0.337	Reclamation	3.49
Oryzihumus	0.004	0.337	Reclamation	4.36
Terrabacter	0.005	0.337	Reclamation	4.09
Mucilaginibacter	0.006	0.337	Reclamation	3.72
Oceanobacillus	0.007	0.337	Reclamation	2.66
Janibacter	0.007	0.337	Reclamation	3.97
Sphingomonas	0.008	0.337	Reclamation	5.2
Deinococcus	0.009	0.337	Reclamation	2.74
Rhodanobacter	0.012	0.337	Reclamation	3.61
Dokdonella	0.013	0.337	Reclamation	3.43
Segetibacter	0.014	0.337	Reclamation	3.9
Phycicoccus	0.014	0.337	Reclamation	3.73
Dyella	0.016	0.337	Reclamation	3.73
Fulvimonas	0.017	0.337	Reclamation	3.06
Streptomyces	0.019	0.337	Reclamation	4.41
Clostridium sensu stricto *1*	0.021	0.337	Reclamation	3.08
Opitutus	0.026	0.380	Reclamation	3.42
Arthrobacter	0.026	0.380	Reclamation	3.82
Flavisolibacter	0.026	0.380	Reclamation	4
Methylobacterium	0.034	0.383	Reclamation	3.63
Candidatus *Koribacter*	0.034	0.383	Reclamation	3.8
Jatrophihabitans	0.034	0.383	Reclamation	4.04
Clostridium sensu stricto 12	0.040	0.383	Reclamation	2.75
Burkholderia-Caballeronia-Paraburkholderia	0.005	0.337	Reclamation	4.43
Rubrobacter	0.003	0.337	Reference	-3.6
Vicinamibacter	0.007	0.337	Reference	-3.49
Chitinophaga	0.014	0.337	Reference	-2.97
Lechevalieria	0.018	0.337	Reference	-3.59
Chryseolinea	0.020	0.337	Reference	-2.08
Sphingomicrobium	0.020	0.337	Reference	-2.09
Rhodopirellula	0.020	0.337	Reference	-2.53
Herpetosiphon	0.020	0.337	Reference	-2.56
Hirschia	0.020	0.337	Reference	-2.8
Flavitalea	0.029	0.383	Reference	-3.05
FFCH5858	0.030	0.383	Reference	-2.64
Virgisporangium	0.030	0.383	Reference	-2.67
SWB02	0.032	0.383	Reference	-2.83
Candidatus *Protochlamydia*	0.037	0.383	Reference	-2.54

Table 3.S4: Relative abundance of dominant bacterial genera across reclamation chronosequence and unmined soil in site Z.

	Relative abundance (%)						
	ReclZ-3	**ReclZ-11**	**ReclZ-15**	**ReclZ-19**	**ReclZ-24**	**unm-Z**	**P-values**
Acidothermus	3.08±0.52	3.57±3.24	7.13±1.42	4.68±3.15	8.37±1.38	1.7±2.05	0.256
Actinoplanes	1.00±0.27^{a}	0.9±0.51^{ab}	0.34±0.34^{b}	0.36±0.12^{b}	0.4±0.29^{ab}	1.06±0.75^{ab}	0.046*
Amycolatopsis	1.78±1.2	3.21±2.2	0.81±0.55	0.54±0.21	0.44±0.33	0.48±0.25	0.635
Blastococcus	1.42±0.91	1.59±0.76	0.58±0.28	0.62±0.26	0.45±0.25	1.17±0.49	0.294
Bradyrhizobium	1.77±0.46	1.07±0.45	1.8±0.65	1.11±0.47	1.04±0.64	1.32±1.06	0.548
Bryobacter	2.59±0.45^{ab}	3.27±0.45^{b}	2.4±0.77^{b}	1.6±0.88^{a}	4.4±1.51^{b}	1.19±0.61^{b}	0.043*
Candidatus Solibacter	1.49±0.55	0.86±0.23	1.45±0.18	0.76±0.21	1.48±0.5	1.13±0.88	0.737
Crossiella	0.63±0.3	1.76±2.16	0.81±0.4	1.47±1.2	2.62±0.84	0.65±0.34	0.361
Jatrophihabitans	2.51±0.79	2.18±0.95	1.6±0.59	1.24±0.48	1.15±0.28	0.98±0.66	0.150
Micromonospora	0.4±0.2	0.4±0.15	0.46±0.44	0.55±0.28	0.26±0.19	1.72±0.62	0.064
Mycobacterium	2.28±0.83	2.07±0.86	2.29±0.89	1.66±0.36	2.83±0.93	1.86±0.32	0.827
Nocardioides	0.62±0.47	0.29±0.25	0.51±0.41	0.67±0.59	0.1±0.07	2.13±1.41	0.191
Pseudarthrobacter	1.28±0.84	1.63±1.25	0.44±0.21	0.96±1.04	0.14±0.15	1.93±1.1	0.569
Pseudonocardia	0.62±0.28	1.64±1.49	0.8±0.47	1.26±0.54	1.07±0.35	2.37±0.68	0.100
Chlorobacteria RB41	0.16±0.21	0.51±0.64	2.18±1.38	4.13±1.84	0.65±0.54	2.5±2.39	0.081
Singulisphaera	2.41±0.71	4.59±1.99	2.29±0.41	2.78±0.92	2.99±0.76	2.05±1.42	0.395
Solirubrobacter	0.62±0.18	0.96±0.41	1.08±0.19	1.49±0.44	1.44±0.38	1.67±0.64	0.523
Sphingomonas	1.29±1.01	0.8±0.58	0.31±0.16	0.28±0.1	0.31±0.01	0.7±0.4	0.071
Streptomyces	1.66±0.49	1.07±0.63	3±1.03	2.66±1.57	1.06±1.16	1.81±0.65	0.236
Variibacter	0.81±0.15	0.38±0.27	1.31±0.49	0.72±0.32	0.69±0.36	0.99±0.3	0.051
Other	20.37±4.28	23.32±4.13	15.52±1.49	17.01±3.25	15.68±3.03	16.41±1.77	0.204
Ambiguous taxa	2.77±0.85	1.94±0.86	4.14±0.77	4.69±1.53	2.32±0.8	4.47±0.55	0.213

Uncultured	35.43±6.01	27.82±8.36	37.51±5.48	37.95±3.34	40.15±4.83	31.72±5.39	0.607

*Significant at a probability level of 0.05 based on Kruskal-Wallis H test. Values are mean± SD. Only dominant genera (average relative abundance ≥1% in at least one group) are shown. Others, unclassified and uncultured all refer to a group of 16S rRNA gene sequences which could not be confidently assigned into a taxonomic group at the genus or higher taxonomic ranks. See also Figure 3.12.

Table 3.S5: Relative abundance of dominant fungal genera across reclamation chronosequence and unmined soil in site Z.

Genus	Relative abundance (%)						P-value
	ReclZ-3	ReclZ-11	ReclZ-15	ReclZ-19	ReclZ-24	unm-Z	
Cercophora	0±0^{b}	0±0^{b}	0.01±0.02^{b}	0±0^{b}	0±0^{b}	2.26±2.34^{a}	0.000*
Cladophialophora	1.92±1.53^{a}	0.77±0.65^{abc}	0.18±0.1^{c}	0.52±0.27^{ab}	0.20±0.19^{bc}	0.90±0.79^{ab}	0.050
Coniosporium	0.65±1.00^{a}	1.58±2.85^{a}	0.01±0.03^{b}	0±0^{b}	0.14±0.32^{b}	0.01±0.02^{b}	0.007*
Curvularia	3.3±1.56^{a}	5.77±4.51^{a}	3.43±1.84^{a}	2.24±1.16^{ab}	1.31±0.79^{bc}	0.7±0.46^{c}	0.005*
Echria	0.04±0.03^{a}	0.24±0.45^{a}	0.04±0.03^{a}	0.07±0.05^{a}	4.58±10.13^{a}	0±0^{b}	0.038*
Endocarpon	0±0^{b}	0.01±0.02^{b}	0±0^{b}	1.16±2.48^{a}	0±0^{b}	0±0^{b}	0.030*
Helicoma	0.02±0.02^{b}	0.01±0.02^{b}	0.01±0.01^{b}	0.01±0.02^{b}	0.01±0.01^{b}	1.56±1.79^{a}	0.010*
Neoascochyta	2.02±4.01^{a}	0.23±0.23^{a}	0.18±0.25^{a}	0.05±0.06^{a}	0.01±0.01^{a}	0.06±0.08^{a}	0.104
Oidiodendron	1.74±2.10^{a}	1.32±1.91^{a}	12.99±19.15^{a}	0.91±1.51^{a}	1.09±2.35^{a}	0.29±0.34^{a}	0.384
Penicillium	4.7±6.99^{a}	3.4±4.84^{a}	3.69±2.82^{a}	8.96±10.42^{a}	3.45±3.18^{a}	7.75±2.83^{a}	0.383
Plenodomus	0±0.01^{a}	0±0^{a}	0.01±0.02^{a}	1.56±3.47^{a}	0±0.01^{a}	0.01±0.01^{a}	0.785
Rasamsonia	1.38±2.03^{ab}	0.49±0.69^{b}	1.10±0.58^{ab}	1.14±1.30^{ab}	1.48±0.72^{a}	0±0^{c}	0.010*
Talaromyces	0.45±0.26^{b}	0.12±0.09^{c}	1.99±1.14^{a}	1.36±1.21^{a}	0.23±0.15^{bc}	0.36±0.26^{b}	0.001*
Thielavia	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.78±1.67^{a}	1.45±3.17^{a}	0.02±0.02^{a}	0.268
unidentified	22.02±10.17^{a}	28.35±6.72^{a}	20.98±6.41^{a}	23.11±10.19^{a}	16.60±7.29^{a}	14.55±9.83^{a}	0.157
Other	55.83±5.7^{ab}	52.68±4.51^{b}	51.63±11.14^{b}	49.41±11.18^{b}	64.25±5.7^{a}	61.63±7.06^{a}	0.047*

*Significant at a probability level of 0.05 based on Kruskal-Wallis H test. Values are mean± SD. Values with different subscript letters are significantly (P < 0.05) different based on Fisher's LSD. Only dominant genera (average relative abundance ≥1% in at least one group) are shown. Others and unidentified refer to a group of ITS2 sequences which could not be confidently assigned into a taxonomic group at the genus or higher taxonomic ranks. See also Figure 3.14a.

Table 3.S6: Tax4Fun statistics for the functional prediction of soil bacterial communities between site and soil histories (2016 samples)

Soil group	Average FTUs	†1-Average FTUs
ReclX	0.89±0.02[a]	0.11±0.02[a]
RefX	0.86±0.03[a]	0.14±0.03[a]
ReclY	0.81±0.04[a]	0.19±0.04[a]
RefY	0.82±0.01[a]	0.18±0.01[a]
ReclZ	0.84±0.01[a]	0.16±0.01[a]
RefZ	0.85±0.04[a]	0.15±0.04[a]

FTU, Fraction of OTUs which were not mapped against KEGG organisms. †Fraction of OTUs which mapped onto the KEGG organisms is obtained by subtracting FTUs from 1. Differences in FTUs or 1-FTUs amongst "treatments" are not significant ($P > 0.05$).

Table 3.S7: FTU for prediction of bacterial along chronosequence

Sampled Area	Average FTU	1-Average FTU
ReclZ-3	0.55±0.04[a]	0.47±0.01[a]
ReclZ-11	0.51±0.05[a]	0.49±0.05[a]
ReclZ-15	0.55±0.03[a]	0.45±0.03[a]
ReclZ-19	0.53±0.06[a]	0.47±0.06[a]
ReclZ-24	0.48±0.04[a]	0.52±0.04[a]
unm-Z	0.53±0.01[a]	0.45±0.04[a]

FTU, Fraction of OTUs which were not mapped against KEGG organisms. †Fraction of OTUs which mapped onto the KEGG organisms is obtained by subtracting FTUs from 1. Differences in FTUs or 1-FTUs amongst "treatments" are not significant (Kruskal-Wallis, $P > 0.05$).

Table 3.S8: Significance of terms (physicochemical properties) in the CCA model of Figure 3.11.

Constraints	F	Pr. (>F)
Sand	2.2023	0.136
Silt	5.5790	0.013*
Clay	1.6856	0.199
BD	4.3089	0.022*
pH	25.7254	0.001***
Moist	1.0265	0.360
OM	1.8720	0.164
EC	5.4034	0.015 *
Cl	1.5691	0.221
NO_3	2.0760	0.174
NO_2	1.4687	0.260
PO4	1.3124	0.272
Na	3.4884	0.041 *
K	0.9829	0.393
Ca	4.5304	0.026 *
Mg	1.0584	0.376
CEC	1.5399	0.222

*Significant at the 0.05 probability level; ***Significant at the 0.001 probability level

Table 3.S9: Significance and contribution of physicochemical properties to the bacterial and fungal communities under a stepwise forward CCA model (Figure 3.17)

Soil properties	Bacterial community				Fungal community		
	Df	Chi Square	F	*Pr* (>F)	Chi Square	F	*Pr* (>F)
pH	1.000	0.110	6.044	0.001***	0.075	2.406	0.001***
Nit.	1.000	0.044	2.403	0.008***	0.095	3.052	0.001***
NH4	1.000	0.018	0.974	0.406	0.046	1.468	0.035*
P	1.000	0.029	1.613	0.058	0.048	1.536	0.020*
K	1.000	0.022	1.197	0.180	0.034	1.108	0.224
Ca	1.000	0.027	1.503	0.081	0.039	1.247	0.098
Mg	1.000	0.017	0.957	0.463	0.034	1.098	0.287
Na	1.000	0.021	1.133	0.243	0.039	1.239	0.110
Sand	1.000	0.020	1.108	0.267	0.039	1.261	0.108
Silt	1.000	0.023	1.280	0.147	0.032	1.040	0.341
Clay	1.000	0.032	1.773	0.039*	0.035	1.123	0.256
C	1.000	0.020	1.121	0.245	0.036	1.162	0.166
SV	1.000	0.025	1.352	0.120	0.038	1.218	0.112
BD	1.000	0.017	0.938	0.466	0.032	1.043	0.371
Residual	15.000	0.273	NA	NA	0.467	NA	NA

*Significant at the 0.05 probability level; ***Significant at the 0.001 probability level

Table 3.S10: Significance and contribution of physicochemical properties to the bacterial and fungal communities across the post-coal mining reclamation soil chronosequence under a stepwise forward CCA model (Figure 3.S8)

		Bacterial community			Fungal community		
Soil properties	**Df**	**Chi Square**	**F**	***Pr* (>F)**	**Chi Square**	**F**	***Pr* (>F)**
pH	1	0.066	3.323	0.001***	0.063	1.892	0.001***
Nit.	1	0.033	1.648	0.017*	0.057	1.705	0.002**
NH4	1	0.018	0.925	0.582	0.042	1.262	0.068
P	1	0.037	1.882	0.006**	0.050	1.509	0.011*
K	1	0.021	1.065	0.303	0.037	1.117	0.225
Ca	1	0.030	1.498	0.041*	0.040	1.190	0.115
Mg	1	0.024	1.207	0.167	0.039	1.185	0.153
Na	1	0.020	0.997	0.428	0.035	1.053	0.311
Sand	1	0.026	1.325	0.101	0.042	1.268	0.067
Silt	1	0.028	1.406	0.053	0.042	1.265	0.073
Clay	1	0.021	1.070	0.310	0.038	1.148	0.173
C	1	0.024	1.211	0.163	0.043	1.282	0.063
SV	1	0.020	1.020	0.397	0.034	1.027	0.400
BD	1	0.021	1.042	0.346	0.031	0.938	0.588
Foliar	1	0.020	1.033	0.392	0.036	1.073	0.292
Basal	1	0.025	1.270	0.113	0.036	1.084	0.251
Litter	1	0.018	0.910	0.589	0.028	0.849	0.822
Residual	7	0.138	NA	NA	0.233	NA	NA

*Significant at the 0.05 probability level; **Significant at the 0.01 probability level; ***Significant at the 0.001 probability level

Table 4.S1: Pair-wise comparison for soil AMF community

	Bray-Curtis dissimilarity						Unifrac Distance					
	Unweighted			Weighted			Unweighted			Weighted		
Site Pairs	**R^2**	***P-value***	***P* (fdr-adjusted)**	**R^2**	***P-value***	***P* (fdr-adjusted)**	**R^2**	***P-value***	***P* (fdr-adjusted)**	**R^2**	***P-value***	***P* (fdr-adjusted)**
Unmined vs. Rec11	0.300	0.008*	0.021*	0.291	0.021*	0.033*	0.353	0.006*	0.038*	0.286	0.064	0.146
Unmined vs. Rec15	0.224	0.021*	0.032*	0.226	0.032*	0.033*	0.228	0.02*	0.043*	0.140	0.273	0.293
Unmined vs. Rec19	0.307	0.0068	0.021*	0.308	0.021*	0.030*	0.253	0.028*	0.050	0.195	0.135	0.184
Unmined vs. Rec03	0.356	0.003*	0.021*	0.326	0.021*	0.033*	0.353	0.008*	0.038*	0.224	0.078	0.146
Unmined vs. Rec24	0.328	0.009*	0.021*	0.316	0.021*	0.030*	0.269	0.01*	0.038*	0.246	0.089	0.148
Rec11 vs. Rec15	0.256	0.037*	0.046	0.250	0.046	0.050	0.235	0.01*	0.038*	0.346	0.016*	0.126
Rec11 vs. Rec19	0.175	0.086	0.099	0.182	0.099	0.067	0.166	0.08*	0.109	0.254	0.03*	0.126
Rec11 vs. Rec03	0.220	0.036*	0.046	0.211	0.046	0.061	0.245	0.018*	0.043*	0.179	0.2	0.242
Rec11 vs. Rec24	0.089	0.729	0.729	0.117	0.729	0.378	0.128	0.251	0.294	0.224	0.033*	0.126
Rec15 vs. Rec19	0.178	0.011*	0.021*	0.205	0.021*	0.033*	0.124	0.307	0.307	0.208	0.073	0.146
Rec15 vs. Rec03	0.285	0.013*	0.022*	0.278	0.022*	0.033*	0.267	0.018*	0.043*	0.213	0.042*	0.126
Rec15 vs. Rec24	0.257	0.01*	0.021*	0.270	0.021*	0.030*	0.168	0.052	0.078	0.180	0.105	0.158
Rec19 vs. Rec03	0.134	0.287	0.308	0.125	0.308	0.323	0.137	0.266	0.294	0.142	0.210	0.242
Rec19 vs. Rec24	0.209	0.011*	0.021*	0.218	0.021	0.030	0.130	0.274	0.294	0.120	0.368	0.368
Rec03 vs. Rec24	0.294	0.005*	0.021*	0.270	0.021	0.030	0.233	0.030*	0.050	0.226	0.042*	0.126

*****Significant at $P < 0.05$. fdr-adjusted, false discovery rate-adjusted. Pair-wise comparison was performed using the "PairwiseAdonis" function in the Vegan package of R software.

Table 4.S2: Pair-wise comparison for roots AMF community

	Bray-Curtis dissimilarity						Unifrac Distance					
	Unweighted			Weighted			Unweighted			Weighted		
Site pairs	**R^2**	***P*-value**	***P* (fdr-adjusted)**	**R^2**	***P*-value**	***P* (fdr-adjusted)**	**R^2**	***P*-value**	***P* (fdr-adjusted)**	**R^2**	***P*-value**	***P* (fdr-adjusted)**
Rec03 vs. Rec11	0.223	0.019*	0.095	0.120	0.025*	0.125	0.130	0.033*	0.083	0.113	0.467	0.584
Rec03 vs. Rec15	0.202	0.126	0.288	0.305	0.107	0.357	0.307	0.236	0.337	0.299	0.738	0.820
Rec03 vs. Rec19	0.093	0.427	0.57	0.598	0.382	0.566	0.571	0.178	0.297	0.299	0.074	0.370
Rec03 vs. Rec24	0.273	0.016*	0.095	0.120	0.019*	0.125	0.120	0.021*	0.070	0.100	0.039*	0.370
Rec11 vs. Rec15	0.121	0.456	0.57	0.598	0.558	0.620	0.590	0.675	0.750	0.726	0.973	0.973
Rec11 vs. Rec19	0.196	0.103	0.288	0.305	0.205	0.410	0.430	0.011*	0.055	0.030	0.295	0.503
Rec11 vs. Rec24	0.098	0.545	0.601	0.634	0.454	0.567	0.590	0.443	0.554	0.555	0.302	0.503
Rec15 vs. Rec19	0.128	0.432	0.57	0.598	0.419	0.567	0.571	0.173	0.297	0.299	0.257	0.503
Rec15 vs. Rec24	0.078	0.886	0.886	0.868	0.949	0.949	0.947	0.758	0.758	0.746	0.354	0.506
Rec19 vs. Rec24	0.192	0.144	0.288	0.328	0.192	0.410	0.430	0.007*	0.055	0.030	0.218	0.503

*Significant at $P < 0.05$. FDR-adjusted, false discovery rate adjusted. The pair-wise comparison was performed using the "PairwiseAdonis" function in the Vegan package of R software.

Table 4.S3: Average relative abundance (%) of AMF genera in soil and root sample

	Site	*Archaeospora*	*Acaulospora*	*Diversispora*	*Scutellospora*	*Claroideoglomus*	*Glomus*	*Paraglomus*	Unclassified
Soil	Rec03	1.29±1.16	4.51±4.37	0±0	2.96±2.68	8.14±5.58	66.93±12.19	11.63±5.57	4.54±6.50
	Rec11	0.36±0.63	1.72±2.11	0±0	8.68±14.67	6.07±3.05	77.67±13.54	4.44±2.76	1.06±1.09
	Rec15	0.79±0.83	0.74±0.78	0.65±1.24	7.25±7.63	4.90±2.74	68.56±14.58	8.90±2.71	8.21±12.81
	Rec19	2.53±1.62	5.05±2.92	0.03±0.07	1.76±0.83	5.90±6.21	74.85±10.31	6.00±2.91	3.87±3.82
	Rec24	0.75±0.69	0.95±0.51	0±0	12.07±18.35	3.47±0.84	76.85±19.00	5.68±1.70	0.23±0.17
	Unmined	0.012±0.03	1.18±1.498	0.51±0.50	0.29±0.41	0.84±0.82	82.33±17.56	3.92±2.75	10.91±13.32
Root									
	Rec03	0.04±0.07	10.02±3.38	0±0	4.186±3.116	0.19±0.43	80.91±2.86	3.22±1.82	1.44±1.59
	Rec11	0.23±0.22	9.05±5.97	0.456±0.546	3.13±3.05	2.47±2.66	77.19±9.80	7.04±0.73	0.44±0.54
	Rec15	0.05±0.06	5.197±3.32	0±0	2.47±2.39	1.08±0.95	74.91±6.37	12.90±8.32	3.40±4.99
	Rec19	0.14±0.21	6.99±3.62	0±0	1.32±0.92	1.92±3.62	73.78±14.90	15.2096±13.09	0.65±0.58
	Rec24	0.05±0.11	2.52±1.36	0±0	1.18±0.89	0.18±0.27	87.65±6.27	7.59±4.21	0.82±0.44

Table 4.S4: Correlation between alpha diversity indices and environmental parameters within post-mining chronosequence

	Soil				Roots			
	SR	***H´***	***J´***	**D**	**SR**	***H'***	***J´***	**D**
pH	0.03	0.05	-0.07	-0.04	-0.07	0.36	0.38	-0.42*
Nitrate	-0.27	-0.43*	-0.49*	0.41*	-0.17	0.07	0.14	-0.04
NH_4^+	-0.17	-0.34	-0.39	0.33	-0.35	-0.06	0.25	-0.07
P	0.20	0.18	0.11	-0.01	-0.09	0.11	0.07	-0.12
K	0.02	0.12	0.03	-0.03	-0.32	-0.07	0.24	-0.05
Ca	0.21	0.22	0.11	-0.22	-0.09	0.3	0.38	-0.34
Mg	0.14	0.29	0.18	-0.28	-0.23	0.15	0.34	-0.24
Na	-0.03	-0.08	-0.08	0.13	0.06	0.13	0.02	-0.05
Sand	-0.08	-0.28	-0.22	0.22	0.29	0.2	-0.1	-0.11
Silt	0.28	0.44*	0.30	-0.26	-0.22	-0.22	-0.03	0.2
Clay	0.03	0.20	0.15	-0.19	-0.3	-0.15	0.17	0.05
C	0.36	0.22	0.12	-0.08	0.03	0.18	0.23	-0.19
SV	0.19	0.23	0.11	-0.22	-0.12	0.25	0.38	-0.31
BD	-0.03	-0.12	-0.14	0.04	-0.16	-0.02	0.24	-0.13
Foliar	-0.40	-0.25	-0.16	0.21	-0.36	-0.57*	-0.25	0.55*
Basal	-0.16	-0.21	-0.11	0.16	-0.12	-0.45*	-0.38	0.49*
Litter	-0.17	-0.54*	-0.47*	0.32	-0.23	0.03	0.28	-0.08

*Significant at $P < 0.05$; SR, species richness; D, Dominance; *H'*, Shannon-Wiener Index; *J´*, Pieolu's evenness

Table 4.S5: Significance (permutation tests) of environmental factors in the reduced CCA model for soil (Fig. 6a) and root (Fig. 6b) AMF communities

	Soil			Roots		
Environmental variables	**Chi-square**	**F**	***P* (>F)**	**Chi-square**	**F**	**Pr. (>F)**
pH	0.009	1.872	0.131	0.005	0.527	0.729
Nitrates	0.020	4.481	0.016*	0.008	0.776	0.517
NH4	0.001	0.280	0.840	0.003	0.291	0.870
P	0.002	0.480	0.689	0.017	1.613	0.227
K	0.002	0.422	0.729	0.004	0.382	0.807
Ca	0.004	0.857	0.433	0.004	0.392	0.789
Mg	0.011	2.372	0.087	0.012	1.130	0.352
Na	0.009	2.003	0.134	0.003	0.325	0.825
Sand	0.007	1.487	0.236	0.012	1.109	0.359
Silt	0.002	0.501	0.650	0.005	0.524	0.684
Clay	0.020	4.394	0.018*	0.004	0.409	0.778
C	0.008	1.649	0.208	0.019	1.876	0.162
SV	0.011	2.329	0.097	0.023	2.176	0.121
BD	0.017	3.632	0.026*	0.003	0.286	0.877
Foliar	-	-	-	0.016	1.545	0.235
Basal	-	-	-	0.013	1.284	0.285
Litter	-	-	-	0.027	2.602	0.069

*****Significant at $P < 0.05$. BD, Bulk density; SV, sum of exchangeable bases (Ca, Mg, Na, K). –Plant cover estimates were unrealistic for the unmined maize field; hence parameters were excluded from the combined (unmined and reclamation soil) constrained ordination).

Table 4.S6: Correlation of AMF genera with environmental variables in soil

	Archaeospora	***Acaulospora***	***Diversispora***	***Claroideoglomus***	***Glomus***	***Paraglomus***	***Scutellospora***
pH	-0.26	-0.15	-0.51**	0.00	0.37	-0.51**	-0.58**
Nitrates	-0.44	-0.18	-0.61*	0.32	0.32	-0.32	-0.47*
NH4	-0.20	-0.15	-0.33	0.25	0.24	-0.20	-0.32
P	-0.31	-0.09	-0.59**	0.30	0.42*	-0.13	-0.40*
K	-0.23	0.00	-0.28	0.06	0.28	-0.32	-0.57**
Ca	-0.09	0.00	-0.31	0.02	0.25	-0.32	-0.45*
Mg	-0.10	0.01	-0.21	-0.12	0.16	-0.29	-0.38*
Na	0.06	0.18	0.10	-0.39*	-0.17	0.13	0.21
Sand	-0.27	-0.22	-0.31	0.30	0.08	-0.04	0.12
Silt	0.23	0.05	-0.03	-0.06	0.13	-0.09	-0.29
Clay	0.21	0.21	0.35	-0.42*	-0.13	0.08	-0.03
C	0.09	0.03	-0.27	0.21	0.26	0.02	-0.28
SV	-0.11	0.00	-0.30	-0.01	0.24	-0.33	-0.47*
BD	0.30	0.38*	0.00	0.33	-0.03	0.14	0.13

*Significant at $P < 0.05$; **Significant at $P < 0.01$. BD, Bulk density; SV, sum of exchangeable bases (Ca, Mg, Na, K). Spearman correlation was performed on the log-transformed species relative count and environmental data using the agricolae package of R software.

Table 4.S7: Correlation of AMF genera with environmental variables in roots

	Archaeospora	*Acaulospora*	*Diversispora*	*Claroideoglomus*	*Glomus*	*Paraglomus*	*Scutellospora*
pH	0.15	0.18	-0.05	0.19	-0.45*	0.39	0.10
Nitrates	0.11	0.18	-0.13	0.27	-0.40	0.18	0.07
NH4	0.22	0.16	0.02	0.20	-0.40	0.09	-0.02
P	0.10	0.18	0.16	-0.20	-0.29	0.33	-0.13
K	0.14	0.37	-0.19	0.14	-0.41	0.03	0.14
Ca	0.21	0.24	0.04	0.14	-0.44*	0.27	0.08
Mg	0.28	0.24	0.01	0.18	-0.35	0.01	0.02
Na	-0.01	0.18	0.05	0.05	-0.25	0.48*	-0.10
Sand	0.03	-0.11	0.25	-0.02	0.14	0.06	-0.11
Silt	0.06	0.04	-0.15	-0.24	0.05	-0.19	-0.11
Clay	0.03	0.09	-0.23	0.12	-0.16	-0.10	0.08
C	0.14	0.32	0.09	-0.09	-0.20	0.14	0.00
SV	0.23	0.26	0.01	0.15	-0.43*	0.20	0.08
BD	-0.17	-0.19	-0.28	-0.03	0.31	-0.35	0.07
Foliar	-0.11	0.06	-0.36	0.03	0.33	-0.72**	0.16
Basal	-0.20	0.21	-0.32	-0.17	0.24	-0.66**	0.24
Litter	0.14	0.21	0.15	0.30	-0.37	0.03	0.21

*Significant at $P < 0.05$. **Significant at $P < 0.01$. BD, Bulk density; SV, the sum of exchangeable bases (Ca, Mg, Na, K). Spearman correlation was performed on the log-transformed species and environmental data using the agricolae package of R software.

Table 5.S1: Mean biomass of earthworms

		Start	Week 1	Week 2	Week 3	Week 4
Mine M	Ctrl.	0.49±0.02^{a}	0.53±0.02^{ab}	0.52±0.02^{ab}	0.49±0.02^{ab}	0.47±0.01^{b}
	Unm-M	0.43±0.02^{d}	0.49±0.04^{bc}	0.48±0.03^{c}	0.48±0.03^{ab}	0.46±0.04^{b}
	Stock-M	0.47±0.02^{ab}	0.55±0.04^{a}	0.56±0.04^{a}	0.54±0.04^{a}	0.53±0.04^{a}
	ReclM-1Y	0.45±0.02^{bcd}	0.50±0.04^{abc}	0.48±0.03^{c}	0.47±0.03^{b}	0.46±0.02^{b}
	ReclM-3YA	0.46±0.03^{abc}	0.47±0.03^{c}	0.46±0.03^{c}	0.45±0.04^{b}	0.46±0.04^{b}
	ReclM-3YB	0.46±0.02^{abc}	0.50±0.03^{abc}	0.48±0.03^{c}	0.46±0.02^{b}	0.46±0.02^{b}
	ReclM-5Y	0.43±0.03^{cd}	0.51±0.03^{abc}	0.50±0.03^{bc}	0.48±0.02^{b}	0.48±0.03^{ab}
	ReclM-10Y	0.46±0.02^{abc}	0.49±0.02	0.48±0.02^{c}	0.46±0.03^{b}	0.45±0.02^{b}
Mine Z	OECD Ctrl	0.49±0.02^{a}	0.53±0.02^{a}	0.52±0.02^{a}	0.49±0.02^{a}	0.47±0.01^{a}
	unm-Z	0.46±0.03^{ab}	0.53±0.05^{a}	0.51±0.05^{a}	0.50±0.06^{a}	0.50±0.05^{a}
	Stock-Z	0.44±0.03^{b}	0.50±0.04^{a}	0.48±0.07^{a}	0.47±0.07^{a}	0.47±0.06^{a}
	ReclZ-3	0.46±0.03^{ab}	0.53±0.04^{a}	0.49±0.05^{a}	0.48±0.05^{a}	0.47±0.05^{a}
	ReclZ-11	0.46±0.03^{ab}	0.54±0.06^{a}	0.50±0.04^{a}	0.48±0.06^{a}	0.48±0.04^{a}
	ReclZ-15	0.46±0.04^{ab}	0.53±0.05^{a}	0.52±0.05^{a}	0.49±0.05^{a}	0.50±0.03^{a}
	ReclZ-19	0.46±0.03^{ab}	0.52±0.03^{a}	0.52±0.03^{a}	0.49±0.03^{a}	0.47±0.03^{a}
	ReclZ-24	0.45±0.03^{ab}	0.54±0.03^{a}	0.53±0.04^{a}	0.50±0.05^{a}	0.50±0.04^{a}

Values are mean values ± SD of five replicates. Values with different superscript letters on the same column within each site (mine M or Z) are significantly different ($P < 0.05$) based on Fisher's least significant difference.

Supplementary Figures

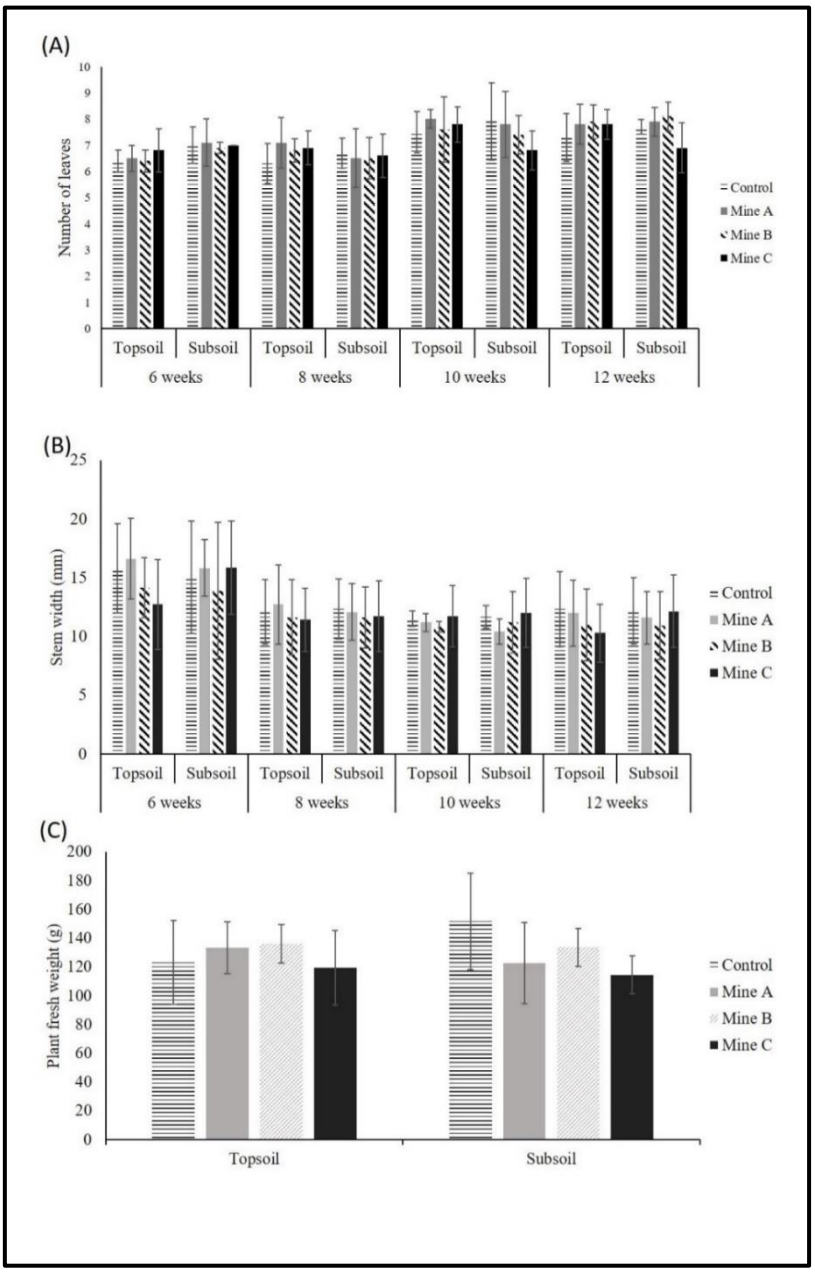

Figure 2.S1: Agronomic performance of maize plants in stockpile soils under greenhouse conditions. (a) The number of leaves (b) Stem width (c) Fresh weight after 12 weeks. Values are means of replicates (n= 5). Error bars indicate standard deviations from the mean. The main effects (site and depth) and interactions did not significantly (P > 0.05) influence stem width, fresh weights and number of leaves.

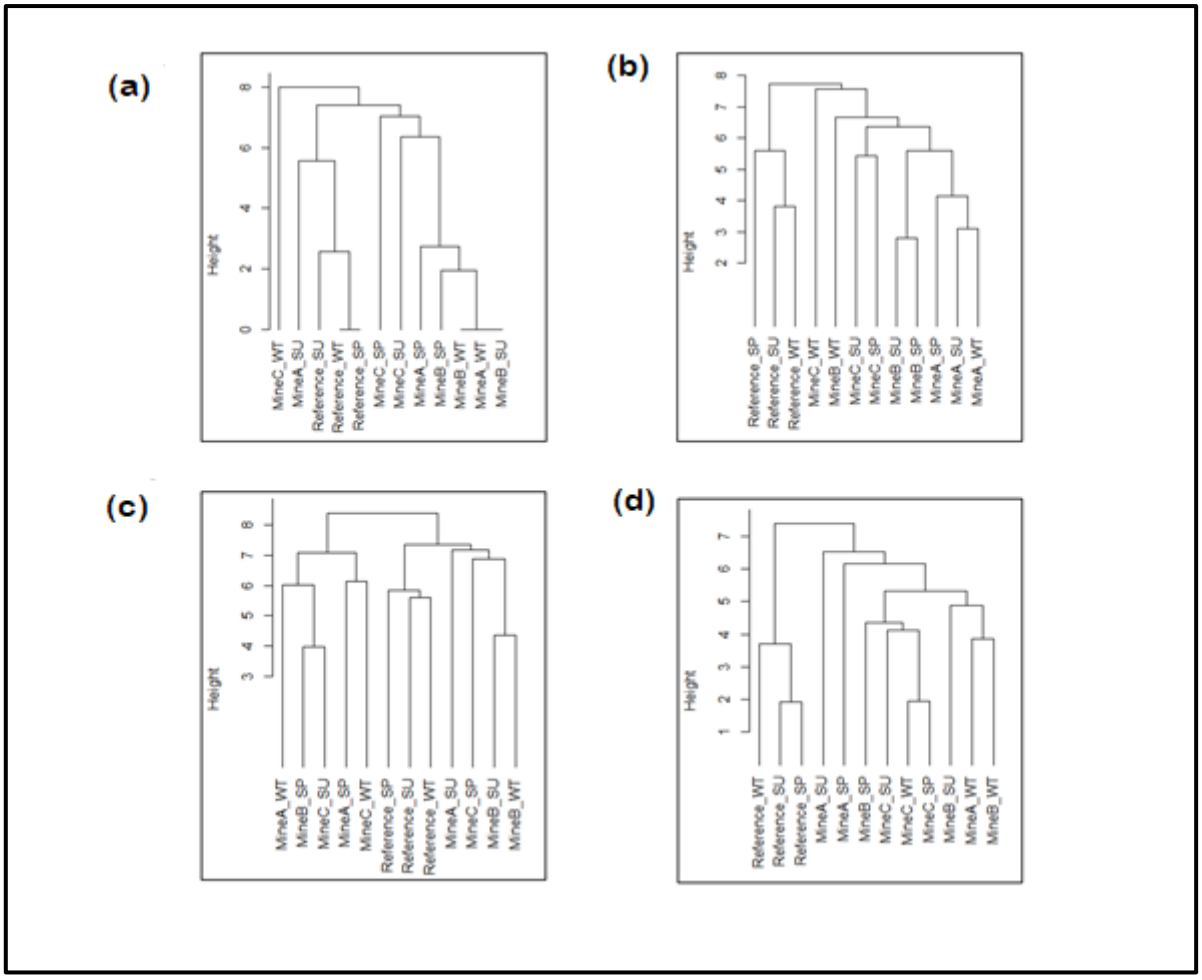

Figure 2.S2: Unweighted hierarchical cluster dendrogram of microbial communities in soils. (a) Topsoil bacterial community. (b) subsoil bacterial community. (c) topsoil fungal community. (d) subsoil fungal community.

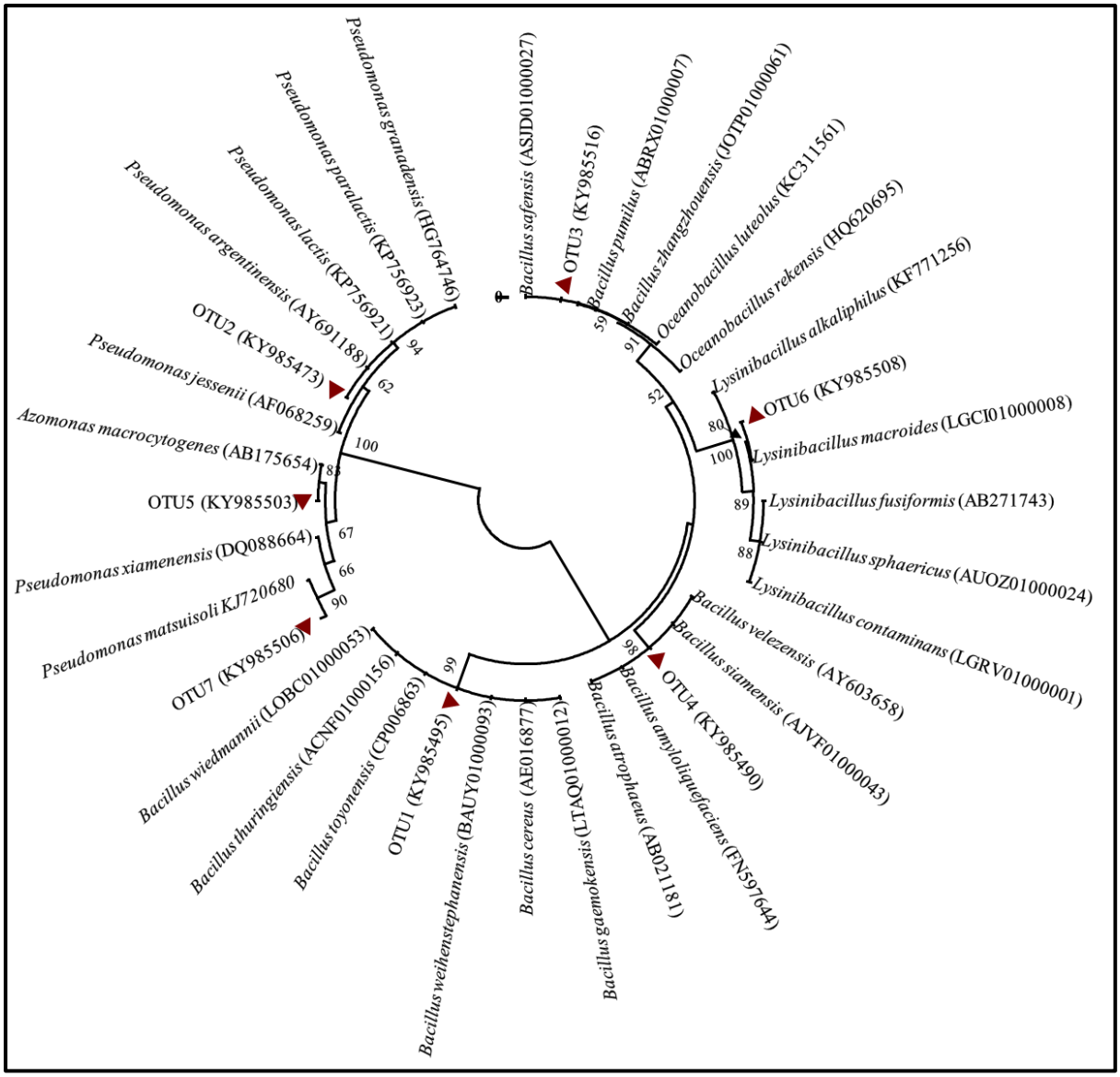

Figure 2.S3: Unrooted Neighbour-joining phylogenetic tree showing evolutionary relatedness of partial 16S rRNA sequences with sequences from the GenBank. The phylogenetic tree was constructed in MEGA software (version 7) with 1000 bootstrap replications. Bootstrap values for branch support less than 50% are not shown. Representative sequences (tips indicated with red circles) for each OTU cluster were used. The accession number of sequences are in parenthesis.

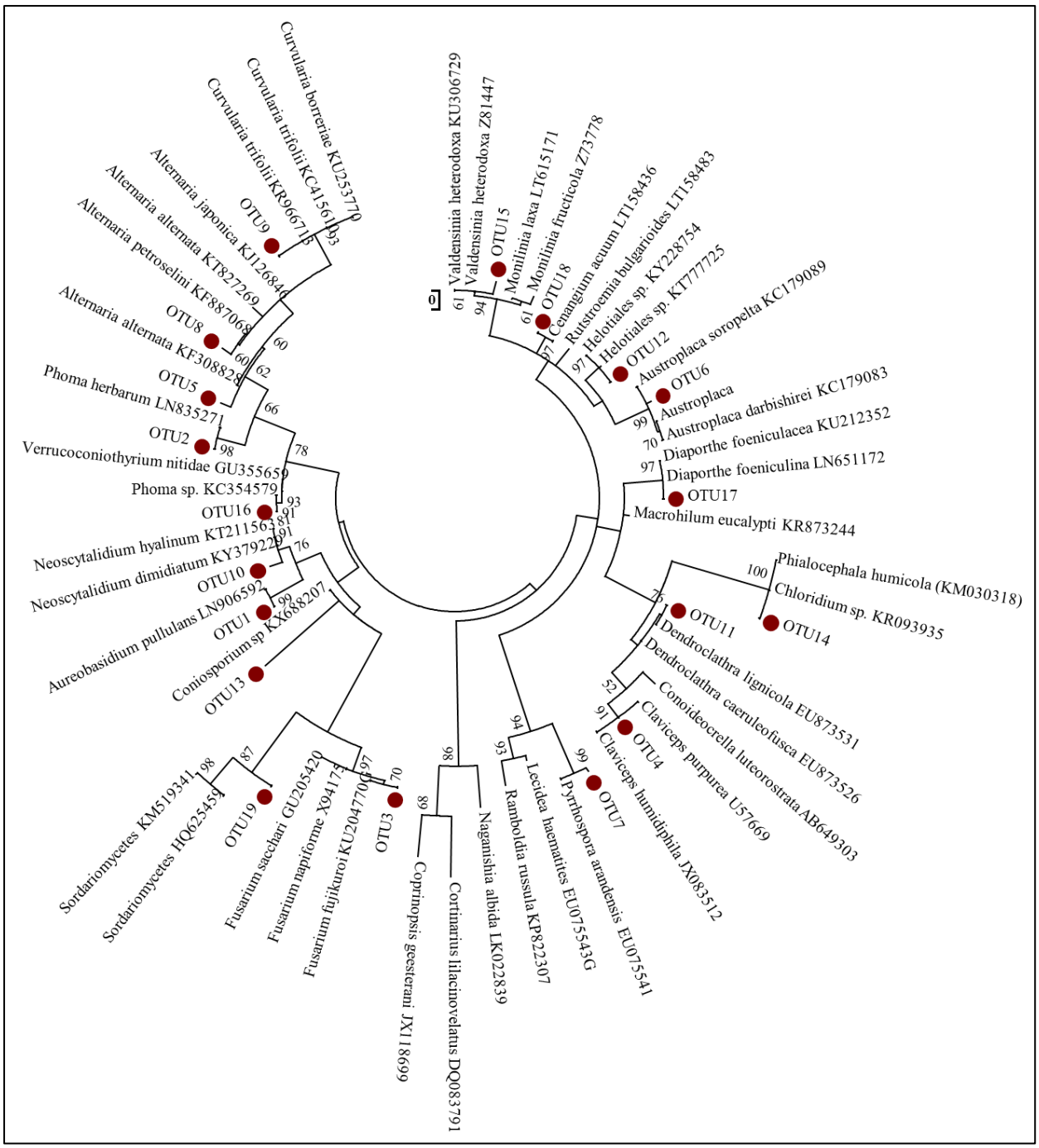

Figure 2.S4: Unrooted maximum likelihood phylogenetic tree showing evolutionary relatedness of fungal ITS2 sequences in soils with sequences from the GenBank. The phylogenetic tree was constructed in MEGA software (version 7) with 1000 bootstrap replications. Bootstrap values for branch support less than 50% are not shown. Representative sequences (tips indicated with red circles) for each OTU cluster were used. The accession number of sequences are in parenthesis.

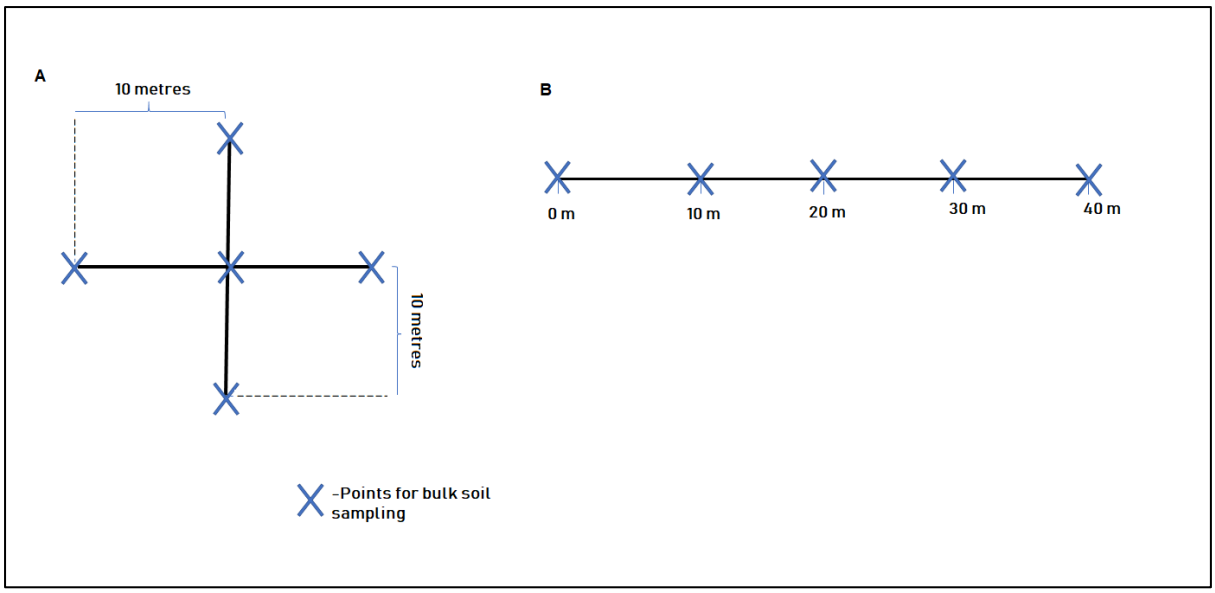

Figure 3.S1. The schematic diagram for soil sampling designs. (a) Cross design. (b) Transect method. The sampling design was aimed at obtaining representative samples and differed based on the topography and dimension of the sites. Transect method was used in site X, while cross designs were applied to site Y and Z in 2016. The transect method was used for the 2017 sampling across a chronosequence.

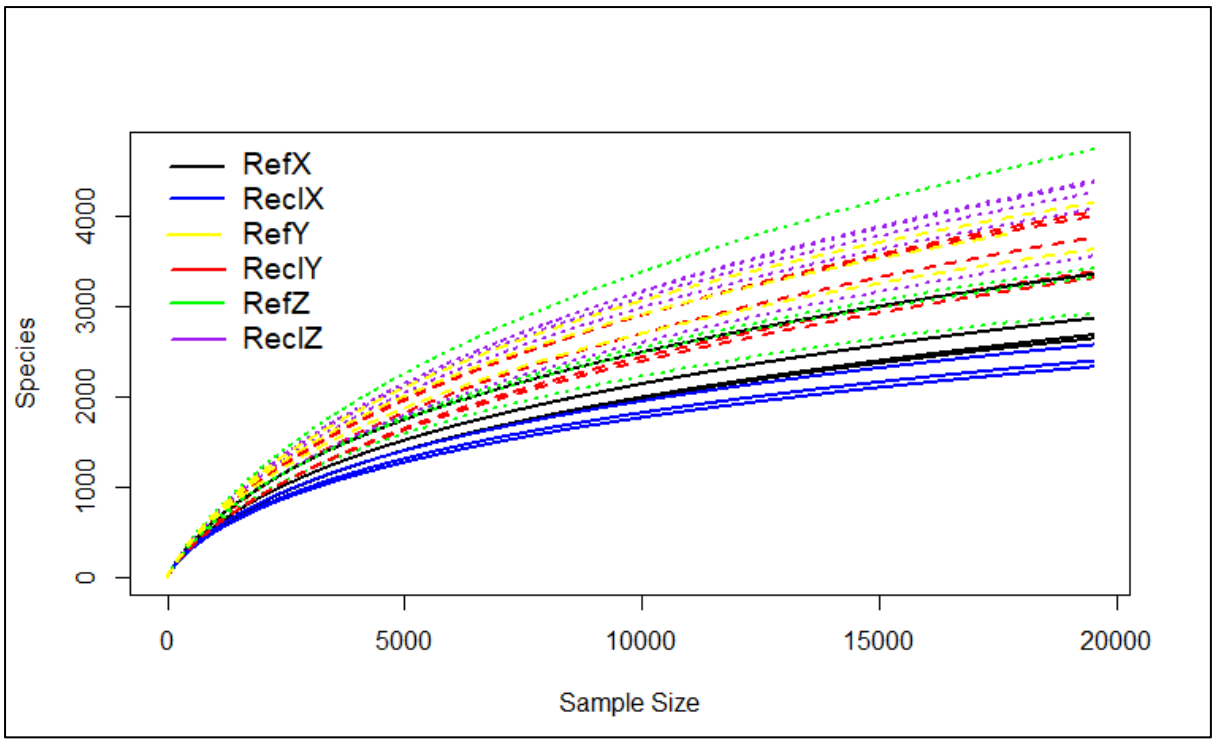

Figure 3.S2. Rarefaction curve. Bacterial communities (97% 16S RNA gene similarity OTUs) were subsampled at a depth of 19500 sequences per sample.

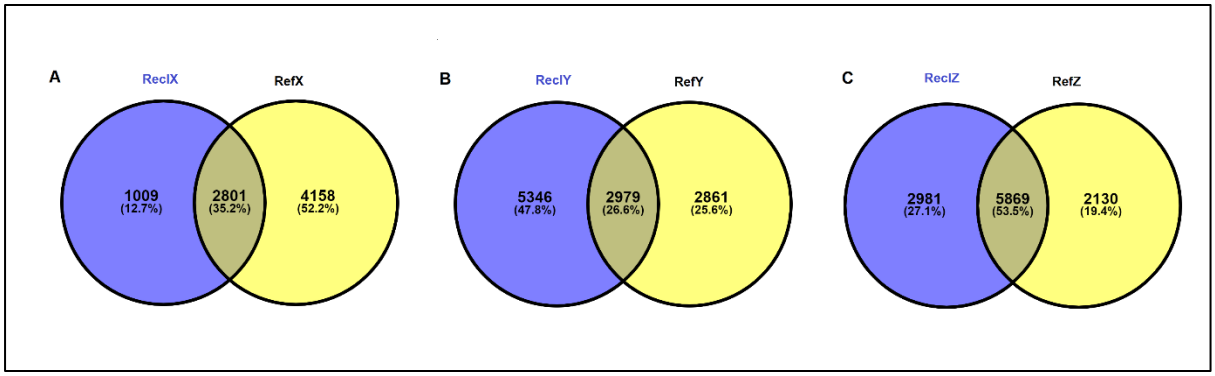

Figure 3.S3: Unique and shared OTUs between and within sites. (A) Site X. (B) Site Y. (C) Site Z. Total number of OTUs per set (or soil cluster) is the sum of unique OTUs in all replicates for each soil group (e.g. site or history). The proportion (expressed in percentage) of OTUs within each subset with respect to the total number of OTUs for any given sets are provided in parenthesis. Venn diagram was constructed by using the online Venny 2.1 software available from http://bioinfogp.cnb.csic.es/tools/venny/ (Oliveros, 2007-2015).

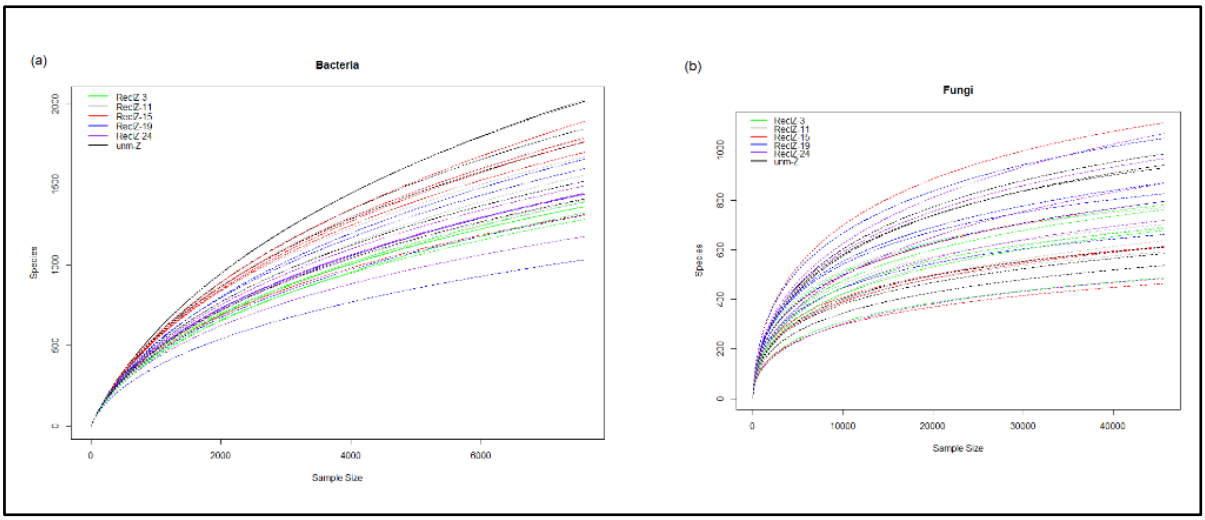

Figure 3.S4: Rarefaction curve for sequence reads analysed across post-coal reclamation chronosequence. (a) Bacterial 16S rRNA gene sequence (b) Fungal ITS2 sequences.

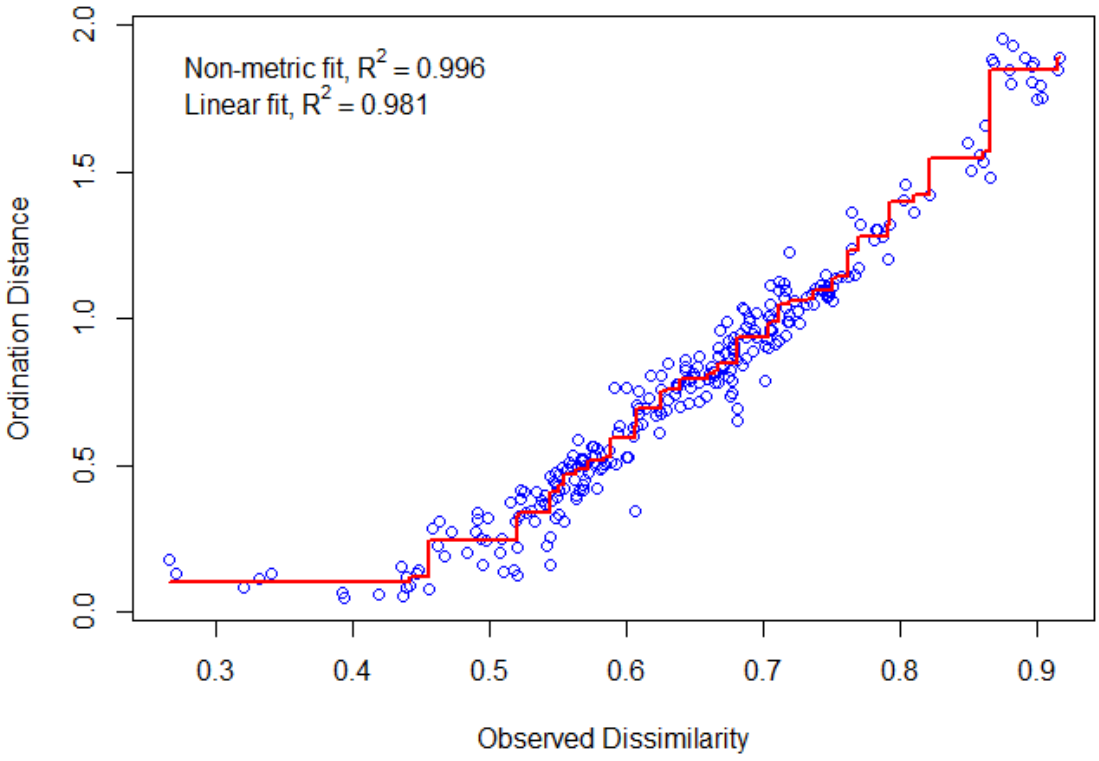

Figure 3.S5: **Stress plot for the non-metric multidimensional scaling plot of Figure 3.5**. Stress plot was generated using the “stressplot ()” function of the Vegan package of R software.

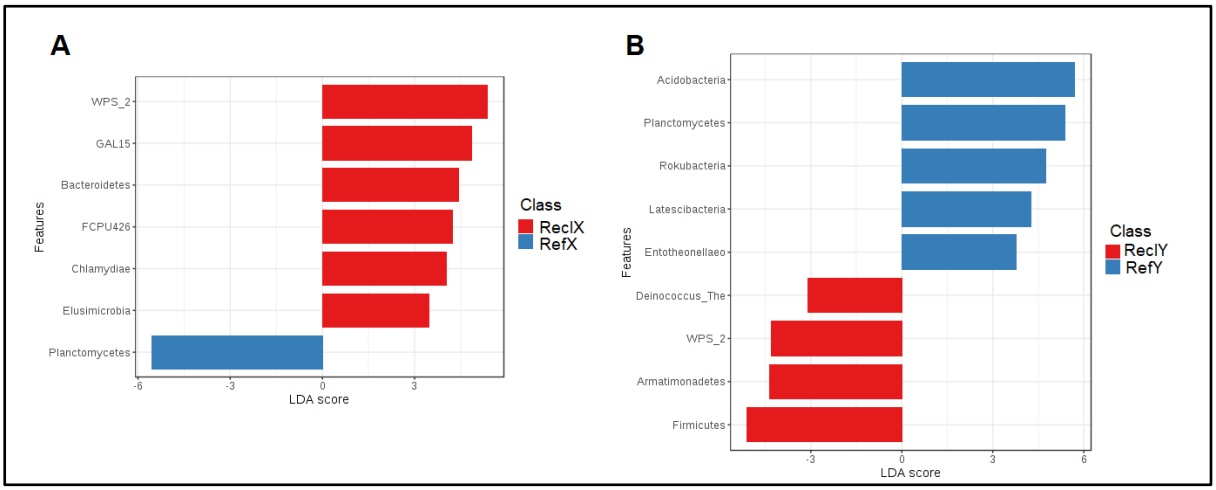

Figure 3.S6: Differentially abundant phyla between bacterial communities of reclamation and reference soils. (A) Differentially abundant phyla (LDA score > 2.0, FDR-adjusted *P*-value < 0.1) in site X. (B) Differentially abundant phyla (LDA score > 2.0, FDR-adjusted *P*-value < 0.3) in Site Y. Differential abundance and bar plots were determined and generated, respectively, using LefSe via the Microbiome Analyst (www.microbiomeanalyst.ca)

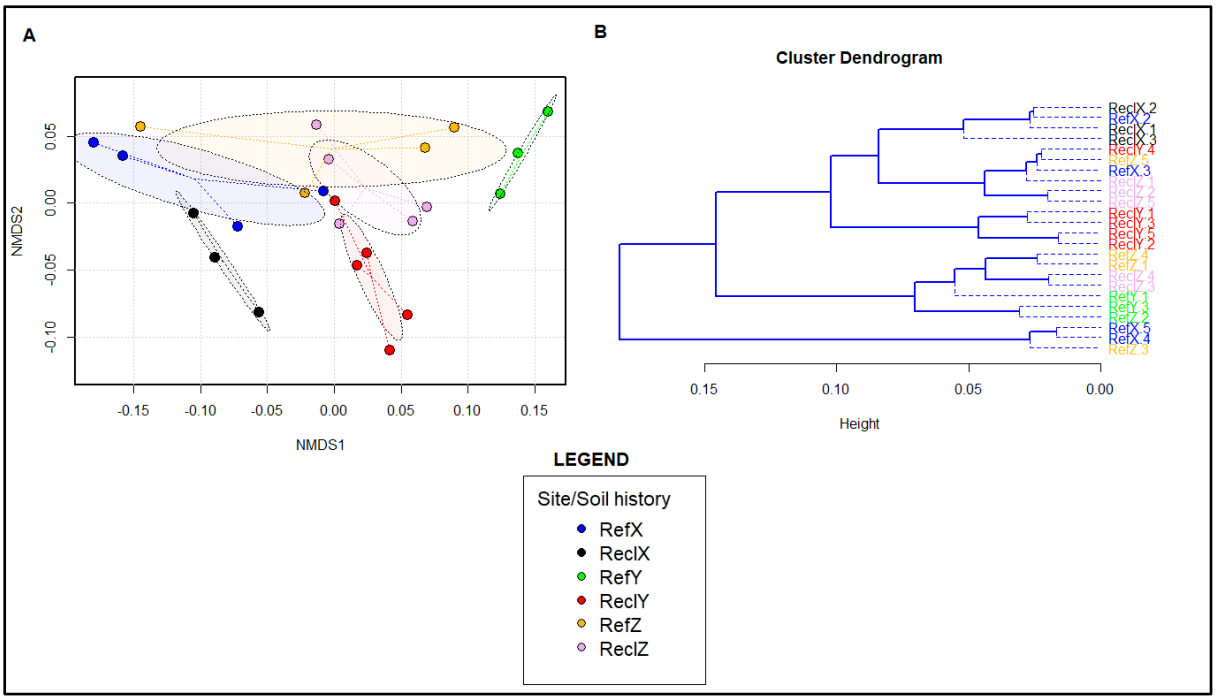

Figure 3.S7: **Bray-Curtis dissimilarity for predicted functional profile of soil bacterial communities.** (A). Non-metric dimensional scaling plot. (B). UPGMA hierarchical cluster dendrogram. Dotted lines in the nMDS plot show the distance of every sample to its group centroids in multivariate space, while ellipses show 95% confidence intervals (standard error) in multivariate space around group centroids. The stress of the nMDS plot is 0.03. Differences in multivariate space are significant for site and history interactions (PERMANOVA R^2 = 7.91%, *P* = 0.045; PERMDISP *P* = 0.016).

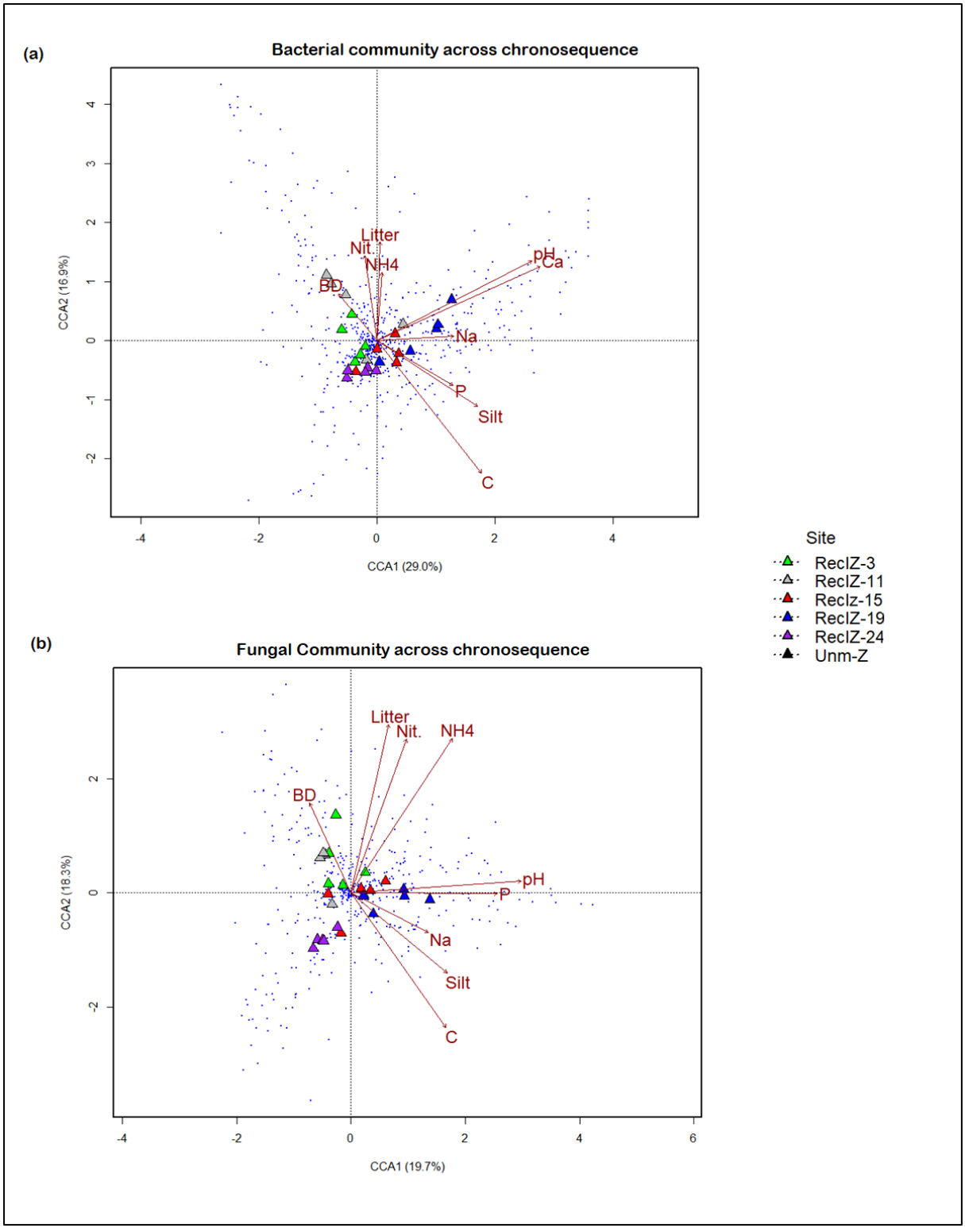

Figure 3.S8: CCA triplot showing the relationship between soil-physicochemical properties and microbial communities along the post-coal mining reclamation chronosequence of Figure 3.2. Only properties which are significant and without high collinearity (VIF > 10) are included in the final CCA model.

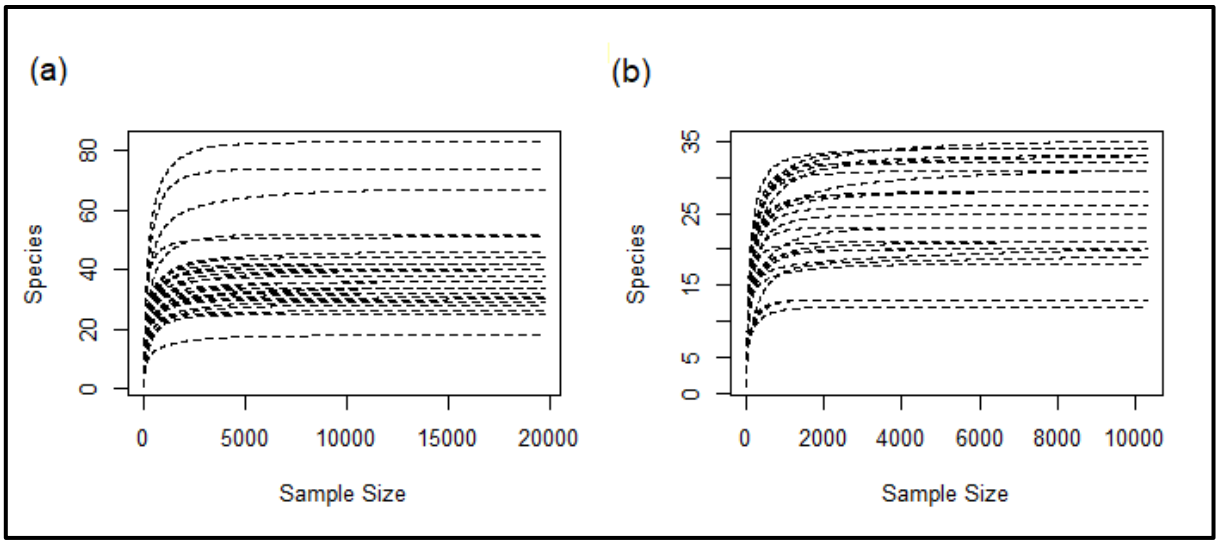

Figure 4.S1: Rarefaction curve for subsampled Glomeromycotan-SSU sequences. (a) Soil. (b) Root. OTU count tables for soil and root AMF community were subsampled to a depth of 19700 and 10300 sequences, respectively.

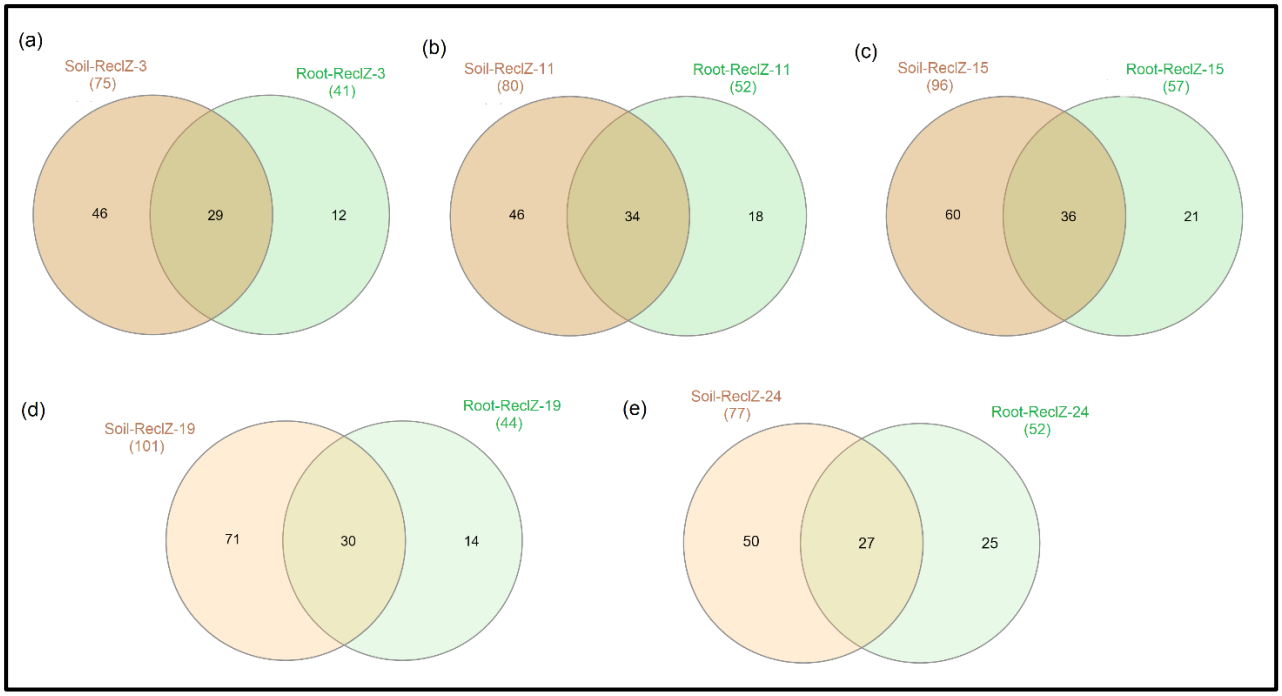

Figure 4.S2: Shared OTUs between soil and roots in reclamation chronosequence. (a) ReclZ-3. (b) ReclZ-11. (c) ReclZ-15. (d) ReclZ-19. (e) ReclZ-24. For this comparison, an OTU count table containing both soil and root samples was rarefied at an even depth of 10300 sequences per sample. Venn diagram was constructed using InteractiVenn (Heberle et al., 2015).

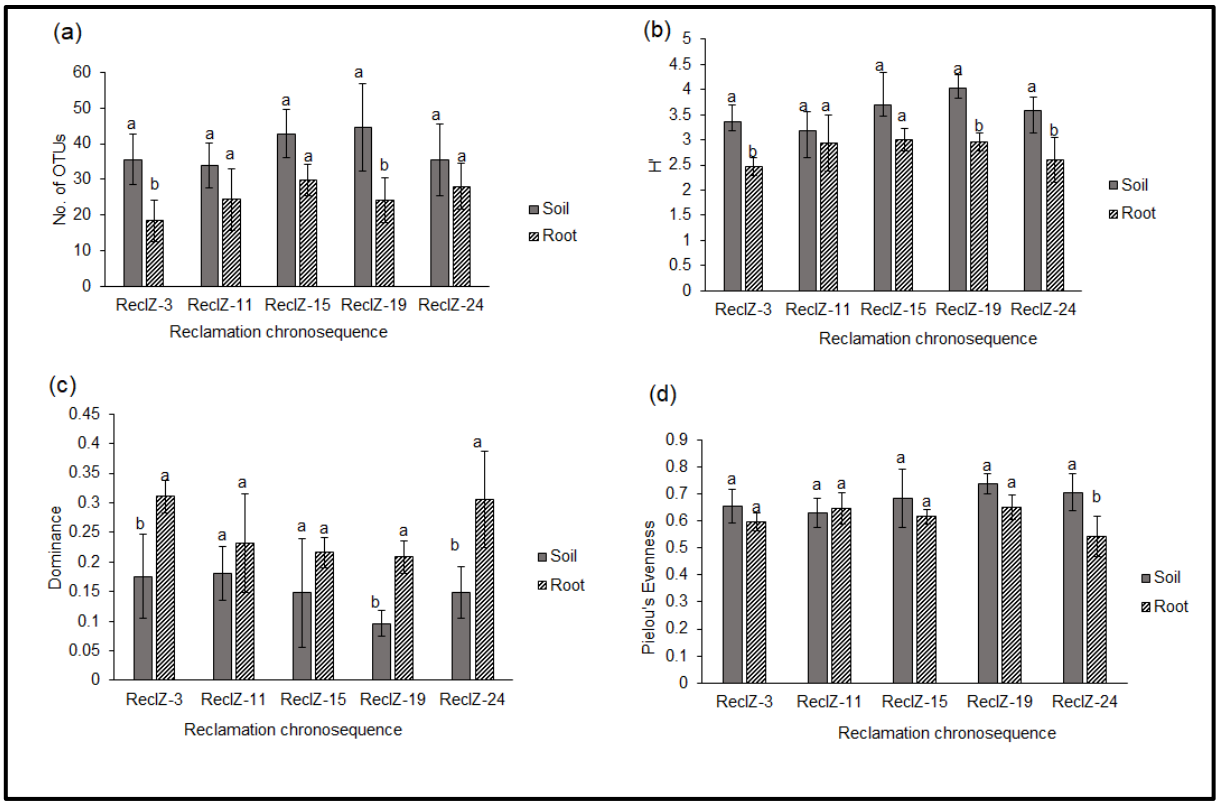

Figure 4.S3: Pair-wise comparison of alpha-diversity indices between the AMF community of soil and root per reclamation site. (a) OTU richness. (b) Shannon-Wiener index. (c) Dominance. (d) Pieolu's evenness. Alpha diversity indices were computed after rarefying the OTU-count table to an even depth of 10300 sequences per sample. Bars with different superscript letters within a reclamation site are statistically different (Mann-Whitney U test, $P < 0.05$).

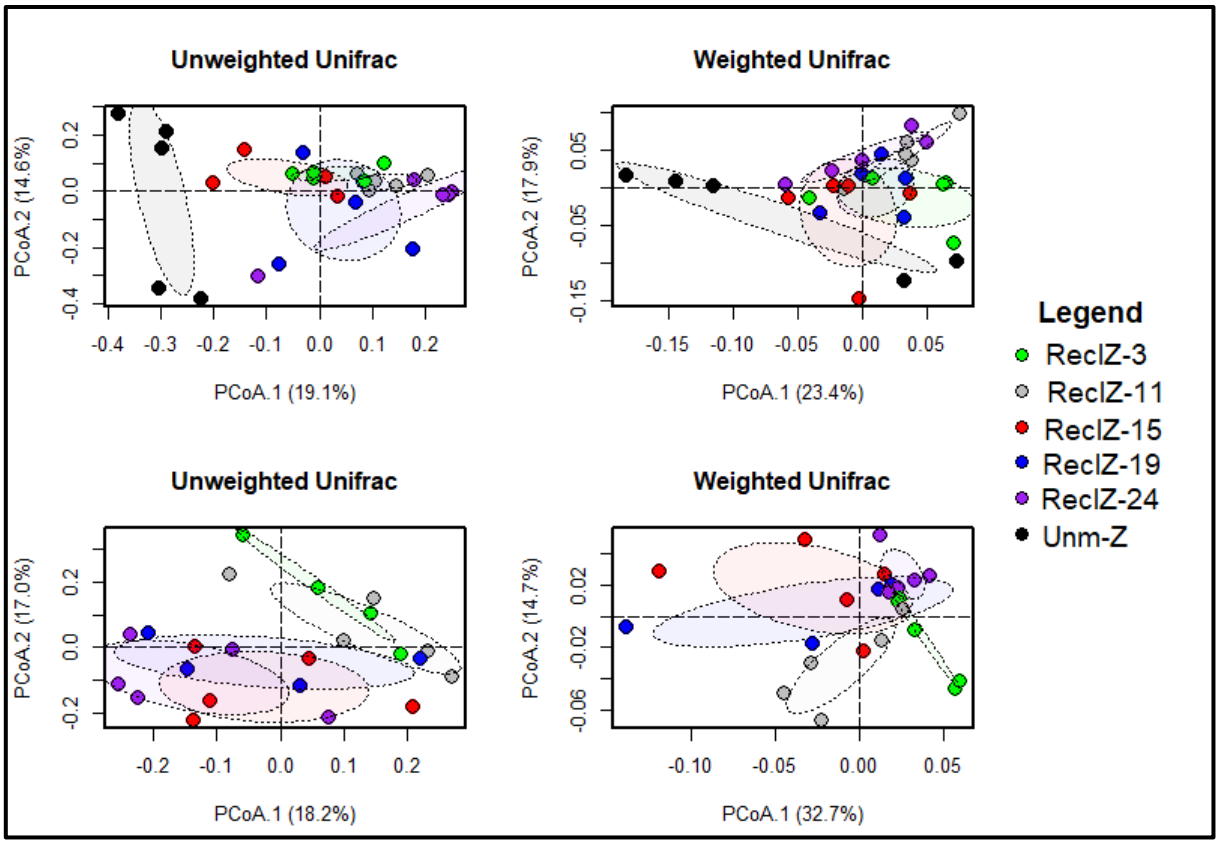

Figure 4.S4: Principal coordinate analysis of the unifrac distances between AMF communities. (a-b) soil (c-d) roots. The ellipses show 95% confidence interval in multivariate space around each group's centroid (median). For soil PERMANOVA (unweighted R^2 = 33.3%, *P* = 0.001; weighted R^2 = 31.7%, *P* = 0.002) and PERMDISP (unweighted *P* = 0.48, weighted = 0.41) indicate true significant differentiation across the chronosequence. Similarly, in roots, PERMANOVA (unweighted R^2 = 28.0%, *P* = 0.006; weighted R^2 = 33.5%, *P* = 0.008) and PERMDISP (unweighted *P* = 0.99; weighted = 0.73) indicate true significant differentiation across the chronosequence. See also Table S2 and Table S3 for pair-wise post hoc comparisons.

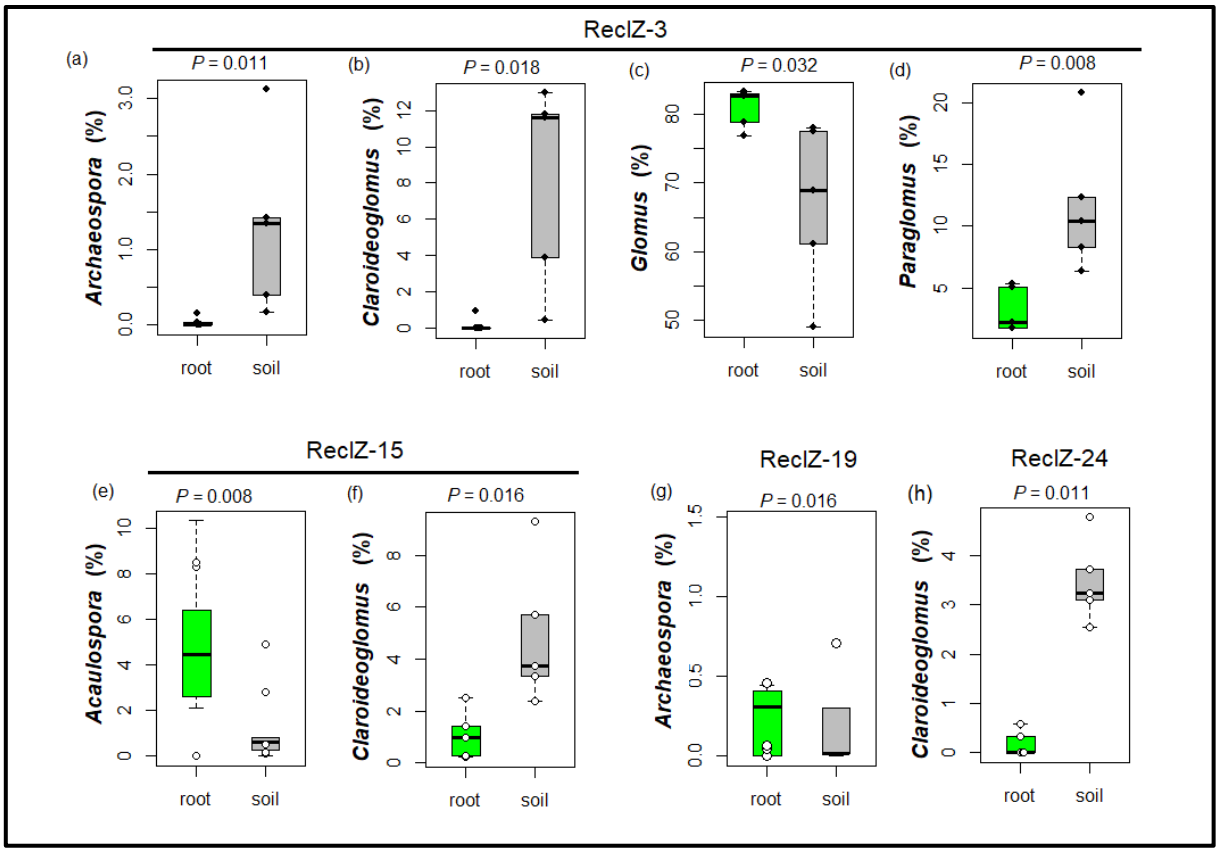

Figure 4.S5: Relative abundance of significantly different (*P* < 0.05) genera between pairs of soil and roots. (a-d) Reclz-3. (e-f) ReclZ-15 (g) ReclZ-19 and (h) ReclZ-24. Significant difference is based on Mann-Whitney U test.