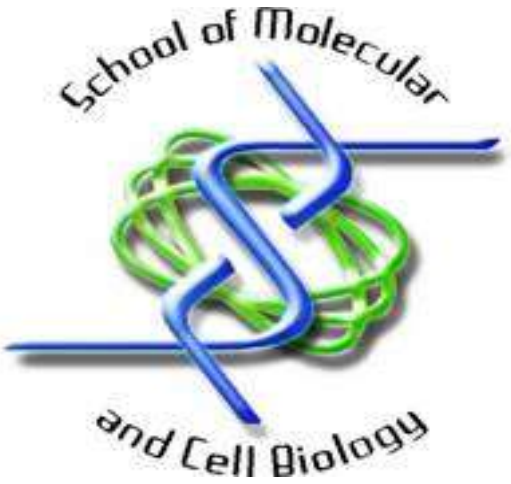

The rhizobiome of *Vachellia* (*Acacia*) woodlands surrounding Witwatersrand gold and uranium mine tailings

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A Thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfillment for the degree of Doctor of Philosophy

Declaration

I, Michelle Toni Buck (Person number: 322062), am a student registered for the degree of Doctor of Philosophy (PhD) in the academic year 2018.

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Abstract

Phytoremediation of mine tailings and surrounding areas provide the most cost-effective means of alleviating their pollutant effects. Research has shown that successful revegetation of mine tailings and surrounding areas can be optimised by providing appropriate microbial symbionts for the plants. Microorganisms are beneficial to plant growth and health which is essential for revegetation and phytoremediation of contaminated sites. The aim of this study was to assess the microbial status of *Vachellia karroo* rhizosphere currently growing on the surrounding areas of two mining operations, namely Vaal River (VR) and West Wits (WW) Operations. Metataxonomy is the study/use of genetic material isolated from field/environmental samples. It has been reported from metataxonomy studies that a large amount of microbial biodiversity had been missed by cultivation-based methods. Metataxonony allows for an unbiased genetic analysis from all members of the sampled community since it is a PCR directed sequencing approach for identification. Root and soil samples were collected in spring, roots were removed and physio-chemical analyses were preformed on the soil, including pH, conductivity, cation exchange capacity and XRF analysis. The soil samples were then prepared for DNA extraction by mixing/bulking 5 g of soil for each niche site and control. Then 500 mg of soil was used to extract DNA using the DNeasy® PowerSoil® Kit (Qiagen). Target genes, namely the 16S rRNA V1-V3 hypervariable region and the ITS2 hypervariable region, were sequenced at Molecular Research LP MR DNA (http://www.mrdnalab.com), Texas. The rhizosphere bacterial communities of *V. karroo* from VR Operations was predominantly comprised of Acidobacteria, Actinobacteria, Bacteroidetes, Verrucomicrobia, Firmicutes, Planctomycetes, Gemmatimonadetes, (between 1 % and 47 % depending on the niche sites and controls) and the remainder of the phyla (Chloroflexi, Armatimonadetes, Nitrospirae, Candidatus Saccharibacteria, candidate division WPS-1 and candidate division WPS-2) were less than 1 %. The numbers all represent the average percentage of sequences across 14 phyla. For WW Operations the bacterial communities were predominantly comprised of Actinobacteria, Acidobacteria, Planctomycetes, Verrucomicrobia, Proteobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes, (between 0.7 % and 41.4 % depending on the niche sites and controls) and the remainder of the phyla (candidate division WPS-1, candidate division WPS-2, Armatimonadetes, Candidatus Saccharibacteria, Nitrospirae and Cyanobacteria/Chloroplast) were around 1 %. The numbers all

represent the average percentage of sequences across 15 phyla. The rhizosphere fungal communities of *V. karroo* from VR Operations was predominantly comprised of Ascomycota (Sordariomycetes, Dothideomycetes, Eurotiomycetes, Ascomycota_unidentified, Leotiomycetes and Incertae_sedis_14); Zygomycota (Incertae_sedis_10); Basidiomycota, Agaricomycetes, Atractiellomycetes, Tremellomycetes (between 0.01 % and 77.1 % depending on the niche sites and controls) and to a lesser degree Glomeromycota (Glomeromycetes) and Fungi_unidentified (between 0.2-6 % depending on the niche sites and controls). The numbers all represent the average percentage of sequences across 12 classes. For WW Operations the fungal communities were predominantly comprised of Ascomycota (Sordariomycetes, Dothideomycetes, Eurotiomycetes, Ascomycota_unidentified, Leotiomycetes and Archaeorhizomycetes), Zygomycota (Incertae_sedis_10), Basidiomycota, Agaricomycetes, Tremellomycetes, Atractiellomycetes, Basidiomycota_unidentified, Fungi_unidentified and Glomeromycota (Glomeromycetes) (between 0.01 % and 91.42 % depending on the niche sites and controls). The numbers all represent the average percentage of sequences across 13 classes. The study represents a first report utilising metataxonomic tools in the analysis of the rhizobiome of *V. karroo* from areas around mine tailings. The results will assist in making decisions about future microbial surveys and applying microbial inoculum in revegetation of mine waste sites and the affected areas.

Key words: mine tailings, revegetation, *Vachellia karroo*, metataxonomy, ITS2, 16S rRNA

Dedication

I would like to dedicate this project to Alexander Spruyt (fondly known as Lexx) who thought up the initial project and who I wish was here to continue his own research.

"This day is remembered and quietly kept,
No words are needed, we shall never forget,
For those we love don't go away,
They walk beside us every day.
Unseen and unheard, but always near,
So loved, so missed, and so very dear."

– Author unknown

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Research output

Conference attendance

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Title: Identification of arbuscular mycorrhizal (AM) fungal genes associated with the tolerance, transport and/or accumulation of non-micronutrient metals found in gold and uranium mine tailings

Authors: M. T. Buck, V. M. Gray and A. J. Valentine

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Auditorium at the School of Public Health, Education Campus, 27 St Andrews Road, Parktown

Title: The rhizobiome of *Vachellia* (*Acacia*) woodlands surrounding Witwatersrand gold and uranium mine tailings

Authors: M. T. Buck, V. M. Gray, P. De Maayer, I. Weiersbye and M. E. C. Rey

Molecular Biosciences Research Thrust Research Day

30 November 2017

Wits Club, West Campus, University of the Witwatersrand

Title: The rhizobiome of *Vachellia* (*Acacia*) woodlands surrounding Witwatersrand gold and uranium mine tailings

Authors: M. T. Buck, V. M. Gray, P. De Maayer, I. Weiersbye and M. E. C. Rey

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List of abbreviations

α	Alpha
Ag	Silver
Al	Aluminium
AMD	Acid mine drainage
AM	Arbuscular mycorrhiza
AMF	Arbuscular mycorrhizal fungi
AMT	Ammonium transporter
ARC	Agricultural Research Council
As	Arsenic
ASV	Anodic stripping voltammetry
Au	Gold
β	Beta
B	Boron
Ba	Barium
bp	Base pair
Br	Bromine
C	Carbon
Ca	Calcium
$CaCO_3$	Calcium carbonate/calcite
CaF_2	Fluorite/calcium difluoride
$CaMg(CO_3)_2$	Dolomite/calcium magnesium carbonate
$CaSO_4$	Gypsum/calcium sulphate
$Ca_5(PO_4)_3Cl$	Chlorapatite
$Ca_5(PO_4)_3F$	Fluorapatite
$Ca_5(PO_4)OH$	Hydroxyapatite
$CaTiSiO_5$	Sphene
Cd	Cadmium
Ce	Cerium
CEC	Cation exchange capacity

Cl	Chlorine
CLR	Carbon Leader Reef
cm	Centimetre
Co	Cobalt
Cr	Chromium
CRG	Central Rand Group
Cs	Caesium
Cu	Copper
CV-AAS	Cold vapour atomic absorption spectrometry
°C	Degrees Celsius
DNA	Deoxyribonucleic acid
ET-AAS	Thermoelectric atomic absorption spectrometry
F	Fluorine
F-AAS	Flame atomic absorption spectrometry
Fe	Iron
FeS	Iron sulphide
$FeTi_3$	Imenite
Fe_2TiO_4	Titanognetite
g	Gram(s)
Ga	Gallium
Ge	Germanium
GF-AAS	Graphite furnace atomic absorption spectrometry
H	Hydrogen
ha	Hectare
HCl	Hydrogen chloride
$HClO_4$	Perchloric acid
Hf	Hafnium
HF	Hydrofluoric acid
Hg	Mercury
HG-AAS	Hydride generation atomic absorption spectrometry
HM	Heavy metal

HPO_4^{2-}	Hydrogen phosphate ion
$H_2PO_4^-$	Dihydrogen orthophosphate ion
hr	Hour(s)
H_2SiO_4	Dihydrogen silicate
H_2SO_4	Sulphuric acid
I	Iodine
ICN	International Code of Nomenclature for Algae, Fungi and Plants
ICP-AES	Inductively coupled plasma-atomic emission spectrometry
ICP-MS	Inductively coupled plasma-mass spectrometry
INAA	Instrumental neutron activation analysis
INVAM	International Culture Collection of Arbuscular and (Vesicular) Arbuscular Mycorrhizal Fungi
ITS	Internal transcribed spacers
k	Kilo
K	Potassium
kg	Kilogram(s)
km	Kilometre(s)
La	Lanthanum
Li	Lithium
LSU	Large subunit
m	Metre
M	Molar
Ma	Mega-annum
MDS	Metric dimensional scaling
mg	Milligram(s)
Mg	Magnesium
mℓ	Millilitre(s)
mm	Millimetre
µ	Micro
µg	Microgram(s)
µℓ	Micro-litre(s)

µm	Micrometre(s)
µS	MicroSemens
Mn	Magnesium
Mo	Molybdenum
MTFs	Mine tailings facilities
N	Nitrogen
Na	Sodium
NAA	Neutron activation analysis
Na_2CO_3	Sodium carbonate
Nb	Niobium
Nd	Neodymium
NGS	Next generation sequencers
NH_3	Ammonia
NH_4^+	Ammonium ions
$NaHCO_3$	Sodium hydrogen carbonate/sodium bicarbonate
NH_4F	Ammonium fluoride
NH4-N	Ammonium
NH_4OAc	Ammonium acetate/ammonium ethanoate
Ni	Nickel
nm	Nanometre(s)
nMDS	Non-metric multi-dimensional scaling
NO3-N	Nitrate
O	Oxygen
OH^-	Hydroxide
OTU	Operational taxonomic unit
P	Phosphorus
Pb	Lead
PCoA	Principle coordinates analysis
PCR	Polymerase chain reaction
PD	Phylogenetic diversity
PEG	Polyethylene glycol

pH	Potential hydrogen
PHB	Polyhydroxybutyrate
PIXE	Particle-induced X-ray emission
PMF	Proton motive force
ppm	Parts per million
Pr	Praseodymium
Pt	Platinum
PTEs	Potentially toxic trace elements
rDNA	Ribosomal deoxyribonucleic acid
Rb	Rubidium
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
S	Sulphur
Sb	Antimony
Se	Selenium
Si	Silicon
SiO_2	Silicon dioxide
Sn	Tin
sp.	Species
spp.	Several species
Sr	Strontium
SSU	Small subunit
t	Tonne
Ta	Tantalum
Te	Tellurium
TE	Trace element
Th	Thorium
Ti	Titanium
TiO_2	Rulite/Titanium(IV) oxide
TP	Toe-paddock

TSFs	Tailings storage facilities
Tl	Thallium
U	Uranium
UO_2	Uraninite
USA	United State of America
V	Vanadium
VCR	Ventersdorp Contact Reef
VR	Vaal River
VR1	Madala site
VR2	Black Reef Site
VR3	East Pay Dam site
VR4	R. G. Williams site
VRC	Vaal River Control
W	Tungsten
WRG	West Rand Group
WW	West Wits
WW1	Tailings Storage Facilities lower slope, over soil, retaining wall
WW2	Toe-Paddock West Wits Old North Tailings Storage Facilities North Toe Paddock – Easter Boundary
WW3	Toe-Paddock West Wits Old North Tailings Storage Facilities North Toe Paddock North-Eastern Boundary
WW4	Varkenslaagte Woodlands Blocks site
WWC	West Wits Control
XRF	X-ray fluorescence
Y	Yttrium
Yb	Ytterbium
Zn	Zinc
Zr	Zirconium

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Chapter 1

Literature review and introduction

1.1 Introduction and background to project

Heavy metal (HM) pollution is toxic not only for the environment but to people as well. Examples of toxic effects for plants include growth inhibition and protein alteration leading to the disruption of enzymatic reactions (Clemens, 2006; Göhre and Paszkowski, 2006). Mining in South Africa has led to the creation of mine tailings which comprise the excess slime (sludge) which is created once the ore and thus mineral, for instance gold (Au), has been extracted from mined rock. In several cases the tailing of South Africa mines include uranium (U), mercury (Hg), lead (Pb) and arsenic (As). When the first mine tailings were created, none of the toxicity and its effects was known, and HM pollution has become a major concern in the last decades. Mine tailings are wind-dispersed and leach HMs and other toxic trace elements into underground water supplies, thus leaving a large contaminated footprint (Straker *et al.* 2007; Weiersbye and Witkowski, 2007). The present research is critical for the successful rehabilitation of existing and newly formed mine tailings.

Mine tailing rehabilitation strategies must focus on developing sustainable and environmentally-friendly techniques, and among the sustainable management technology, phytoremediation, i.e. the use of plants and associated microorganisms is an option, opposed to the expensive and impactant 'dig and dump' options or to the 'no intervention' scenario, that ultimately leave the site out of control (Göhre and Paszkowski, 2006). Phytoremediation can detoxify soil using two main strategies, namely phytostabilization, whereby plants bind HMs in the soil, and phytoextraction, whereby the plants take-up, actively translocate and store HMs in their aerial parts (Göhre and Paszkowski, 2006).

Until fairly recently, mine tailings have been rehabilitated with HM-intolerant, exotic (to South Africa, the area or the biome), grassland plant species, which only survive over the period of irrigation and fertilisation, for example only 5 % of the 400 species introduced to two mine fields in the North-West and Free State Provinces survived after 6 years (Straker *et al*., 2007). The predominant plant types that naturally colonise mine tailings are slow-growing, woody, semi-

woody and perennial (Weiersbye *et al.*, 2006). *Vachellia* (*Acacia*) spp. were chosen for this study as they are indigenous, are able to colonise mine tailings and are useful in increasing soil fertility through their decomposition of foliage and through nitrogen fixation (Smit, 1999).This latter feature should aid in the self-maintenance of the plant community as well as support colonisation by other plant species. However, *Vachellia* spp. are slow growing plants and thus it is necessary to identify methods that optimise their growth rate. One such method is the colonisation of the *Vachellia* spp. root material with compatible and HM-resistant arbuscular mycorrhiza (AM) fungal species. AM fungi provide the host plant with an increased capacity to absorb water and nutrients, such as phosphorus, from the soil (Entry *et al.*, 2002), thus AM fungal colonisation alleviates nutrient stress of the host plant and consequently increases plant growth (Smith and Read, 2008). AM fungi can have wide host ranges (Ruiz-Lozano *et al.*, 1995) but the effects of AM fungal colonisation on the plant are very plant species and even cultivar (of the same plant species) specific. AM fungi also differ in their sensitivities and/or tolerances to various ecological parameters and main soil properties such as pH value, texture and organic matter content. It is noteworthy to mention that the percentage of colonisation, spore density and species diversity of AM in the host plant is directly proportional to the accessible root surface area and thus the length of the root (Straker *et al.*, 2007). Host roots also tend to stimulate the sporulation and growth of AM fungi (Straker *et al.*, 2008). It is imperative that data regarding the species identity of AM fungi associated with different host plant species and in different soil types is collated and communicated between researchers (especially those working in different geographical locations) (Schenck and Pérez, 1990).

It is expected that the use of effective growth promoting AM fungi combined with naturally colonising rhizobia (nitrogen-fixing bacteria) will enhance the growth rate as well as improve the host plants' resistance to low nutrient soils and HM pollution. Mine tailing land restoration or erosion control programmes can be implemented using the benefits of mycorrhizal symbioses which allow for increased soil aggregation by the extramatrical hyphal network (Tisdall, 1994) which will increase plant diversity (Dodd and Thomson, 1994). Thus identification of natural AM fungal colonisers of *Vachellia* spp. is necessary in order to select for AM fungal species tolerant to the conditions found in mine tailings so as to optimise the growth of planted *Vachellia* spp. through inoculation and thus enhance the rehabilitation of mine tailings.

The rationale behind this project is to improve rehabilitation of mine tailings through the application of biological methods and to stimulate further research activity leading to practical applications. The project objectives required the collection of samples pertaining to two differently rehabilitated gold and uranium mine regions in the Gauteng and North-West Provinces and the identification of microorganisms naturally associated with *Vachellia karroo*. The soil chemistry and characteristics were also analysed to provide background information.

1.2 Heavy metal pollution and the consequences

HMs are considered to be metals with a density/specific mass above 5 $g.cm^{-3}$ (Hooda, 2010; Nies, 1999) and include copper (Cu), zinc (Zn), cadmium (Cd), Pd, iron (Fe), nickel (Ni), silver (Ag), thorium (Th), U, Hg and As (Clemens, 2006; Hooda, 2010; Kabata-Pendias, 2011; Kapoor and Virarghavan, 1995; Khan, 2005; Nies, 1999) with the total being 53 of the periodic elements (Nies, 1999). Several metals are essential for plants and other organisms, but they become toxic when present in excess (Clemens, 2006; Göhre and Paszkowski, 2006; Hooda, 2010; Weiersbye *et al.*, 1999).

Trace metals typically occur in soils with concentrations lower than 100 $mg.kg^{-1}$ or < 0.1 % and in general many of the metals occur in even lower concentrations (Hooda, 2010; Leyval *et al.*, 1997); for instance, the Zn concentration tends to be 80 ppm or 36.6 $mg.kg^{-1}$, Cd occurs in concentrations of 0.1-0.2 ppm or 0.4 $mg.kg^{-1}$ and Pb concentration is often 15 ppm or 33.6 $mg.kg^{-1}$ (Göhre and Paszkowski, 2006; Oliveira and Pampulha, 2006). When trace elements are introduced from anthropogenic sources at concentrations which exceed the regional geochemical background composition and cause negative effects on the resident organism populations, the soil is considered to be anthropogenically polluted (Kabata-Pendias, 2011) (i.e. to exceed naturally-occurring tolerance ranges). Therefore, for example, a naturally metal enriched soil (such as serpentine derived soil or the natural reef outcrops of the Witwatersrand Basin) that hosts populations of organisms tolerant to the prevailing metal-rich conditions is not considered “anthropogenically polluted”, whereas the same soil (or any soil) in receipt of elevated contaminants from an anthropogenic source (e.g. deep rock mining or smelting), which exceeds

the tolerance range of the component organisms, is. Examples of HM concentrations when found in polluted soils include Zn > 20 000 ppm, 8.3 % or 165.5 $mg.kg^{-1}$; Cd > 14 000 ppm, 863 $\mu g.g^{-1}$ or 1.1 $mg.kg^{-1}$ and Pb > 7 000 ppm or 270.8 $mg.kg^{-1}$ (Göhre and Paszkowski, 2006; Leyval *et al.*, 1997; Oliveira and Pampulha, 2006).

The relevant areas of pollution for agriculture and thus humans involve soil and water; however, soil will be the main target in this study as there is limited new arable land with the increase in the human population (Clemens, 2006; Xavier and Boyetchko, 2002). Soils, which are a target for a large variety of various contaminants, are polluted through a range of actions, which may either be anthropogenic or natural in origin (Clemens, 2006; Kavamura and Esposito, 2010; Oliveria and Pampulha, 2006). Anthropogenic actions include mining activities, liming materials, application of sewage sludge in agriculture, chemicals used in agriculture and industrial activities (Del Val *et al.*, 1999; FließBach *et al.*, 1994; Göhre and Paszkowski, 2006; Khan, 2005; Oliveira and Pampulha, 2006; Rösner *et al.*, 2001; Tack, 2010; Valsecchi *et al.*, 1995) and natural sources include proximity to an ore body (Glick, 2003). More specifically, industrial activities include waste disposal, waste incineration and vehicle exhausts (Khan, 2005) and mining activities include mine tailings storage facilities (TSFs) (Weiersbye and Wikowski, 2007), uncontrolled release of acid mine drainage (AMD) and wind-blown tailings material, as well as a large contaminated footprint on the subsurface from reclaimed tailings material (Rösner *et al.*, 2001; Weiersbye and Witkowski, 2007). All these sources cause accumulation of HMs in soils and present a threat to food safety and possible health risks as a result of soil-to-plant transfer of the HMs (Khan, 2005).

HM loadings of soil particularly affect biological processes (Kelly and Tate, 1998). The effects on organisms from HMs in the soil depend on several factors including HM solubility and organism sensitivity. The physical and chemical characteristics of soil determine the mobility, solubility and speciation of HMs (Borůvka and Drábek, 2004; Kabata-Pendias, 2011; Kabata-Pendias and Pendias, 1992; Khan, 2005; Khan, 2006; Tack, 2010). In general HMs have a low solubility due to their strong interaction with soil particles (Clemens, 2006; Clemens *et al.*, 2002). Changes in soil main chemical properties such as acidification or variation of organic matter content, can vary the HMs solubility (Borůvka and Drábek, 2004; Del Val *et al.*, 1999;

Kabata-Pendias, 2011), and can be therefore immobilized within the different soil solid phases (Valsecchi *et al.*, 1995) and are non-biodegradable (Göhre and Paszkowski, 2006; Kelly and Tate, 1998; Leyval *et al.*, 1997) and thus linger in the soil for extended periods (FließBach *et al.*, 1994) depending on the metal (Kabata-Pendias, 2011).

Some of the negative effects on plants of high levels of HMs involve protein modification affecting enzymatic reactions, replacement of other vital elements leading to deficiency, changes in plasma membrane permeability and functionality (Göhre and Paszkowski, 2006), oxidative stress (Clemens, 2006) and any of the heavy metal ions may form unspecific complex compounds in the cell leading to toxic effects (Kavamura and Esposito, 2010; Nies, 1999). Some plant symptoms associated with excess HMs are growth retardation, chlorosis, root browning, disturbance of plant water balance, inhibition of stomatal opening, cell cycle arrest and effects on both photosystems (Clemens, 2006; Göhre and Paszkowski, 2006). HMs in the soil also decrease the functional diversity of soil organisms and microorganisms (Arriagada *et al.*, 2007; Kavamura and Esposito, 2010). As well as these detrimental effects of HMs, they have also been found to be associated with the development of cancers (Türkdoğan *et al.*, 2002).

1.3 Gold slimes dams in South Africa

1.3.1 History

Gold bearing reefs were first discovered in the Witwatersrand basin in 1886 at Langlaagta by George Harrison (Adamson, 1972; Foster, 1993; Janisch, 1986). Full scale mining started in 1887 in the Central Rand and later, gold fields were established in the Orange Free State in 1951 (gold was discovered here late 1940s), and gold mines were established in Klerksdorp in 1952 and Evander in 1958 (Adamson, 1972; Foster, 1993). Gold in the Witwatersrand basin is comprised of conglomerate deposits, as opposed to nugget or vein formation or alluvial deposits, which tend to be low grade (Adamson, 1972). The conglomerate reef bands are characterised by the presence of ovoid pebbles, mainly vein quartz, that make up approximately 70 % of the overall volume and are embedded in a mineralogically complex matrix (Janisch, 1986). The matrix that contains the pebbles consists of a variety of minerals, including pyrite, chromite, uraninite, kerogen and secondary quartz to name a few (Janisch, 1986).

Certain reefs, for instance, the Vaal Reef, Carbon leader (Main Reef Group) and Basal Reef (Orange Free State) are characterised by thin, discontinuous layers of kerogen being present at their base (Adamson, 1972; Janisch, 1986). Kerogen is a carbonaceous material comprised of hydrocarbons, organic sulphur and oxygen compounds (Foster, 1993). Kerogen always contains uraninite and is often associated with gold, which is found on its surface, as well as filling open spaces between filaments and cleats (Foster, 1993; Janisch, 1986). U, in the form of uraninite, is best preserved in the kerogen (Foster, 1993). Radioactive materials, such as uraninite, occur more frequently in the conglomerates than gold does and a large proportion of gold is associated with these materials (Adamson, 1972). Gold is often embedded in the uraninite in irregular pits and cracks and miniscule veins of gold may sometimes traverse from surrounding masses of gold into the uraninite (Adamson, 1972; Foster, 1993).

Due to the conglomerate, the gold when mined, is intimately associated with a matrix and thus requires intensive extraction procedures whereby the ore undergoes some kind of milling in order to release the contained gold particles before concentration (Adamson, 1972). Concentration techniques include gravity concentration, amalgamation, cyanidation and flotation and these allow for the comminution of the ore in order to extract the gold from the surrounding debris (gangue) (Adamson, 1972).

The original milling in the Witwatersrand used stamp milling and the stamp-mill product was screened followed by being passed over amalgamation plates, which recovered approximately 75 % of the gold from the ore (Adamson, 1972; Janisch, 1986). Later, in the 1890s, the stamp milling was improved with the addition of screening by bar grizzleys and the screened coarse fraction was subjected to hand sorting and jaw or gyratory crushing (Janisch, 1986). The extraction process was further improved, in 1890, with the introduction and implementation of the MacArthur-Forest cyanidation and precipitation process, which improved the recovery of the gold from 75 % to 90 % (Adamson, 1972; Janisch, 1986). However, surplus slime (fine material of less than 50 μm), a result of early processes, that was slow to settle in water in the leaching tanks was impeding the flow of these tanks and thus was separated and placed on tailings (Janisch, 1986). However, the slime contained 4-5 g/t of gold and in 1894 the natural-settlement

decantation process was introduced by J. R. Williams (Adamson, 1972; Janisch, 1986). In this process lime was added to assist slime settlement, which was then followed by the addition of cyanide solution to the thickened slime, which was then transferred to a second tank and circulated (Adamson, 1972; Janisch, 1986). This process dissolved most of the gold, which was then precipitated either using Zn metal or a Zn-Pb couple (Adamson, 1972).

Slime dams (also referred to as mine tailings or mine dumps) are created in order to dispose of remaining slime as it has no function in industry. These slime dams have also been used to dispose of excess cyanide solution and water from underground, and manganese sludges from uranium plants (Adamson, 1972).

1.3.2 Consequences

Slimes dams have become a prominent feature in South Africa. This is problematic as soil is a biologically active surface layer that provides a medium for the roots of plants and provides most of the nutrients that they require (Bradshaw and Chadwick, 1980) and slime dams cause the land and soil to become derelict. Originally plants could not grow on the slimes dams (new methods for the restoration of slimes dams have since been developed) and wind would send clouds of dust into the air and would cause traffic problems due to poor visibility and factories that process food to halt work on windy days (Bradshaw and Chadwick, 1980). In addition to these problems, rivers also silted up and more than one lake was destroyed (Bradshaw and Chadwick, 1980). Plants could not grow on the slimes dams as they mainly comprise of quartzite (roughly 95 % silica), have low nutrient content and contain 1.5-3.5 % pyrite (iron sulphide (FeS_2)) which is acidifies when oxidised (Bradshaw and Chadwick, 1980; Clausen, 1973). Initially the slime dams have a pH value above pH 10, due to the liming step in the gold extraction process; however, this decreases over a period of a few months (roughly 2-4) as the pyrite becomes oxidised to produce sulphuric acid (H_2SO_4) (Bradshaw and Chadwick, 1980; Hutnik and Davis, 1973). The acidity is leached into the surrounding area, down underground (Bradshaw and Chadwick, 1980; Clausen, 1973) and flows into the nearest stream (Clausen, 1973). The decrease in pH increases the solubility of heavy metal compounds and minerals, for instance, Fe, Cu and Zn (Hutnik and Davis, 1973).

1.4 Phytoremediation

Until recently mine tailings have been treated with non-tolerant, exotic, grassland plant species, which only survived over the period of irrigation and fertilisation (Straker *et al.*, 2007; Weiersbye *et al.*, 2006) as well as the less effective method of covering the surface of the mine tailings with waste rock (Weiersbye *et al.*, 2006). Irrigation and fertilisation methods require extensive amounts of resources and time and are expensive (Straker *et al.*, 2007). It has since been determined that only 5% of the 400 species introduced to these sites survived after 6 years (Straker *et al*., 2007) and thus in the long run these plantings are unsuccessful post irrigation and fertilisation. 'Grassing', as the revegetation method is referred to, was selected as a result of the speed with which the grass cover is established and not on its survivability or as an efficient control of erosion (Weiersbye *et al.*, 2006). More recent techniques attempt to encourage predominant growth of perennial, mycotrophic plant species so that stable plant communities may be created (Straker *et al.*, 2008).

Phytoremediation is an alternative form of remediation that makes use of plants to extract, stabilise, sequestrate or decontaminate terrestrial environments (Kavamura and Esposito, 2010; Leung *et al.*, 2007). It is a cost effective form of remediation, is environmentally friendly (Kotrba *et al.*, 2009) and has advantages over microbial bioremediation as plants have higher biomasses with lower nutrient requirements (Kotrba *et al.*, 2009). Plants that naturally grow on HM contaminated soils and are generally used in this method may be categorized into one of three groups, namely excluders, accumulators or indicators (Leung *et al.*, 2007). Tolerance of plants to pollution in soils depends on the bioavailability of HMs and the plants' adaptations, whether biological, physiological or chemical (Göhre and Paszkowski, 2006; Hildebrandt *et al.*, 2007; Leung *et al.*, 2007).

In the remediation of HM contaminated soils only two options are available due to the inability of HM to be degraded; namely removal of the metals (phytoextraction) or sequestration in nonbioavailable forms (phytostabilization) (Göhre and Paszkowski, 2006; Kelly and Tate, 1998). Phytoremediation can be further divided into 6 subgroups according to the method and/or nature of the contaminant; however, not all of them apply to HM remediation. These subgroups are as

follows: phytoextraction, which is absorption and removal of the contaminant by the roots and concentration of the contaminant in the harvestable plant parts; phytodegradation is the degradation or mineralization of organic contaminants by specific enzyme activity of plants and associated microbes; phytostabilisation is where the contaminants are immobilised or their mobility and bioavailability is reduced through the incorporation into lignin or soil humus, thus reducing spread of pollutants through erosion; rhizofiltration is the absorption, concentration and/or precipitation of contaminants from polluted water by plant roots; phytostimulation is the promotion of root growth to encourage the development of rhizosphere microorganisms that are able to degrade contaminants; phytovolatilisation is the volatilisation of some ions of the periodic elements of subgroups II, V and VI, such as arsenic, through absorption of these molecules by the root, conversion into less toxic forms and releasing them into the atmosphere (Glick, 2003; Kavamura and Esposito, 2010; Khan, 2005; Kotrba *et al.*, 2009; Leyval *et al.*, 1997).

The major issue with phytoremediation is the slow time by which the process is performed (Khan, 2005). This is due to the slow growth and small size of metallophytes and therefore it would be necessary to optimise the process with the current plants or select for fast growing metallophytes with extensive root systems (Khan, 2005) or the combination of both. And, although the long treatment times involved with phytoremediation is an issue, the main concern is the persistence of the elevated metal concentrations in the soil.

1.5 The genus *Vachellia*

The genus *Vachellia* comprises woody legumes and is the largest genus of the Mimosoideae sub-family and the second largest genus in the Leguminosae family (Carr, 1976; Ross, 1972; Smit, 1999). Acacias appear as trees (single-stemmed) or shrubs (multi-stemmed) that may be scandent (Ross, 1972; Smit, 1999). The branches have spines or curved prickles (Figure 1.1 row C images B1 and B2 and row D image B) and the foliage occurs as bipinnate leaves (Figure 1.1 row A) (Ross, 1972; Smit, 1999). Flowers (Figure 1.1 row C) may appear as racemes or panicles or may be axillary or fasciculate with the overall display either being spicate (Figure 1.1 row C image A) or capitates (Figure 2.1 row C images B1 and B2) (Ross, 1972; Smit, 1999). The fruit or pod,

containing smooth, hard walled, un-winged seeds, varies in appearance and is generally dehiscent (Figure 1.1 row B) (Ross, 1972; Smit, 1999). Keys for the identification vary depending on the author and publication; however, a simple method for grouping the *Vachellia* species uses the difference in shape and position of the spines (Figure 1.1 row C image B1 arrow) and/or prickles (Figure 1.1 row C image B2 arrow) to separate the genus into 5 groups, namely group 1 to group 5 (Smit, 1999).

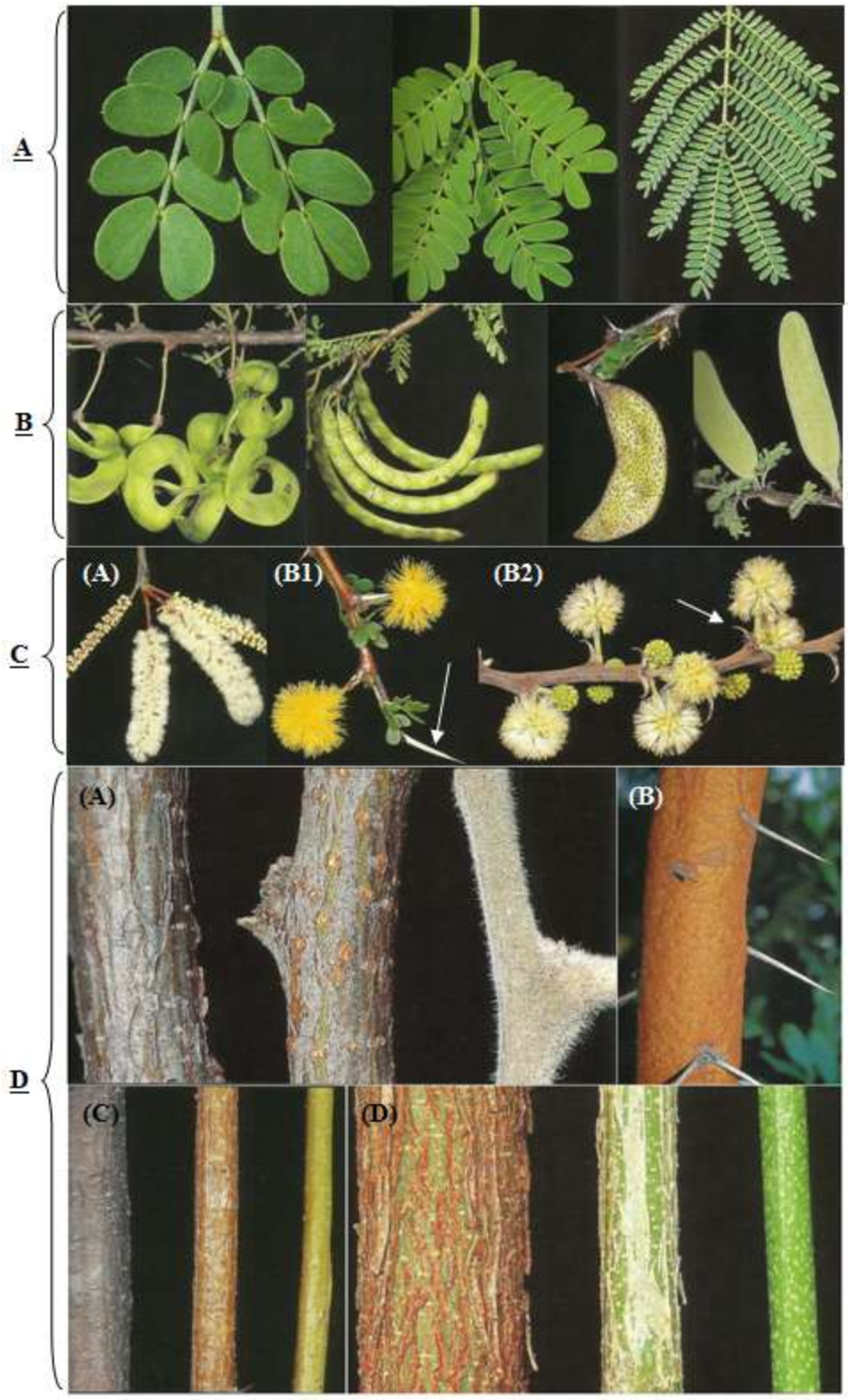

Figure 1.1: Row A shows different *Acacia* leaves; row B shows the variation in the *Acacia* pods; row C demonstrates the flower shapes as well as the difference in appearance of spikes (B1 arrow) and prickles (B2 arrow) and row D shows different appearances of trunks and branches. Illustrations taken from Smit (1999).

1.5.1 Natural habitats

The habitat of the genus depends on the species with some species able to inhabit a wide variety of habitats while others are restricted to specific ecological niches (Carr, 1976; Smit, 1999). More specifically, acacias are generally found in the tropical and sub-tropical areas of South Africa (S. A.) (Carr, 1976) with the majority being found in the savanna biome, which is the largest of the seven biomes in S. A. (Figure 1.2) covering an area of 408 876 km^2 (Smit, 1999).

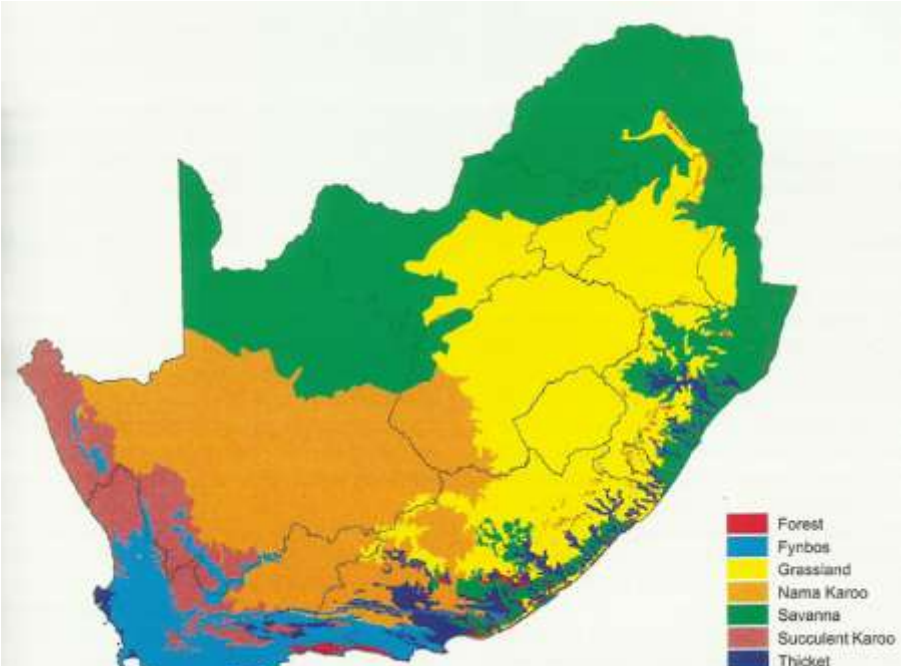

Figure 1.2: The 7 biomes of South Africa. Illustration taken from Smit (1999).

1.5.2 Uses

The tree or shrub leaves, shoots, flowers and pods act as a source of nutrients for herbivores, be it domestic or game. The flowers are also useful for insects. Since acacias are legumes they tend to enhance the soil fertility as a result of nitrogen fixation by symbiotic rhizobia and also enhance soil fertility by providing litter for decomposition. Anthropogenic uses include the use of the wood as a source of fuel in cooking and heating, as fence-posts/poles, for the production of furniture and charcoal and in years gone by as railway sleepers and mining timber; the tannins

are used in the leather tanning industry and parts of the trees may be used as food or medicine as well as there being several more uses for the trees (Smit, 1999).

1.5.3 *Vachellia karroo*

In S. A. *Vachellia karroo* (Hayne) Banfi & Galasso, formerly *Acacia karroo* Hayne, is a prevalent woody plant species that exhibits a diverse variety of morphological characteristics (Ross, 1971a,b; Taylor and Barker, 2012). *V. karroo* also has common names, such as sweet thorn (English), soetdoring (Afrikaans), umuNga (isiZulu and Xhosa), Mooka (Tswana) and Mookana (north Sesotho) (Taylor and Barker, 2012). *V. karroo* grows throughout southern Africa and its growth location ranges from Southwestern Cape up north into Namibia, Angola, Botswana, Zambia and Zimbabwe (Taylor and Barker, 2012). *V. karroo* is considered to be a pioneer species, that is the first species (usually a hardy species) to colonise a bare, previously disturbed and impaired ecosystem as the first step in ecological succession that may ultimately lead to a biodiverse steady-state ecosystem community (Duram, 2010; Lawrence, 2005). It is capable of rapidly invading grassland grazing areas which have been overgrazed and is thought to be a significant ecological indicator as an invader of South African grasslands (O'Connor, 1995).

Although the morphology or vegetative of *V. karroo* is diverse, likely due to environmental factors (Archibald and Bond, 2003; Ross, 1971a,b; Taylor and Barker, 2012), the plant can occur has a bushy shrub, commonly with multiple stems, or as a single-stemmed tree that can reach a height of 22 m. As it is frequently thin and infrequently branched; having an irregularly (often) rounded or occasionally flattened crown. The trunk of *V. karroo* can reach a diameter of 750 cm and the bark can be dark brown, brownish-black to black, reddish-brown with a rough texture, frequently fissured; or the bark can be white to pale greyish-white or greyish-brown with a smooth texture. The plants with lighter bark tend to be green when young with distinct transversely elongated lenticels. The young branchlets vary from reddish- to purplish- to blackish-brown or white to yellowish- to greyish-brown with a smooth or minutely flakey texture and may be distinctly or indistinctly lenticellate and may be sparse (glabrous) to barely pubescent. The stipules (spines) appear in pairs below the nodes and can either be straight or

slightly curved, white or matching the stem colour and are generally 0.4-4.5 to 10 cm long; they are sometimes swollen and markedly elongated to 25 cm long and these elongated spines are often joined basally. The entire plant tends to be exceedingly spinescent. The leaf's petiole length is between 5-18 mm and may be glabrous to barely pubescent with a rounded or oval adaxial gland often present, position variable and occasionally somewhat stalked. The leaflets occur in 6-15 pairs (even up to 24 pairs), range from 2.8-12.5 mm in length and 1-5 mm in width, and shapes can vary between linear, linear-oblong to obovate-oblong, base oblique, apex rounded to sub-acute or acute with all margins being entire. The flower clusters (inflorescence) are capitate located on axillary peduncles, are usually fascicle (bundle of structures) or occasionally occur singularly, and ultimately form terminal racemes. The flowers are a bright yellow colour (Ross, 1971a).

Microbial association

A tripartite association occurs between legumes, mycorrhizal fungi and rhizobial bacteria. In this regard it becomes important to review these microbes as examples of microorganisms that will occur in the rhizobiome of *V. karroo*.

1.6 Mycorrhizal fungi

Mycorrhizas are classified into seven classes, namely; arbuscular mycorrhizas, ectomycorrhizas, ectendomycorrhizas, arbutoid mycorrhizas, monotropoid mycorrhizas, ericoid mycorrhizas and orchid mycorrhizas (Smith and Read, 2008). Mycorrhizal fungi form symbiotic relationships in or on host plants roots and are thus ecologically significant (Entry *et al.*, 2002). In the symbiotic relationship the fungus is provided with soluble carbon sources from the host plant and in turn provides the host plant with an increased capacity to absorb water and nutrients, such as P, from the soil (Entry *et al.*, 2002). It has been reported in the literature that 90 to 95 % of all land plants at some point form some sort of mycorrhizal association and thus it may be suggested that the mycorrhiza itself and not the roots are the organs for nutrient uptake by plants (Bago *et al.*, 2000; Smith and Read, 2008). It is noteworthy to mention that not only does the fungus facilitate nutrient uptake of the host plant it also improves the host plant health through improved

resistance to various biotic and abiotic stresses (Lovato *et al.*, 1996; Xavier and Boyetchko, 2002).

1.6.1 Arbuscular mycorrhizal fungi (AM fungi)

AM fungi, unlike ectomycorrhizal fungi, which ensheath the host root, fail to induce obvious changes on the root surface (Norris *et al.*, 1994), but form structures within the host plant roots. The characteristic structure formed by AM fungi is the arbuscule, hence the name AM. These structures form within the cortical cells of the host plant roots. Another structure, which may be used as a diagnostic for AM symbioses along with arbuscules, is the storage vesicle, which may form within or between the cells. Other intraradical structures may be produced by the AM including intracellular hyphal coils, which on occasion may form in the absence of arbuscules. The formation of the various structures and overall development of the AM depends on and is determined by both the host plant and the fungus (Smith and Read, 2008). Figures 1.3 and 1.4 demonstrate the various structures that may be formed within the tissue of the host plant post colonisation.

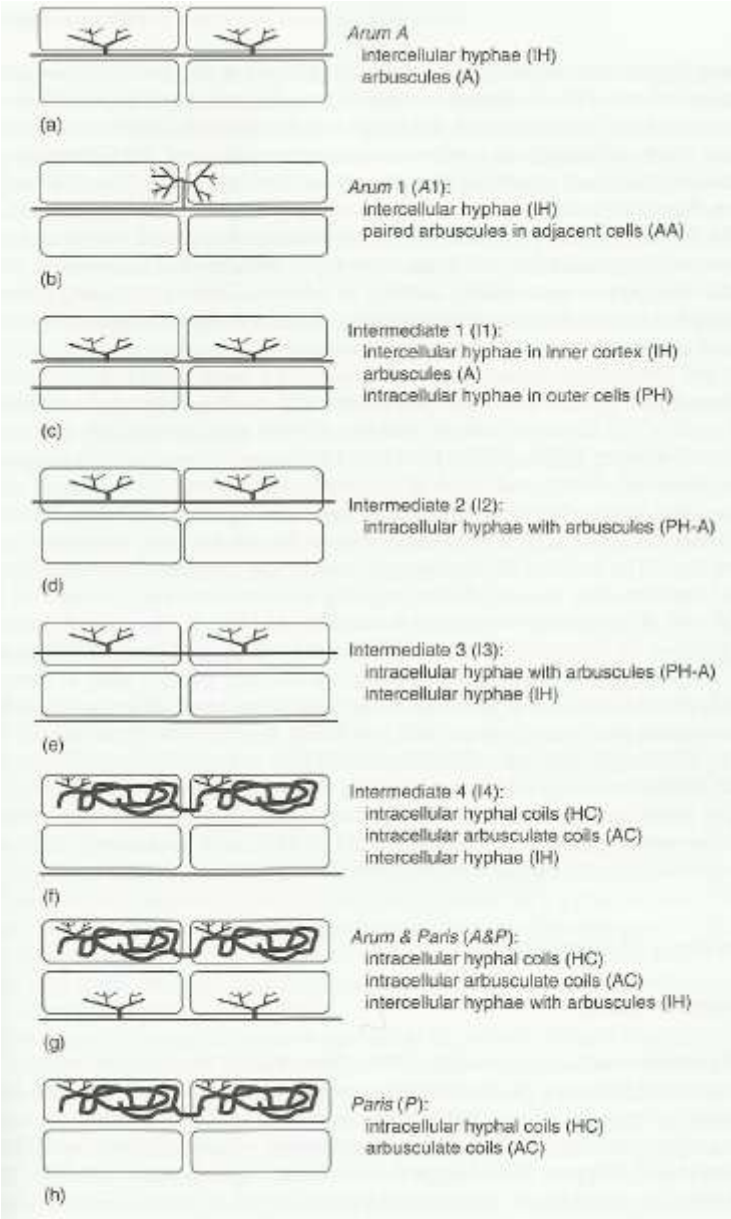

Figure 1.3: Schematic diagrams of various structures and colonisation types that may appear in the roots of different host plant species, colonised by different arbuscular mycorrhizal fungi. Illustration taken from Smith and Read (2008).

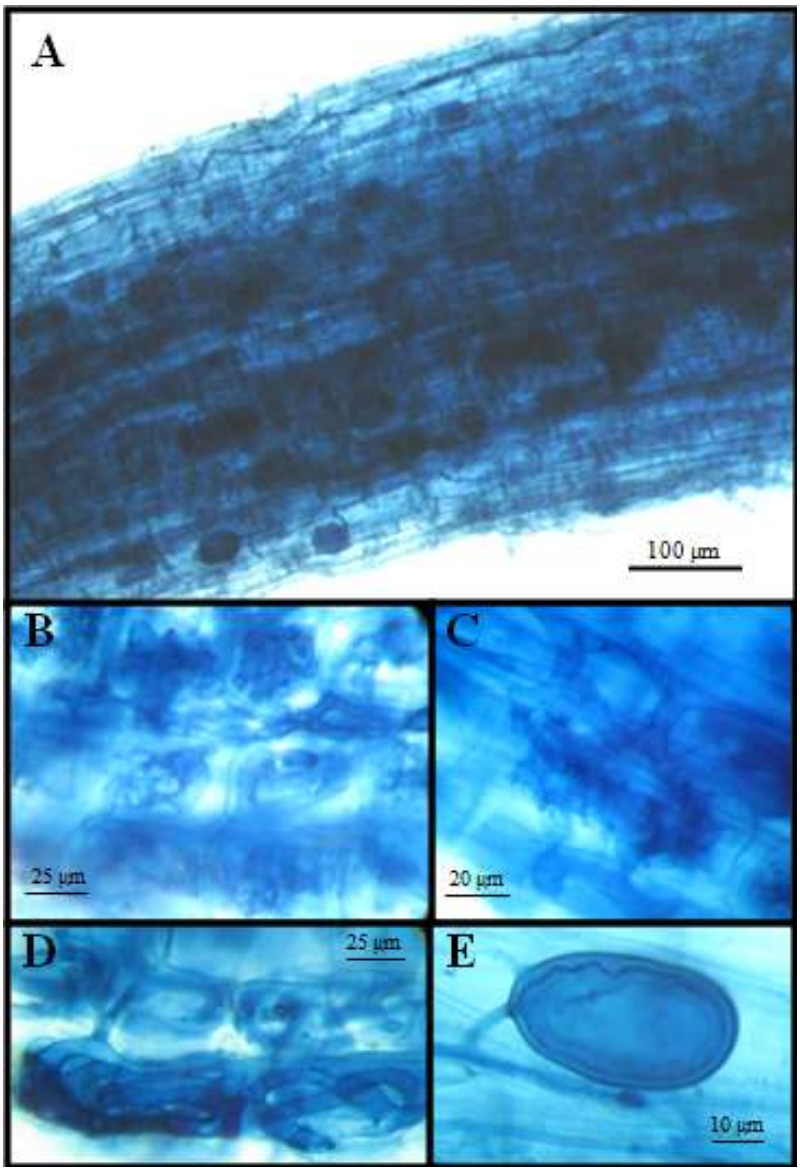

Figure 1.4: Light micrographs of intraradical fungal structures (Buck, 2011). A) Overall view of a root colonised by AM fungi. B) Root cells colonised by arbuscules. C) Young arbuscule. D) Hyphal coils. E) Vesicle attached to a hypha.

AM fungi may either be considered *Arum*-type or *Paris*-type mycorrhizas depending on the intraradical structures that they develop. *Arum*-type mycorrhizas demonstrate rapid development of intercellular hyphae that fan out from the point of penetration and extend longitudinally along intercellular spaces in the root cortex. *Arum*-type mycorrhizas form highly branched arbuscules by penetrating cortical cells with short side-branches, developing from the hyphae in the intercellular spaces, which then branch dichotomously. These arbuscules have a short life-span and the oldest arbuscules are located around the point of cortical penetration and the young and

immature arbuscules are increasingly further away. This type may also produce hyphal coils when penetrating the exodermal cell layers of the root, however, hyphal coils are not a major component of cortical colonisation (Smith and Read, 2000; Smith and Read, 2008).

Paris-type mycorrhizas are characterised by extensive growth of intracellular coiled hyphae within the cortex which develop directly from cell to cell. Arbuscules may grow from these coils and there is barely any intercellular growth (Smith and Read, 2000; Smith and Read, 2008). *Paris*-type mycorrhizal infection units grow slower than *Arum*-type infection units (Smith and Read, 2000) but are denser and compact (Smith and Read, 2008). Vesicles may be formed by both types as the infection units age, however, members of the Gigasporaceae develop auxiliary cells on the extraradical mycelium instead of vesicles (Smith and Read, 2008).

Spores produced by the fungus are extraradical (except in the case of *Rhizophagus intraradices* which produce their spores intraradically; INVAM) and are very large being up to 500 µm in diameter and are complex consisting of abundant storage lipids, some carbohydrate, and are multinucleate containing large numbers of nuclei, with estimations ranging from 800 to 35 000 in a spore depending on the species (Hosny *et al.*, 1998; Smith and Read, 2008) (Figure 1.5). Spore walls are thick and resistant, containing chitin and in some cases ß-1,3 glucan (Gianinazzi-Pearson *et al.*, 1994; Lemoine *et al.*, 1995). The physical appearance of spores tends to vary among species of arbuscular mycorrhiza. Figure 1.6 shows the variation between spores of differing species. The degree of sporulation varies between AM fungal species (Pearson and Schweiger, 1993) as well as being influenced by environmental conditions, soil nutrient levels, host and host colonisation level (Brundrett, 1991; Douds, 1994; Menge *et al.*, 1978; Stutz and Morton, 1996).

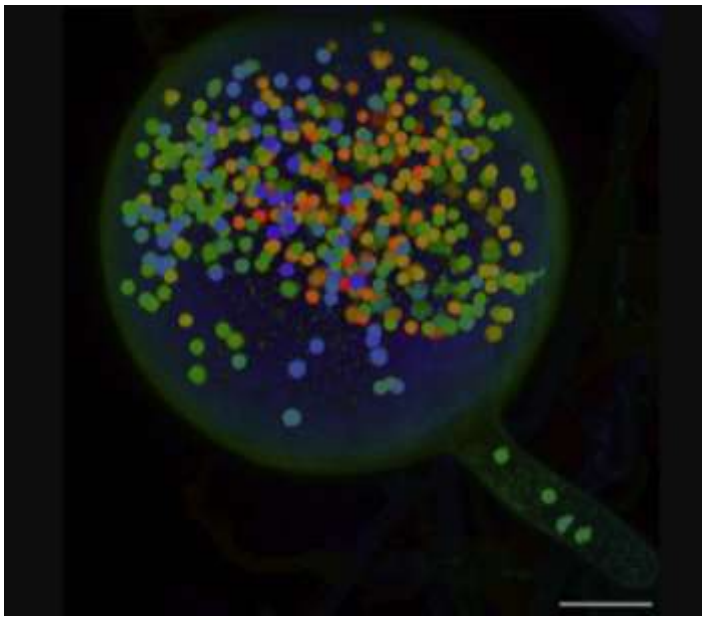

Figure 1.5: Confocal micrograph demonstrating a typical multinucleated, asexual spore of *Glomus diaphanum*. Nuclei were stained with Sytogreen fluorescent dye. Bar: 10 µm. Illustration taken from Denison and Kiers (2011).

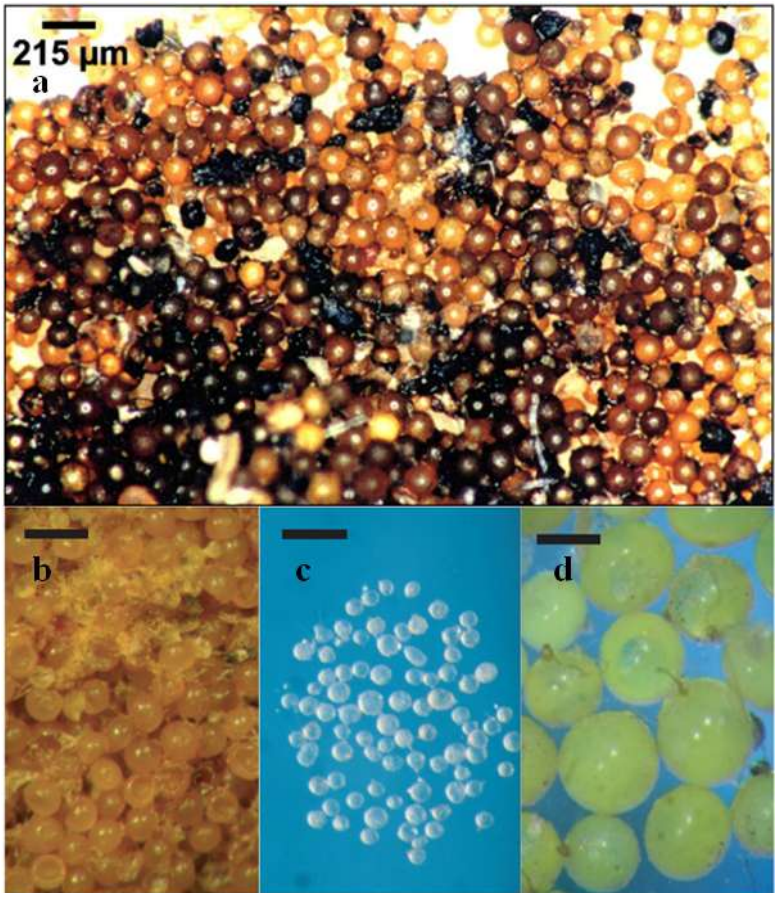

Figure 1.6: Differences in spore size, shape and overall appearance of different arbuscular mycorrhizal species. Illustrations taken from Straker *et al.* (2010) (a) and Young (2012) (b-d). (a) A collection of *Glomus etunicatum* spores of varying ages. (b) Spores of *Diversispora*. (c) Spores of *Paraglomus*. (d) Spores of *Gigaspora*. Bars for images b-d, 200 µm.

Spores are the best described source of inoculum of AM fungi and have been used in the identification and classification of AM fungi. Spore and sporocarps are able to survive passing through the gut of various mammals, birds and invertebrates and can be dispersed locally with the aid of animal movements. Spores are resistant to damage and are able to survive extended periods of time while remaining dormant and have the capacity to be dispersed by water and wind (Smith and Read, 2000; Smith and Read, 2008). Spores may also be distributed through the burrowing habits of large and small animals (Smith and Read, 2000).

1.6.1.1 Morphological classification of AM fungi

It is crucial to note that taxonomy is a comparative science (Redecker *et al.*, 2013). Original classifications were usually based on microscopic anatomically observable attributes, phenotypically distinct cell wall constituents of AM fungal spores and hyphal attachments (Schenck and Pérez, 1990) as well as the development of the spores and wall constituents (Morton and Benny, 1990; Morton and Bentivenga, 1994; Walker, 1983, 1992). This was done on broken spores mounted on microscopic slides and viewed under a light microscope.

This classification system was demonstrated with the use of murographs (Figures 1.7 and 1.8), which summarise, simplify and standardise the classification descriptions (Pfleger and Linderman, 1994; Schenck and Pérez, 1990; Smith and Read, 2000). The spore wall characteristics noted during the classification process include wall layer number, orientation, pigmentation, thickness, ornamentations and histochemical reactions (Pfleger and Linderman, 1994; Schenck and Pérez, 1990). Other morphological characters used during the traditional classification of AM fungi include spore shape (Figure 1.9), size and colour, hyphal attachment, shape and type of occlusions, sporocarp occurrence, shape, size and colour and peridium occurrence and characteristics (Giovannetti and Gianinazzi-Pearson, 1994; Schenck and Pérez, 1990).

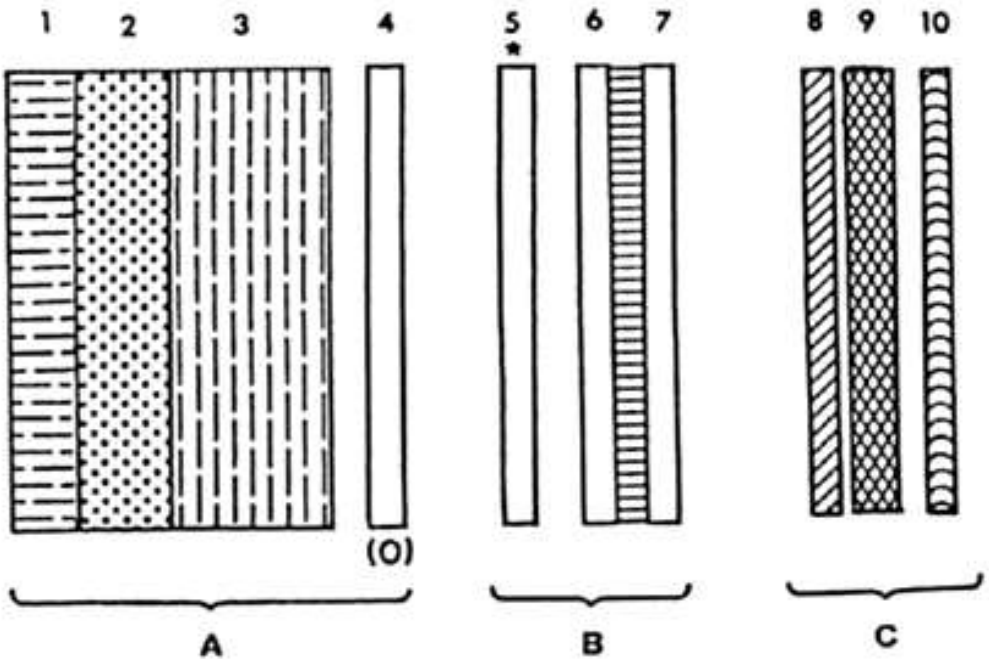

Figure 1.7: Murograph of wall type, number, group and position in an AM fungus spore. O: ornamented surface and *: difficult to see. The lines that connect two walls demonstrate a cementing layer. Walls are numbered consecutively from the outer to inner layer. 1: expanding, 2: evanescent, 3: laminated, 4-7: unit, 8: membranous, 9: coriaceous and 10: amorphous walls. Each wall group is bracketed in parentheses with consecutive letters from inner to outer wall. Illustration taken from Morton (1988).

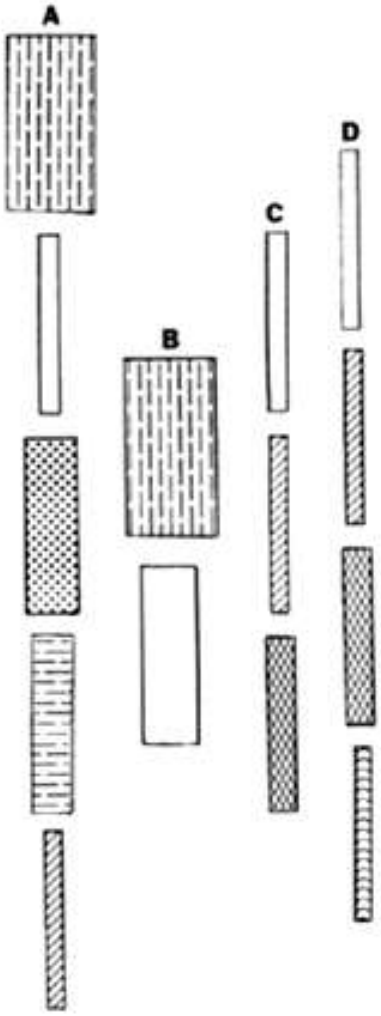

Figure 1.8: Demonstration of the relative positions of different wall types in AM fungal spores. The columns represent different wall groups with A: outer walls, B: structural walls, C: inner walls and D: innermost walls. Any combination of walls in a column can form a wall group. Illustration taken from Morton (1988).

Figure 1.9: The range of shape of spores in taxa of all genera of AM fungi. A: globose, B: sub-globose, C: ovoid, D: obovoid, E: ellipsoid, F: pyriform, G: irregular, H: oblong, I: reniform, J: fusiform and K: clavate. Illustration taken from Morton (1988).

This classification process is problematic due to limited morphological differentiation whereby some AM fungal species form more than one spore morph (Krüger *et al.*, 2012), field samples generally consist of spores in bad condition, either through association with microorganisms that erode spore walls or cause pitting or perforations in the walls (Bhattachrjee *et al.*, 1982; Walker *et al.*, 1984), or mechanical damage either at the sampling site or through the extraction process (Schenck and Pérez, 1990). Spore walls may also be difficult to distinguish individually as they may be colourless, wrinkled, overlapped or folded and may also remain attached to each other after being manipulated (Pfleger and Linderman, 1994), which overall may lead to misinterpretations during the classifications.

Detailed identification and classification involves long-term and intensive study of many isolates and developmental stages of an organism (Redecker *et al.*, 2013). Developmental stages or biological characters consist of spore germination, AM fungal infectivity, symbiotic efficiency, infection patterns, spore formation (ontogeny of spores), edaphic requirements and environmental tolerance (Giovannetti and Gianinazzi-Pearson, 1994). Spore germination is useful for the discrimination between *Gigaspora* and *Scutellospora*, in the family *Gigasporaceae* (Giovannetti and Gianinazzi-Pearson, 1994; Redecker *et al.*, 2013). Spore germination between isolates may vary depending on host root stimuli, pH, moisture, temperature, nutrient content, dormancy, rate and extensiveness of hyphal production (Gianinazzi-Pearson *et al.*, 1989; Giovannetti *et al.*, 1991; Hepper and Smith, 1976; Siqueira *et al.*, 1985; Tommerup, 1983). Spore and sporocarp ontogeny is used to differentiate between *Acaulospora* and *Entrophospora* (Ames and Schneider, 1979), although *Entrophospora* is not definitively categorised into a particular family as it is often referred to as a sister genus to *Acaulospora*, as it was originally placed in *Acaulosporaceae* and is still so on the INVAM website and there is insufficient evidence to be placed in an alternative family to *Entrophosporaceae* (Schüßler and Walker, 2010).

For the information from the taxonomic classification of AM fungi to be beneficial to the scientific community the information needs to be descriptive, readily available and updated regularly. Since 1990, the information has been available online on the INVAM (International Culture Collection of Arbuscular and (Vesicular) Arbuscular Mycorrhizal Fungi) website, which

was created by Professor J. B. Morton of West Virginia University, USA (INVAM). This germplasm resource provides researchers with sets of voucher specimens, namely photographs, slides, vials of intact spores (Pfleger and Linderman, 1994) and this is particularly important for researchers that do not have the facilities or the time to carry out these analyses. As well as vouchers, INVAM maintains a large number of isolates, including those not described (INVAM).

In order to classify and name AM fungi in a manner that can be replicated one must use standardised recommendations and rules. These recommendations and rules are specified in the "International Code of Nomenclature for Algae, Fungi and Plants (ICN)" (McNeill *et al.*, 2012; Miller *et al.*, 2011) and are generally referred to as "The Code" (Redecker *et al.*, 2013). The Code regulates nomenclature (the application and administration of names) and provides a nomenclatural framework for taxonomy (the science of defining, classifying and arranging the names in a hierarchical system) (Redecker *et al.*, 2013). Unfortunately, names applied using the rules, which may be published, have no similarity to an ordinary phylogeny-based classification, which in turn can cause problems particularly when classification is meant to reflect evolutionary relationships (Redecker *et al.*, 2013). It is noteworthy to mention that the Code relies on concrete evidence especially if new taxa are created.

1.6.1.2 Bio-molecular identification and classification of AM fungi

Recently the classification of AM fungi has undergone a major revision and many of the fungi that were originally classified as AM fungi have been reclassified and placed in different genera (Redecker *et al.*, 2013). This revision has been based on the fact that the original classification of these fungi was incomplete and has since been improved with the development of new DNA-based techniques and the reassessment of previous identifications and cultures.

Newer classification techniques make use of molecular methods, including DNA analysis, isozyme polymorphism and protein profiles (Giovannetti and Gianinazzi-Pearson, 1994). These analyses are useful since morphologically similar isolates may be genetically and physiologically different (Krishna, 2005). DNA analyses focus mainly on ribosomal genes (Krishna, 2005). The

advantage of using DNA in analyses is that there are no problems associated with gene expression because the genotypes are analysed directly (Redecker *et al.*, 2003).

The molecular method most commonly practised at present for the systematics and identification of AM fungi is DNA/PCR analysis of the ribosomal genes (Krishna, 2005; Redecker, 2000; Redecker *et al.*, 2003). This genome region has genes that are available in a high copy number and have variable as well as highly-conserved sectors, which allows for the characterisation of taxa at different levels (Redecker *et al.*, 2003). It is noteworthy to mention that the ribosomal DNA (rDNA) in single AM fungal spores is highly polymorphic (due to the spores being multinucleate), which is in contrast to other fungi in that variable rDNA sequences, for instance internal transcribed spacers (ITS), are usually identical within a species (Redecker *et al.*, 2003). ITS regions (two) are variable non-coding regions nested within the rDNA repeat between the SSU, 5.8S and LSU rRNA genes (Gardes and Bruns, 1993). The ITS regions have very high levels of polymorphism (Clapp *et al.*, 1995; Redecker *et al.*, 1997) and rarely is the same ITS sequence recovered twice from a single spore (Lanfranco *et al.*, 1999).

Originally Simon *et al.* (1992) designed the PCR primer VANS1, which was used for the analysis of three glomalean 18S (small) subunit (SSU) sequences, which were available at the time. However, later studies revealed that the VANS1 annealing site is not well conserved within the Glomerales (Clapp *et al.*, 1999; Redecker *et al.*, 2000; Schüßler *et al.*, 2001) as well as several of the newer classified ancestral lineages of the Glomerales do not have the annealing site (Redecker *et al.*, 2000). Other problems with creating primers for AM fungi include primers only being specific for single isolates and the primers may not exclude non-AM fungi (Redecker, 2000). The exclusion of other organisms is vital in the phylogenetic analysis of AM fungi since the fungal tissue tends to be embedded within host roots and it is likely that the host, pathogenic or saprophytic fungi will be co-detected (Redecker *et al.*, 2003). Another SSU analysis study by Helgason *et al.* (1998) involved the construction of the primer AM1, which amplified most but not all glomalean fungi. Other studies have analysed the 28S (large) ribosomal subunit (LSU), for instance van Tuinen *et al.* (1998) were able to design several specific primers which targeted the highly variable D2 region of the LSU, each for a different AM fungal species. In a study by Redecker *et al.* (1997) it was demonstrated that a minute amount of fungal biomass could be

used to perform PCR/restriction analysis of ITS regions of rDNA, which allowed for species of the Glomerales to be distinguished and that the fragment patterns created were highly reproducible. Unfortunately, the variation of rDNA sequences within single spores limits the resolution of this method when closely related species are compared (Redecker, 2000). It is important to mention that as the understanding of the phylogeny of a group of organisms improves then more reliable primers can be designed (Lee *et al.*, 2008).

Currently the preferred and more advantageous PCR method used for AM phylogenetic analyses and AM fungal identification is nested PCR. It is advantageous as it has high specificity and sensitivity, which allows for the simultaneous detection of different fungi within the same root fragment and can technically allow for the detection of an unlimited number of different fungi if the sequence targeted in the nested reaction is sufficiently discriminating between AM fungi and it can also be performed on crude DNA isolates and stained root fragments. The initial PCR reaction is carried out directly on the biological material using general/non-specific primers which allows for a wide range of analyses using specific primers to be performed on a single sample, thus a small sample of colonised root can be analysed for the identification of a variety of AM fungi. The product of the initial PCR may be diluted in order to reduce the problems associated with the presence of plant components that may reduce amplification efficiency (van Tuinen *et al.*, 1998).

At present the primer pair that is used and known to amplify most AM fungal species as well as exclude non-AM fungi is AML1 and AML2 (Lee *et al.*, 2008), which are used for the nested PCR after the initial PCR with the eukaryotic primer pair NS1 and NS4 (White *et al.*, 1990). These primers amplify the SSU rRNA gene which has a low polymorphism (Simon *et al.*, 1993) and allows for the differentiation between fungal genera and species. The disadvantage of these primers is the length of the product, which is insufficient for reliable species resolution of all AM fungi, particularly within certain families (Krüger *et al.*, 2009). In the study by Krüger *et al.* (2009) AM fungal primers were designed to include the ITS regions and part of the LSU region so as to provide species level resolution and reliable phylogenetic analyses from the PCR products. The primer sets that were designed consisted of a mixture of primers for both the first PCR reaction primers, SSUmAf and LSUmAr, and the nested PCR reaction primers, SSUmCf

and LSUmBr, to allow for the amplification of all AM fungal taxa. The final product length of these primers is almost twice the length of the product produced by the primer pair AML1 and AML2 (Krüger *et al.*, 2009; Lee *et al.*, 2008).

The current nomenclature of AM fungi is as follows (Redecker *et al.*, 2013; Schüßler *et al.*, 2001; Schüßler and Walker, 2010) (Figure 1.10):

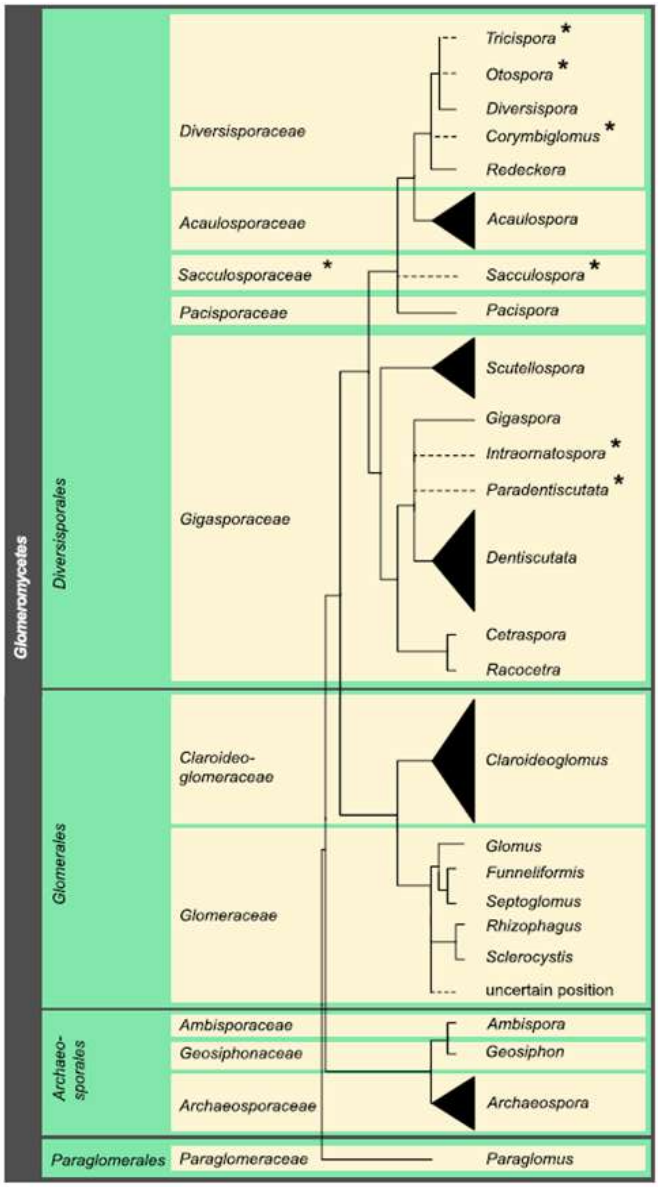

Figure 1.10: Demonstration of the consensus classification of the Glomeromycota. Dashed lines: genera of uncertain taxonomic position and *: insufficient evidence. Note: Neither Enterosporaceae nor *Enterospora* appears on this image. Illustration taken from Redecker *et al.* (2013).

Glomerales: The fungi are generally hypogeous, occasionally epigeous, with the symbioses being endomycorrhizal accompanied by vesicles and/or arbuscules and asexual spores (Morton and Benny, 1990; Schüßler *et al.*, 2001). Hyphae are generally coenocytic, however, septa may form in older hyphae as the cytoplasm is withdrawn or when the fungus cuts off resting spores (Morton and Benny, 1990; Schüßler *et al.*, 2001). Fungi produce asexual chlamydospores (glomoid spores; Morton and Redecker, 2001) (Morton and Benny, 1990; Schüßler, 2002). This

order differs from other AM fungi by having the SSU rRNA gene sequence signature motif YTRRY}2-5} RYYARGTYGNRCARCTTCTTAGAGGGACTATCGGTGTYTAACCGRTGG which corresponds to the homologous position 1353 of the *Saccharomyces cerevisiae* SSU rRNA sequence J01353 (Schüßler *et al.*, 2001).

Glomeraceae: (Phylogenetic *Glomus* group A; Schüßler and Walker, 2010). The fungi are mostly hypogeous, occasionally epigeous (Rani *et al.*, 2006). Fungal mycelium is generally coenocytic, rarely septate (Rani *et al.*, 2006). Mycorrhizal symbiosis develops vesicles that are generally intraradical but have the potential to become thick-walled spores in some species (Morton and Benny, 1990). This family differs from other families in this order by having the specific, but degenerate, SSU rRNA gene sequence motif TGTYADGGCAYYRCACYGG (INVAM; Schüßler and Walker, 2010)

Claroideoglomeraceae: (Phylogenetic *Glomus* group B; Schüßler and Walker, 2010) The fungus forms a mycorrhizal symbiosis with vesicle formation being varied depending on the environment and arbuscule formation (INVAM; Schüßler and Walker, 2010). Glomoid spores form in soil or, very occasionally, individually in decaying roots (Schüßler and Walker, 2010). This family differs from other families in this order by having specific SSU rRNA gene sequence motifs CAGYYGGGRAACCRACTAAA, ATTKRCACATCGGTCGTGC, CYTAAGGGGYATGAACYRGTGTAGTSA and TAAAAYRGGACGGCATGATTCTATT (Schüßler and Walker, 2010). In this family the species were separated from previous inclusion in the genus *Glomus* because they grouped in a distinct clade based on SSU rRNA sequences (INVAM). Unfortunately rDNA-based species-level resolution is not as reliable as in other glomoid clades, which may be due to the continued ancestral polymorphisms in gene repeats (VanKuren *et al.*, 2012).

Diversisporales: The fungi are hypogeous, with the symbioses being endomycorrhizal accompanied by arbscules, rarely producing vesicles, and may produce hypogeous auxiliary cells (Schüßler *et al.*, 2001; Walker and Schüßler, 2004). Fungus may produce complex spores within a sporiferous saccule (acaulosporoid spores; Morton and Redecker, 2001) or from a bulbous base on soporiferous hyphae or the fungus may produce glomoid spores (Schüßler *et al.*, 2001; Walker and Schüßler, 2004). This order differs from other AM fungi by having the specific SSU rRNA gene sequence signature motifs GGGTTTH and TYACCGGRAGGTRT which

correspond to the homologous positions 234 and 1495, respectively, of the *S. cerevisiae* SSU rRNA sequence J01353 (Walker and Schüßler, 2004).

Gigasporaceae: The fungus forms a mycorrhizal symbiosis with arbuscules (Morton and Benny, 1990). The fungi produce large, globose to subglobose, asexual spores, generally greater than 200-300 μm (Bentivenga and Morton, 1996; INVAM; Morton and Benny, 1990; Rani *et al.*, 2006), which are borne singly in the soil (Morton and Benny, 1990).

Acaulosporaceae: The fungi form an endomycorrhizal symbiosis with the production of intraradical arbuscules and ellipsoid, irregular or lobed vesicles (Morton and Benny, 1990). Glomoid spores are produced (INVAM; Morton and Benny, 1990; Rani *et al.*, 2006).

Pacisporaceae: The only genus in this family is *Pacispora* and thus the character description will be based on genus characters. The fungus forms a mycorrhizal symbiosis accompanied by vesicles and arbuscules (INVAM). The fungus produces globose, subglobose to ellipsoid, rarely irregular spores in the soil singly on the terminal ends of hyphae and the production of sporocarps is unknown at this point (Oehl and Sieverding, 2004).

Diversisporaceae: The fungus produces glomoid spores (INVAM, Walker and Schüßler, 2004). This family was constructed only due to the divergence of the SSU gene sequence divergence from other clades (INVAM). This family differs from other families in this order by having specific SSU rRNA gene sequence signature motifs GGCTCATTYGRRTYTS, ACYCATTRYCAGGCTTAAT and TTGGCATTTAGYCA which correspond to the homologous positions 487, 648 and 1389, respecitively of the *S. cerevisiae* SSU rRNA sequence J01353 (Walker and Schüßler, 2004) and the SSU rRNA gene sequence signature motifs YVRRYW}1-5}NGYYYGB and GTYARDYHMHYY}2-4}GRADRKKYGWCRAC which correspond to the homologous positions 658 and 1346, respectively of the *S. cerevisiae* SSU rRNA sequence J01353 (Schüßler *et al.*, 2001).

Sacculosporaceae: The fungus produces spores within the hyphal neck of closely adherent terminal or intercalary soporiferous saccules and the production of sporocarps is unknown at present (Oehl *et al.*, 2011). Phylogenetic position is uncertain at present pending more sampling and sequence analyses (Redecker *et al.*, 2013).

Entrophosporaceae: The fungus has an endomycorrhizal symbiosis accompanied by vesicles and arbuscules (Oehl *et al.*, 2011). Spores produced by the fungus either follow the glomoid development or entrosporoid development (Oehl *et al.*, 2011) (which is distinguished from

acaulosporoid development since the spores develop within and not laterally on the saccule subtending hypha; Kaonongbua *et al.*, 2010). Family is of uncertain phylogenetic position due to the lack of reliable evidence to substantiate its position as being its natural phylogenetic position and may in the future be combined with other groups (Schüßler and Walker, 2010).

Paraglomerales: The fungi are hypogeous, with the symbioses being endomycorrhizal forming arbuscules and intraradical mycelium and rarely producing vesicles (Schüßler *et al.*, 2001). Spores are glomoid and lack pigmentation (Schüßler *et al.*, 2001). This order differs from other AM fungi by having the SSU rRNA gene sequence signature motif GCGAAGCGTCATGGCCTTAACCGGCCGT which corresponds to the homologous position 703 of *S. cerevisiae* SSU rRNA sequence J01353 (Schüßler *et al.*, 2001). However, it is noteworthy to mention that this premise is based on only two species and may thus be subject to modification as more species are identified (Schüßler *et al.*, 2001).

Paraglomeraceae: This family is based entirely on molecular data and evidence, however, the only genus in this family is *Paraglomus* and thus the character description is based on genus characters. Molecular character studies of this family, including monoclonal antibody specificities and fatty acid profiles has not produced enough data and evidence to position the family in relation to other glomalean taxa (Rani *et al.*, 2006). The fungus forms a mycorrhizal symbiosis with arbuscules but development of vesicles is not known at present (Morton and Redecker, 2001). The fungus produces glomoid spores (INVAM; Rani *et al.*, 2006). The sequences from the SSU rRNA gene indicate that all the members of this family are an ancient group within Glomales and are distant from other glomalean families (Redecker *et al.*, 2000). This family's, and members of Archaeosporaceae, SSU rRNA is uniquely amplified by the primer ARCH 1311, sequence TGCTAAATAGCCAGGCTGY (Morton and Redecker, 2001; Redecker, 2000).

Archaeosporales: The fungi are hypogeous, with the symbioses being mycorrhizal, accompanied by arbscules and may or may not produce vesicles, or endocytosymbiotic, with the fungus associating with photoautotrophic prokaryotes (Schüßler *et al.*, 2001). These fungi produce glomoid spores in loose clusters or singly and acaulosporoid complex spores singly (Schüßler *et al.*, 2001). This order differs from other AM fungi by having the specific SSU

rRNA gene sequence signature motif YCTATCYKYCTGGTGAKRCG which corresponds to the homologous position 691 of the *SD. Cerevisiae* SSU rRNA sequence J01353 (Schüßler *et al.*, 2001).

Geosiphonaceae: The only genus in this family is *Geosiphon* and the genus has only one species, *Geosiphon pyriformis* (INVAM; Rani *et al.*, 2006; Schüßler, 2002; Schüßler *et al.*, 1994) thus the character description will be based on genus and species characters. The fungus is hypogeous, however, does not form mycorrhizal structures within host roots but associates with endosymbiotic cyanobacteria in the genus *Nostoc* (INVAM; Rani *et al.*, 2006; Schüßler, 2002; Schüßler *et al.*, 1994; Smith and Read, 2008). Molecular analyses suggest that Geosiphonaceae is more closely related to Archaeosporaceae and Paraglomaceae according to its position on phylogenetic trees (Schüßler *et al.*, 2001). The mycelium is coenocytic and unicellular multinucleated 'bladders', of about 1-2 mm in size, develop at hyphal tips, which harbour the cyanobacteria (Rani *et al.*, 2006; Schüßler, 2002; Schüßler *et al.*, 1994). The fungus produces asexual, glomoid spores (resemble AM fungal spores) individually or in loose clusters in the soil (INVAM; Schüßler, 2002; Schüßler *et al.*, 1994). The fungus has only been found occasionally (roughly five times) in the Spessart mountains in Germany (INVAM; Rani *et al.*, 2006; Schüßler, 2002; Schüßler *et al.*, 1994). Although this family is not known to form a mycorrhizal symbiosis, the rDNA evidence places it in a clade within the order Archaeosporales (Schüßler *et al.*, 2001).

Ambisporaceae: The only genus in this family is *Ambispora* and thus the character description will be based on genus characters. The fungus develops an endomycorrhizal symbiosis accompanied by arbuscules but vesicles have not yet been observed in the symbiosis (INVAM; Walker *et al.*, 2007a; Walker *et al.*, 2007b). Dimorphic spores produced by the fungus either follow the glomoid development or acaulosporoid development but both may be present and develop concurrently (INVAM; Walker *et al.*, 2007a; Walker *et al.*, 2007b). This family/genus is separated from other families/genera in the Archaeosporales by its SSU rRNA gene characteristics, for example, the gene sequence motif CAAAACCAATCTCGTCTTCGGGC (Walker *et al.*, 2007a).

Archaeosporaceae: The only genus in this family is *Archaeospora* and thus the character description will be based on genus characters. The fungus forms an endomycorrhizal symbiosis with intraradical hyphae and arbuscules and the structures are similar to the structures of Archaeosporaceae and Paraglomaceae (Walker *et al.*, 2007a). Vesicle formation has not been

observed in cultures yet (Morton and Redecker, 2001). This family of AM fungi produce small, hyaline monomorphic (acaulosporoid) or dimorphic (acaulosporoid and glomoid) spores (Morton and Recker, 2001; Walker *et al.*, 2007a). Spores develop in the soil, very occasionally in roots, and appear globose to sub-globose to irregular (Walker *et al.*, 2007a). This family's, and certain members of Paraglomaceae, SSU rRNA is uniquely amplified by the primer sequence TGCTAAATAGCCAGGCTGY (Redecker, 2000).

1.6.1.3 Nutrition and influence on plants

AM fungi form part of the rhizosphiome associated with plant roots, that play vital role for their metabolism (Kavamura and Esposito, 2010). These organisms found to exist in mutualistic cooperation with plant roots are known as rhizosphere microorganisms (Kavamura and Esposito, 2010). The rhizosphere composition is highly influenced by the type of plant, quantity and composition of root exudates and different root zones (Marschner *et al.*, 2004; Yang and Crowley, 2000). The rhizosphere determines a specific plant interaction and interactions with other rhizosphere microorganisms and the environment (Kavamura and Esposito, 2010). An example of this is AM fungi being able to modify soil microbial communities in the rhizosphere either directly or indirectly through changes in root exudate patterns (Barea *et al.*, 2005) as well as enhancing the soil enzyme activities (Wang *et al.*, 2006).

AM fungi have nutrient (N, P, micronutrients such as Mo, K) scavenging activities and acquire water from the soil (Ames *et al.*, 1983; Chen *et al.*, 2005; Fitter *et al.*, 2011; Govindarajulu *et al.*, 2005; Harley, 1989; Li *et al.*, 1991; Smith and Read, 2008), whereas the majority of C acquisition (as carbohydrates) is attributable to the host plant (Fitter *et al.*, 2011; Smith and Read, 2008). It is noteworthy to mention that C acquisition from the soil and transfer to the host plant from the fungus occurs only when the host plant has an abnormally restricted C supply, as is the case in achlorophyllous plants (Bidartondo *et al.*, 2002). However, in the case of the majority of AM fungal species the fungus cannot complete its life cycle without the symbiosis, suggesting that they are entirely dependent on the host for organic C (Smith and Read, 2008).

Nutrient transfer between the symbionts occurs across symbiotic interfaces (Smith and Read, 2008). The structure, location and composition of the interfaces vary, for instance the interface may be intracellular involving hyphal coils, arbuscules (*Paris*-type mycorrhizas) or arbusculate coils (*Arum*-type mycorrhizas) or intercellular involving hyphae growing in the intercellular spaces of the root cortical cells; however, all involve plasma membranes of both symbionts which are separated by an apoplastic interfacial compartment (Smith and Read, 2008). During the exchange of nutrients between the fungus and host plant the fungus provides the host with nutrients acquired from the soil through the external (extraradical) hyphae such as P, Zn and N (low mobility mineral nutrients) and in return the host provides the fungus with photosynthates (Bolan, 1991; Fitter *et al.*, 2011; Jakobsen *et al.*, 2001; Marschner, 1995; Smith and Read, 2008; Thingstrup *et al.*, 2000). P has a low mobility due to its associations with soil cations, such as Ca^{2+}, Fe^{2+} and Al^{3+} (Smith and Read, 2008; Tinker and Nye, 2000) and N also has low mobility in soil as NO3-N (in well drained soils) and NH4-N (Smith and Read, 2008). P is most readily available to plants at pH 6.5; however, at lower pH values the P associated with Al and Fe decreases in solubility and at higher pH values the P associated with Ca decreases in solubility (Smith and Read, 2008). AM fungi tend to transfer any nutrients that will result in a local sugar flux as well as any excess nutrients that they can spare, which is usually phosphate and when there is an adequate supply, N in the form of ammonium. The host tends to increasingly provide sugars to the region of elevated nutrient supply and some of the sugars diffuse into the apoplast where they may be secured by the fungus (Fitter *et al.*, 2011).

AM fungi are generally considered extensions of the host plant root system (Hodge *et al.*, 2010) by increasing the surface area of the uptake system allowing for greater nutrient assimilation and access. It is thus safe to say that due to hyphae effectively using a larger volume of soil than only roots, mycorrhizal roots are able to acquire more nutrients from nutrient-deficient soils than non-mycorrhizal roots (Entry *et al.*, 2002; Smith and Read, 2008). This is particularly important with regards to P since the transfer of P ions is faster across cell membranes than through the soil, which depends on diffusion, and as a result depletion zones develop around plant roots. Due to this, the rate of P uptake of the roots is determined by the slow diffusion rate, which is avoided by AM fungi extraradical mycelium which acquires nutrients beyond the depletion zone (Fitter *et al.*, 2011; Smith and Read, 2008). It has also been shown that nutrient availability affects AM

fungal colonisation, with low nutrient levels corresponding to increased colonisation and vice versa (Douds and Chaney, 1986; Smith and Read, 2008). This may be related to fungus and host plant interactions with each other with regards to arbuscule and vesicle numbers, content of P, starch and soluble sugars in the host root, spore populations and C allocation (Douds and Chaney, 1986).

AM fungal vesicles are responsible for nutrient storage as well as for propagating AM fungi (Biermann and Linderman, 1983; Jabaji-Hare *et al.,* 1984) and are able to store and provide the host with P if there is lingering P limitation (Olsson *et al.*, 2011). Vesicles are the preferential storage bodies over extraradical spores which may undergo predation (Olsson *et al.,* 2011). Generally P is stored as polyphosphate (a linear polymer consisting of orthophosphate residues linked together by high energy phosphoanhydride bonds) in the vesicles (Olsson *et al.,* 2011; Smith and Read, 2008). The concentration of P within vesicles is nearly double that of Ca (second most abundant element analysed in the study performed by Olsson *et al.,* 2011). Vesicles contain nearly 2-4 times the amount of P when compared with plants, namely 0.4-0.7 % and 0.2 % respectively. Larger vesicles contain more P and there is a high variation of P concentration between vesicles (Olsson *et al.*, 2011). The concentration of P in roots is decreased in soils that contain high amounts of NaCl (Allen and Cunningham, 1983), which may be due to the acidic property of Cl and polyphosphate in cells and there tends to be a negative correlation between the two elements (Olsson *et al.*, 2011). However, arbuscules in mycorrhizal roots contain high amounts of P without any detectable Cl and vice versa in non-mycorrhizal roots (Schoknecht and Hattingh, 1976).

The majority of the nutrient transfer, with regards to P and N, between AM fungi and the host plant occurs across the arbuscule-cortical cell interface. However, it is unclear as to where the carbohydrate transfer occurs (Fitter *et al.*, 2011; Smith and Read, 2008). The transfer of uncharged hexoses could be passive with direction being controlled by concentration gradients across the interface sustained by consumption and production of the hexoses. In lieu of this, the efflux from the host is passive after which influx into the fungus makes use of active proton co-transport that requires maintenance of proton motive force (PMF) across the fungal membrane (Smith and Read, 2008). Photosynthates (hexoses) transferred to AM fungi are very quickly

assimilated into trehalose and glycogen, which creates a buffer against excessive glucose build-up in the cytoplasm (Shachar-Hill *et al.*, 1995; Solaiman and Saito, 1997). Lipids are the main organic C pool in AM fungi (Cooper and Lösel, 1978). Approximately 4-20 % of total photosynthate is transferred to AM fungi during the symbiosis (Smith and Read, 2008). However, it is noteworthy to mention that the production of extraradical hyphae uses less C per unit length than the production of roots (Tinker, 1975) and the small diameter of the extraradical hyphae allow for the penetration of soil pores too small for roots thus reducing the development of depletion zones (Smith and Read, 2008).

The extraradical mycelium may significantly affect plant interactions with the metal ions in the rhizosphere due to its high sorption capacity for Cd, Zn and Cu (Gonzalez-Chavez *et al.*, 2002; Joner *et al.*, 2000; Pawlowski and Charvat, 2004). As well as influencing organic and inorganic nutrient relationships in plants, arbuscular mycorrhiza influence water relations and carbon cycling in plants (Entry *et al.*, 2002).

The second best documented element taken up by AM fungi and transferred to the host plant is N. As mentioned previously N is taken up by the extraradical mycelium either as nitrate ions or ammonium but it is transported within the fungus as arginine, is then probably broken down to urea and finally transferred to the host plant as ammonia with the left over C skeletons being recycled back into the AM fungal stores (Bago *et al.*, 2001; Govindarajulu *et al.*, 2005). The ammonia may be transferred to the plant with the aid of a plant ammonium transporter (AMT), identified in *Lotus japonicas*, which is mycorrhiza-specific and is preferentially expressed in cells containing arbuscules (Guether *et al.*, 2009). N fixation and nodulation in legumes colonised by AM fungi is increased as well as N concentrations in tissues (Barea and Azcón-Aguilar, 1983). Legumes colonised with AM fungi in low P soils have increased assimilation of N_2 in rhizobial root nodules (Smith and Read, 2008).

It has been well established that plants co-existing in a symbiotic associations with AM mycorrhizae are able to increase P obtained per unit area by elevating shoot P concentration, which is attributable to mycorrhizal colonisation and the increased shoot biomass which in turn is linked to P uptake and the positive effect of P on photosynthetic carbon fixation. It is

noteworthy to mention that one plant species can affect other plant species within the vicinity with regards to P uptake, AM fungal colonisation and sporulation, however, this varies between plant species and the reverse is true for nonmycorrhizal plants (Chen *et al.*, 2005). Host plant responsiveness to AM fungal colonisation (mycorrhizal dependency) varies greatly between species and is largely influenced by nutrient supply (Smith and Read, 2008).

Ultimately, AM fungal colonisation and nutrient uptake results in alleviating nutrient stress of the host plant and consequently increases plant growth (Smith and Read, 2008).

1.6.1.4 Applications

With regards to nutrient acquisition from the soil, namely, P, C, Cu, Zn, Fe, Mo, K, Ca and so forth, the fungus makes use of both passive and active transport mechanisms (Smith and Read, 2008). Of all the elements taken up by the extraradical mycelium P is the best documented. One mechanism of P uptake by the AM fungal extraradical mycelium includes a high affinity phosphate transporter (Harrison and van Buuren, 1995).

AM fungi are organisms that are able to sequester toxic trace elements or exclude excessive amounts of trace elements (Khan, 2005) and hence aid mycorrhizal plants survive in polluted soils and ultimately play a role in phytostabilisation of toxic trace element (HM) polluted soils (Khan, 2006). The fungi are able to sequester toxic trace elements with the use of glomalin, an insoluble, highly stable, iron-containing glycoprotein (N-linked oligosaccharides) produced by the hyphae (González-Chávez *et al.*, 2004; Wright and Upadhyaya, 1998), which is released into the surrounding soil (Diver *et al.*, 2005), where it tends to attach to soil and helps stabilise soil aggregates (Khan, 2005) or binds HMs in the soil (González-Chávez *et al.*, 2004; Wright and Upadhyaya, 1998). Glomalin's overall structure consists of monomeric structures bound together through hydrophobic interactions (González-Chávez *et al.*, 2004). The release of glomalin is not through passive secretion and usually remains within the hyphal and spore walls (Khan, 2006), where it may immobilise HMs before they enter the fungal-plant system (Khan, 2005).

AM fungi are able to exclude HMs through adsorptions onto their chitinous cell walls (Göhre and Paszkowski, 2006; Zhou, 1999) or increased efflux with the formation of HM complexes extracellularly from organic acid release (Leyval *et al.*, 1997). An additional method that the fungus may employ as a detoxification mechanism is the use of vesicles for storage of toxic compounds (Göhre and Paszkowski, 2006) or chelation of HM intracellularly through the synthesis of ligands; for instance, polyphosphates and metallothioneins, and/or compartmentation within vacuoles (Göhre and Paszkowski, 2006; Leyval *et a.*, 1997). Since AM fungi presents a large surface area in the soil hyphal binding of HMs acts as an important HM sink (Göhre and Paszkowski, 2006).

Due to the above abilities it has been thought that AM may be used in the remediation of mine tailings as mycorrhizoremediation, which is an enhanced form of phytoremediation (Khan, 2006). This idea is further supported from studies showing AM being present on the mine tailings without introduction (Straker *et al.*, 2007; Straker *et al.*, 2008).

1.7 Coexistence of Rhizobia with AM fungi

AM fungi are also able to form tripartite symbiotic associations with nodule-inducing rhizobia and legumes (Ibijbijen *et al.*, 1996; Olesniewicz and Thomas, 1999; Saxena *et al.*, 1997; Zhao *et al.*, 1997). Rhizobia are soil bacteria known for being root-nodule symbionts of legumes (Denison and Kiers, 2011). However, unlike AM fungi, the symbiosis is not obligate for either the rhizobia or the host plant and some can grow endophytically in non-legumes (Ji *et al.*, 2010). There is a vast number of rhizobial cells in the soil and when a host is present only a few infect and colonise the host plant roots. These few cells proliferate to produce millions of cells within each root nodule and some of these rhizobial cells differentiate into bacteroids, which are modified forms that can convert atmospheric N_2 into N forms that the host can use (Denison and Kiers, 2011). Once the nodule senesces, many of the rhizobia escape into the soil (Bottomley, 1992; Moawad *et al.*, 1984). It is noteworthy to mention that in some hosts the bacteroids lose the ability to divide, thus the soil is repopulated with the undifferentiated rhizobial cells from the same nodules (Oono *et al.*, 2009; Oono and Denison, 2010; Oono *et al.*, 2010).

AM fungi and rhizobia both enhance mineral nutrition of the host plant in exchange for carbohydrates supplied by the host plant (Sharma and Johri, 2002). Unlike AM fungi, the rhizobia fix atmospheric N in root nodules with the use of the nitrogenase enzyme (Thorneley, 1992). A synergistic effect of co-inoculation with AM fungi and rhizobia has been reported for different crops and these effects are more noticeable in soils deficient in both P and N (Sharma and Johri, 2002). It is generally accepted that AM fungi enhance P nutrition of legumes, which improves host plant growth and N fixation (Barea and Azcon-Aguilar, 1983; Cluett and Boucher, 1983). Enhancing P nutrition in P deficient soils is essential as P is vital for both root nodulation and N fixation (Zahran, 1999) and with this it can be seen that P deficiency is a factor that limits N fixation by *Rhizobium*. It may be that the *Rhizobium*-plant system is assisted by the presence of AM fungi because they correct any nutrient deficiency (not just P deficiency) in the host plant that might be limiting to *Rhizobium* (O'Hara *et al.*, 1988; Pacovsky, 1986).

Nodules containing more rhizobial cells can fix more N_2, which supports more plant growth and photosynthesis, which ultimately may support more rhizobia. Rhizobial cells are also able to stockpile resources within nodules, including P and Polyhydroxybutyrate (PHB), which may boost future survival (Denison and Kiers, 2011). Nodulated AM fungal plants have enhanced nitrogen production in comparison to non-nodulated AM fungal plants (Ibijbijen *et al.*, 1996).

The number and biomass of nodules has been shown to significantly increase when both microsymbionts are co-inoculated on to the host (Olsen and Habte, 1995; Saxena *et al.*, 1997; Zhao *et al.*, 1997). However, the exception involves plants colonised/inoculated by *Funneliformis mosseae* and *Gigaspora rosea* which have low nodule numbers, demonstrating symbiont antagonism (Schreiner *et al.*, 1997), which may be due to competition for nutrients between AM fungi and rhizobia (Bethlenfalvay *et al.*, 1992). The efficiency of a symbiosis has also been found to depend on the specific combination of *Glomus* species and *Rhizobium* strain, which indicates selective interactions between the two symbionts (Azcon *et al.*, 1991). The specific compatibility between the symbionts can exist both in terms of root colonisation by introduced AM fungi and competitive ability of introduced rhizobia (Saxena *et al.*, 1997). Simultaneous inoculation with AM fungi and rhizobia has, however, been found to control a few of the soil borne plant pathogenic fungi (Dar *et al.*, 1997).

1.8 AM fungi and mine tailing rehabilitation in South Africa

Several studies have been performed in South Africa in order to determine the species and mycorrhizal status of mine tailings. All studies were able to determine the status of AM fungi on gold and uranium mine tailings located in the North West and Free State provinces (Straker *et al.*, 2007; Straker *et al.*, 2008; Spruyt, 2010) as well as on HM contaminated sites in the Gauteng province (Buck, 2011). The HM sites were ABB Zinc, a galvanising plant and Impala Platinum, a platinum refinery and the heavy metals at the sites included zinc and cadmium and platinum respectively, all in salt form (D. Furniss, *pers. comm.*).

It would appear that AM fungi colonisation is affected by host plant; host species, with woody perennials being more mycotrophic than herbaceous annuals, and age of rehabilitation (Straker *et al.*, 2007). There were differences in AM fungal colonisation levels as well as infectivity between zones on mine tailings with the veld and toepaddocks having the highest rates and the steep slopes having the lowest rates (Straker *et al.*, 2007; Straker *et al.*, 2008).

A number of recent studies have used sequence data based on the SSU rRNA gene to identify the AM fungi associated with plants on HM sites. The study of Spruyt (2010) identified species from the *Acaulospora* and *Entrophospora* genera associated with *Tamarix usneoides*, whereas the study performed in 2011 on *T. usneoides* identified species mainly in the *Claroideoglomus* genus from two heavy metal contaminated sites with only one species matching *Acaulospora mellea* and another matching *Glomus eburneum* (Spruyt *et al.*, 2014). Since *Acaulospora mellea* was also identified from *T. usneoides* growing at Mispah in the Free State province in South Africa (Spruyt, 2010), it could be suggested that this species associates with *T. usneoides* under HM conditions. However, the differences in genera associated with *T. usneoides* at the different HM contaminated sites, suggest that HM and soil properties determine which AM fungal species are associated with *T. usneoides* (Buck, 2011).

Another study performed on *Searsia lancea* and *Searsia pendulina* at the Mispah and West Complex sites in the Free State province, identified the genera *Glomus*, *Sclerocystis*, *Diversispora*, *Scutellospora* and *Claroideoglomus* which shows greater AM fungal diversity than

the studies with *T. usneoides* (Spruyt *et al.*, 2014). However, the *Claroideoglomus* (*C. lamellosum*) species identified from *S. pendulina* growing at Mispah had the same species number in the BLAST database (FR773152.1) as that identified from *T. usneoides* growing naturally in the Northern Cape (Spruyt *et al*., 2014), which demonstrates that this species can associate with different hosts as well as having the ability to tolerate HMs. This may be true of many other AM fungal species.

1.9 Taxanomic classification system

The goal/objective of taxonomy (biological classification) is to clarify and organise the vast diversity of organisms into logical units, termed taxa, which have universally approved names and whose members share significant characteristics (Ruggiero *et al.*, 2015). This classification incorporates a large variety of character information, for instance, morphological, physiological and molecular (genes, metagenome and metabolome) (Ruggiero *et al.*, 2015; Willey *et al.*, 2008). Unfortunately, at present there is no consensus between taxonomists for a classification strategy due to new information of characteristics on organisms continually being ascertained, resulting in the requirement of constant revision of classified taxa. Other reasons for a lack of consensus include researchers redefining classification terminology, which have not yet been incorporated universally, as well as characteristics/evidence contradicting and/or being uncertain for the taxanomic relationships (Hennig, 1966; Mayr and Bock, 2002; Ruggiero *et al.*, 2015).

The organisation system in taxonomy involves hierarchical levels/ranks starting with broad characteristics and then refining down to a single level. The first level, life, is comprised of all cellular organisms. The second level, domain/empire, is comprised of Bacteria, Archaea and Eucarya based on SSU rRNAs (Woese and Fox, 1977; Woese *et al.*, 1990). The third level, kingdom, is comprised of several groups/taxa although an agreement on quantity and names is not yet reached. The number of kingdoms ranges from 5 to 8, namely, Fungi, Eubacteria, Archaebacteria, Animalia, Plantae, Protozoa Archezoa and Chromista (Whittaker, 1969; Cavalier-Smith, 1981, 1998, 2004). Although Cavalier-Smith (1981) suggested the following structure of kingdoms and subkingdoms: Fungi (Eufungi and Cilliofungi), Bacteria (Eubacteria and Archaebacteria), Animalia (Parazoa, Mesozoa and Metazoa), Plantae (Biliphyta and

Viridiplantae), Protista (Protozoa and Euglenozoa) and Chromista (Cryptophyta and Chromophyta). The fourth, fifth, sixth, seventh and eighth levels are phylum/division, class, order, family and genus, respectively (Cloete and Atlas, 2006; Lawrence, 2005), and are comprised of many groups, which due to the ever-changing nature of the ranking system it would be impractical to list and describe them all. Finally, the last level, species, is comprised of individual organisms given latin binomial names originating from the classification system created by the Swedish botanist Carolus Linnaeus (Carl von Linné or Caroli Linnæi) (Willey *et al.*, 2008); however, species level may be reduced even further to subspecies, strains, races and varieties (in cultivated plants) (Lawrence, 2005).

Eventually taxonomists will be able to integrate characteristics into an agreed upon standardised system so that life can be categorised into the ranks easily and efficiently.

The classification system can be attributed to three fields or branches, namely classification, nomenclature and identification (Cloete and Atlas, 2006; Willey *et al.*, 2008). Classification specifically involves the organisation of organisms into groups based on apparent evolutionary traits (similarities and differences) (Cloete and Atlas, 2006; Lawrence, 2005). Nomenclature is concerned with assigning names to the taxanomic groups following/in agreement with internationally published rules/standards (Cloete and Atlas, 2006; Willey *et al.*, 2008). Identification comprises the practical side of taxonomy and involves ascertaining whether a certain isolate belongs within a specific recognised taxon (Cloete and Atlas, 2006; Willey *et al.*, 2008).

The process of identification may involve several aspects when considering the method upon which to identify an isolate/organism. One may look at the morphological, physiological and metabolic and ecological characteristics if the isolate is culturable and if the isolate is either culturable or not one may alternatively use genetic characteristics (Willey *et al.*, 2008).

1.10 Studying the rhizobiome

The rhizosphere is comprised of a thin area of soil that immediately surrounds and is influenced by plant roots (Lawrence, 2005; Phillippot *et al.*, 2013), roughly within 2 cm of the root surface (Coats *et al.*, 2014). It is also thought to be the most active matrix on Earth with an abundance of microorganisms and invertebrates (Philippot *et al.*, 2013). The microbiome is comprised of all the gene sequences within a microbiota (Marchesi and Ravel, 2015). Microbiota is the assembly of microorganisms existing in a defined environment (Marchesi and Ravel, 2015). The rhizosphere is a soil hot spot that is chemically modified as compared to the bulk soil, due for example to the nutrient depletion, acidification, Fe solubilisation and precipitation. The rhizobiome, simply stated, is the microbiome of the rhizosphere. Knowing the composition of the rhizobiome is a prerequisite for understanding the ecosystem, individual organisms' purposes and the overall function and activity of the community (Högberg *et al.*, 2001).

The entire nature of the rhizobiome is yet to be elucidated; however, many studies have illuminated some functions performed by microbes in the rhizosphere, for instance, in the case of plant-associated microorganisms, seed germination, seedling vigour, plant development and growth, nutrition, productivity and diseases are greatly affected (Mendes *et al.*, 2013). Due to the microorganisms' drastic influence on plants and/or other higher organisms (such as humans) it is considered that these higher organisms rely, partially or entirely, on their microbiome for specific traits and functions (Mendes *et al.*, 2013; Philippot *et al.*, 2013). The microorganisms receive elements in return and with regards to plants, photosynthetically produced carbon is deposited or released into the immediate surroundings, namely, the rhizosphere and mycorrhizosphere (Berendensen *et al.*, 2012; Frey-Klett *et al.*, 2007; Raaijmakers *et al.*, 2009). Other elements released by plants as rhizodeposits include, other nutrient elements (low-molecular-mass compounds, namely, amino acids and organic acids), border cells, mucilage (polymerised sugar) and exudates (Philippot *et al.*, 2013). Root exudates not only sustain a larger and more active microbial community but also induce a selection in the community composition and microbial metabolic activity (Mendes *et al.*, 2013). Essentially an intricate feedback system between organisms is in place (Coats *et al.*, 2014). The focus of the analysis of the rhizobiome will be metataxonomically (DNA) based as the vast majority of soil microbes are unculturable as

determined by Kellenberger (2001). Unfortunately, the soil sample sizes used in molecular research are frequently less than 1 g, which results in limitations in the research scope to a microscopic version of the habitat that was actually sampled (van Elsas and Boersma, 2011).

1.11 Soil DNA analysis

Soil is difficult to analyse due to it heterogenous nature and the fact that the biogeochemistry and spatial complexity is not fully understood (Mocali and Benedetti, 2010). Soil is also recalcitrant to mixing (Daniel, 2004). The soil processes and properties by which carbon interacts with microorganisms are also not well understood (Mocali and Benedetti, 2010). Girvan *et al.* (2003) illustrated important parameters that affect microbial community composition, these parameters include formation and stabilisation of microaggregates, micropores and clay-organic matter complexes (the dominant structural characteristics of soil matrix) and soil mineral and organic particles actively interact with soil biota. Additionally, the communities of microorganisms in the soil are randomly distributed (Nunan *et al.*, 2002). One study counted and showed that within 1 g soil roughly 10^7 microbial cells were present (Kellenberger, 2001), however, it has been suggested that roughly 0.1-1 % of the total soil microbial population is culturable using current methods (Daniel, 2004; Kellenberger, 2001). Thereby the use of molecular (DNA-based) methods, which do not rely on isolation and cultivation of microorganisms, reduces any bias associated with isolation and cultivation (Daniel, 2004).

Without a DNA extraction kit DNA may be extracted from soil either through direct or indirect techniques, although the DNA extraction method requires optimisation for every soil sample type (Harry *et al.*, 1999; Rajendhran and Gunasekaran, 2008; Zhou *et al.*, 1996). The goal is to extract nucleic acids from cells that have been unbiasedly lysed (Rajendhran and Gunasekaran, 2008). Cell lysis within the sample matrix and DNA isolation from the matrix and cell debris is considered to be a direct DNA extraction technique (Ogram *et al.*, 1987; Rajendhran and Gunasekaran, 2008). Alternatively, the indirect method of DNA extraction involves first the isolation of cells from the soil matrix then the lysis of the cells followed by DNA extraction (Holben *et al.*, 1988; Rajendhran and Gunasekaran, 2008).

The particular steps involved in DNA extraction include cell lysis, DNA extraction, followed by quantitative and qualitative DNA analysis.

Cell lysis, a vital step in the DNA extraction process, is devised to break cell membranes and walls of microorganisms thereby releasing cell contents and DNA. There are several techniques that may be employed in order to achieve cell lysis but generally they are chemical or physical. Chemical (or enzymatic) lysis is relatively precise and can be conceived as a mild method whereas physical (mechanical) lysis indiscriminately lyses cells as well as evenly distributing soil sediment and allows for the lysis buffer to fully permeate the sample. However, no single technique of cell lysis is suitable for all soil sample types and various combinations and alterations of lysis techniques may be required for various soil samples (Rajendhran and Gunasekaran, 2008).

DNA extraction, subsequent to cell lysis and DNA release, relies on the ability to isolate the DNA from contaminating cell contents for instance RNA and proteins. RNase is used in the removal of RNA and proteins may either be removed through deproteinisation using organic solvents or salted-out using saturated salt solutions. DNA is then precipitated using ethanol, isopropanol (Rajendhran and Gunasekaran, 2008) or polyethylene glycol (PEG) (Martin *et al.*, 2001; Rondon *et al.*, 2000), although the choice of solution is dependent on soil type (Rajendhran and Gunasekaran, 2008). The extraction buffer pH plays a pivotal role in recovering metagenomic DNA from soil, with the optimal pH range being 9.0-10.0 (Frostegard *et al.*, 1999). DNA precipitation also serves to discard buffer and any remaining contaminants thus improving the quality and quantity of DNA isolated (Rajendhran and Gunasekaran, 2008). The precipitated DNA is then resuspended in an appropriate solution (usually nuclease-free water) and the DNA concentration and purity is tested.

In the case of soil DNA extraction, additional purification steps may be required, as frequently the extract (crude) contains a noticeable amount of contaminants, such as fulvic or humic acids, that impede downstream analytical methods requiring PCR or hybridization labelling. Unfortunately, purification steps may cause loss of DNA material and should thus be optimised. It is essential that purification steps across samples are standardised since discrepancies in DNA

isolation methods may cause bias in data obtained thus compromising the scientific warrantee of the research (van Elsas and Boersma, 2011).

Efficient methods for testing DNA concentration and purity use spectrophotometry, although this can be prone to bias as contaminating organic compounds interfere with the solution readings. Generally when using spectrophotometric techniques the absorbance reading at an absorbance of 260 nm is considered the DNA concentration and the ratio between an absorbance of 260 nm and 280 nm is used to assess the purity of the DNA sample. Alternatively, densitometric methods using stained agarose gels may be used to estimate DNA concentrations (Rajendhran and Gunasekaran, 2008).

For simplicity, efficiency and consistency, commercially available DNA extraction kits for soil samples have been developed. The majority of researchers make use of extraction kits such as those referred to 'Powersoil' or 'Ultraclean' soil DNA extraction kits, by MoBio, USA and/or the 'Fast DNA Spin' kit for soil, by Bio101, USA (van Elsas and Boersma, 2011). Although the kits may not be suitable for all soil samples, certain steps may be supplemented in order to optimise DNA extraction. Along with soil DNA extraction kits, resin-based DNA purification kits, namely Wizard by Promega, USA, have been developed and when used in conjunction with DNA extraction kits (particularly Powersoil) the DNA yield isolated from a variety of soils was identified as PCR-amplifiable (Inceoglu *et al.*, 2010).

Once all DNA extraction steps have been completed and optimised for proposed research, the DNA can then be subjected to PCR amplification targeting specific sequences (or organisms) using specific or sequenced directly using next generation sequencers (NGS). The currently available NGS technologies/platforms include 454 pyrosequencing (Roche 454 GS FLX); SOLiD (ThermoFisher Applied Biosystems); miSeq, HiSeq2000 and HiSeq2500 (Illumina); and Ion Torrent (ThermoFisher) to list a few (Buermans and den Dunnen, 2014; Levy and Myers, 2016). Currently all available NGS platforms need a certain amount of DNA pre-processing into a library suitable for sequencing. Often the steps include the shearing of high molecular weight DNA into suitable sizes for specific platforms, after which an end polishing step generates blunt ended DNA fragments. Following this certain adapters are ligated to these fragments by either

A/T overhand or direct blunt ligation. A functional library needs to have certain adapter sequences to be added to the 3' and 5' ends. Interestingly each sequence platform makes use of a different set of unique adapter sequences to be compatible with the further steps of the process. Different NGS providers utilise different methods in the preparation of sequence libraries into suitable templates as well as using different methods in signal detection and ultimately reading the DNA sequences. Interestingly these different strategies lead to differences in output capacity for the different platforms (Buermans and den Dunnen, 2014). Following sequencing, sequence data is then analysed through bioinformatic processes, which are mentioned later.

1.12 Research question

Does the diversity of microbial communities associated with engineered and natural *Vachellia karroo (Acacia karroo)* woodlands on the Witwatersrand Basin gold fields vary with exposure to acid mine drainage or between regions?

1.13 Specific objectives

- Select and collect soil and root samples from selected *Vachellia* (*Acacia*) trees.
- Perform soil chemical analysis.
- DNA extraction from pooled soil samples
- DNA quantity and quality checking
- Next generation DNA sequencing
- Metataxonomic analysis and comparison of alpha and beta diversity

1.14 References

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Chapter 2

Site and soil analysis

Introduction

Soil, in the traditional sense, is considered to be a naturally occurring medium that supports the growth of land plants (Eash *et al.*, 2008). More precisely, according to the Soil Science Society of America, soil may be considered the unconsolidated mineral or organic matter on the earth's surface that shows the effects and has been subjected to environmental and genetic factors of macro- and microorganisms, climate (including temperature and water effects), conditioned by relief, acting on parent material over a period of time (Eash *et al.*, 2008). Soil varies substantially and is extremely diverse over the earth's surface. It is distinct from the parent material because of morphological, biological, chemical and physical characteristics and properties, brought about by fragmentation and chemical weathering of rock. Overall, soil formatiom may be summarised using the CLORPT equation proposed by Jenny (1941), S = f(Cl, O, R, P, T), whereby soil types/properties are a function of climate (Cl), biotic effects (O for organisms), topography (R for relief), parent material (P) and time (T). Knowing the properties of the soil in question allows for proper management and strategies for future development (Eash *et al.*, 2008; Rowell, 1994).

2.1 Site descriptions

The Vaal River (VR) Operations site (S 26°54'58.89" E 26°45'7.00"; overall size 23 876 hectares) is surrounded by the following towns: Orkney (within the VR Operations), Klerksdorp (18 km north-west), Potchefstroom (50 km east), Bothaville (45 km south), Leeudoringstad (56 km south-west). The West Wits (WW) Operations site (S 26°26'4.61" E 27°21'37.92"; overall size 3785.8-4176 hectares), which straddles the Gauteng and North West Provinces, is roughly 75 km west of Johannesburg and roughly 5-7 km south of Carletonville. Other nearby towns are Fochville (12 km south) and Potchefstroom (50 km west). Figure 2.1 represents a local map showing the VR and WW Operations sites, and shows the overall spatial distribution of each site and provides the geographical context regarding the proximity of the sites to each other and to the towns in the vicinity; however, the image was created in 2009 and some changes to the landscape may have occurred.

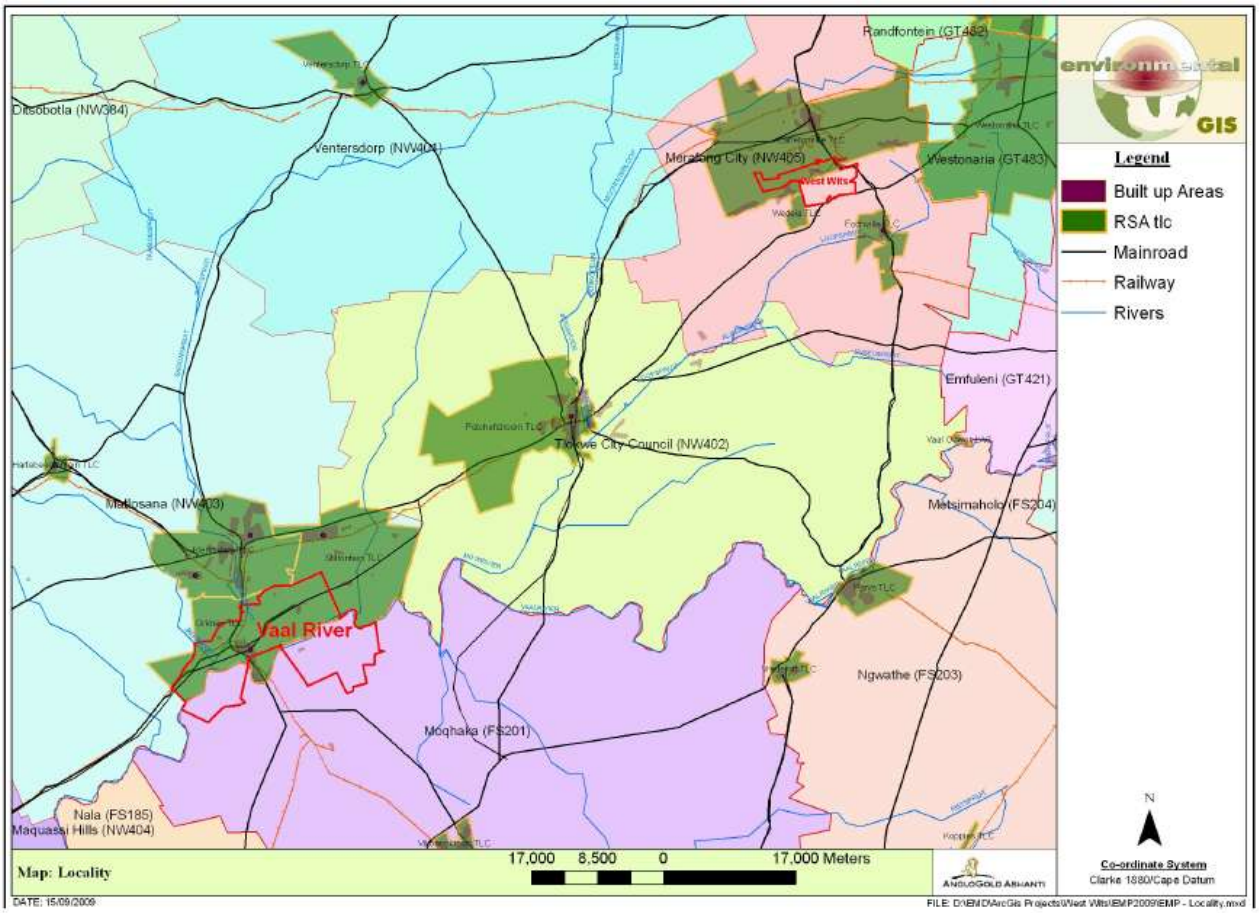


Figure 2.1: A regional map for Vaal River and West Wits Operations within the reference to AngloGold Ashanti (AngloGold Ashanti, 2009).

2.2 Soil history and description

Geologically the WW and VR sites are part of the Witwatersrand basin which is one of the world's most metallogenic gold and uranium deposit regions. The Witwatersrand basin is composed of an oblong north-east to south-west trending basin (350 km x 160 km), underlying southern Gauteng, North-West and northern Free State provinces. Soil sediment deposition in the Witwatersrand basin occurred roughly over 360 million years ago (Ma) between years 3 074 Ma and 2 714 Ma (Proterosoic time period) (Robb and Robb, 1998) onto a granite-greenstone basement (Kaapvaal Craton) (McCarthy and Rubidge, 2005). At the end of that time two micro-continents, the Zimbabwe and Kaapvaal Cratons, collided, resulting in the rupture of the crust and caused a great quantity of lava to erupt. Following this was a duration whereby mountains were built, and stabilization and erosion occurred.

Collectively the sediments, which accumulated within the basin (Figure 2.2), are known as the Witwatersrand Supergroup (Figures 2.2-2.4) and is comprised of the West Rand Group (WRG) (Figures 2.2-2.4) and Central Rand Group (CRG) (Figures 2.2-2.4). The CRG, made up of quartzites and conglomerates, overlays the WRG, made up of shales and quartzites. Black Reef formation (Figures 2.5 and 2.10) in the WW was created through rifting of Kaapvaal Craton (Figure 2.2) roughly 2 650 Ma followed by erosion, after which subsidence of the continent below sea level caused river systems to be buried by beach and shallow-water marine deposits ultimately leading to conglomerate, sandstone and mudstone deposits (McCarthy and Rubidge, 2005). Bacteria then flourished in the shallow sea environment and the arising bacterial growth resulted in the accumulation of more than 1 km in depth of dolomite and large quantities of manganese and iron. Following which these were precipitated by the oxygen released by cyano-bacteria (MacCarthy and Rubidge, 2005). These dolomites form a broad band spanning from Johannesburg, through Carletonville to Orkney and comprise Malmani Subgroup Dolomites (Figures 2.6-2.10) of the Chuniespoort Group (Figures 2.9 and 2.10) and Transvaal Supergroup (Figures 2.9 and 2.10).

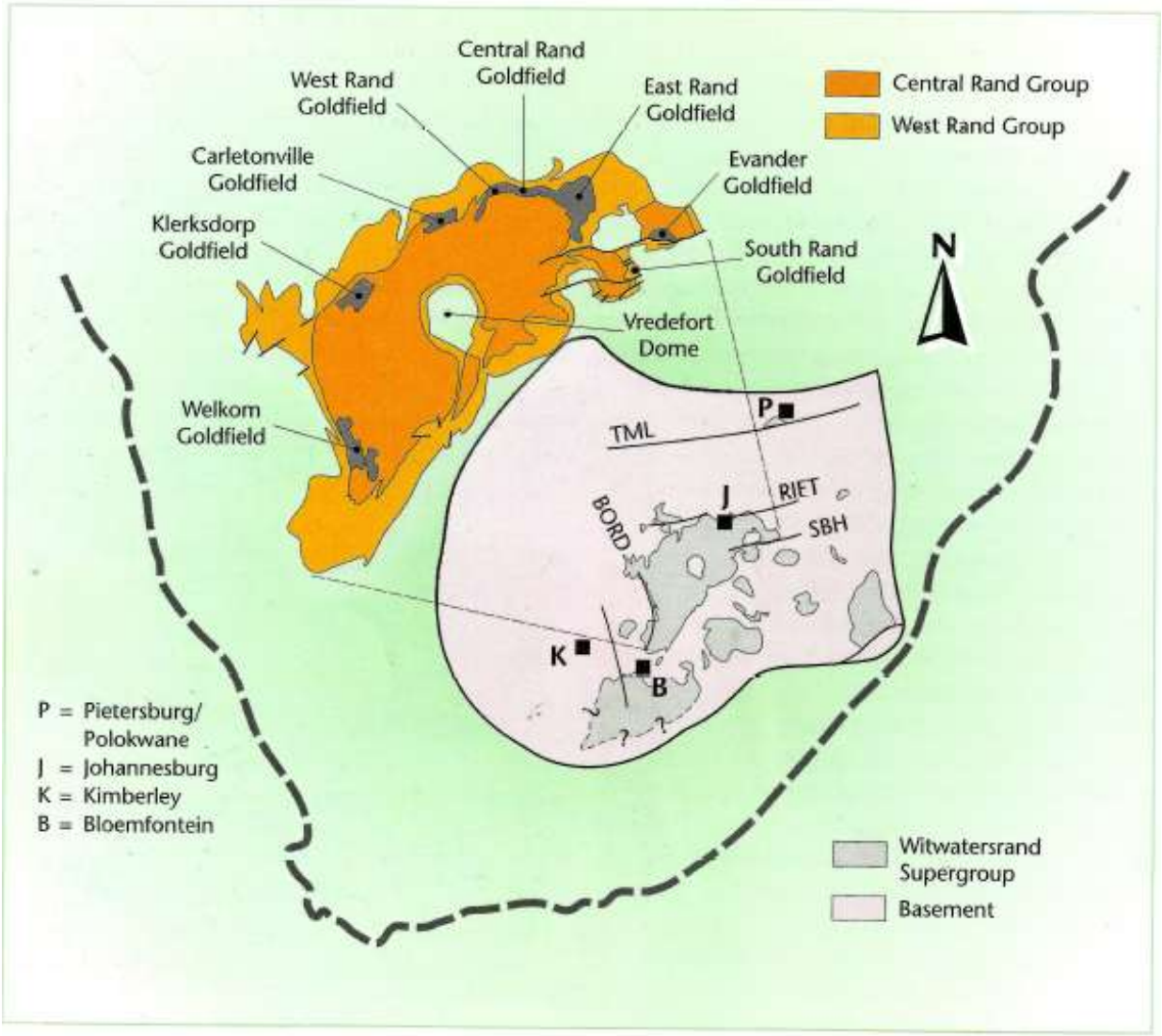


Figure 2.2: A schematic overview of the Witwatersrand supergroup basin within the Kaapvaal Craton with the enlarged inset demonstrating the primary area of preservation of the Witwatersrand Supergoup basin. The major goldfields occur in a curve around the western and northern sides of the basin. The locations of these goldfields were determined by earth movements along faults such as the Thabazimbi-Murchison Line (TML), the Rietfontein Fault (RIET), the Sugarbush Fault (SBH) and Border Fault (BORD). Illustration taken from McCarthy and Rubidge (2005).

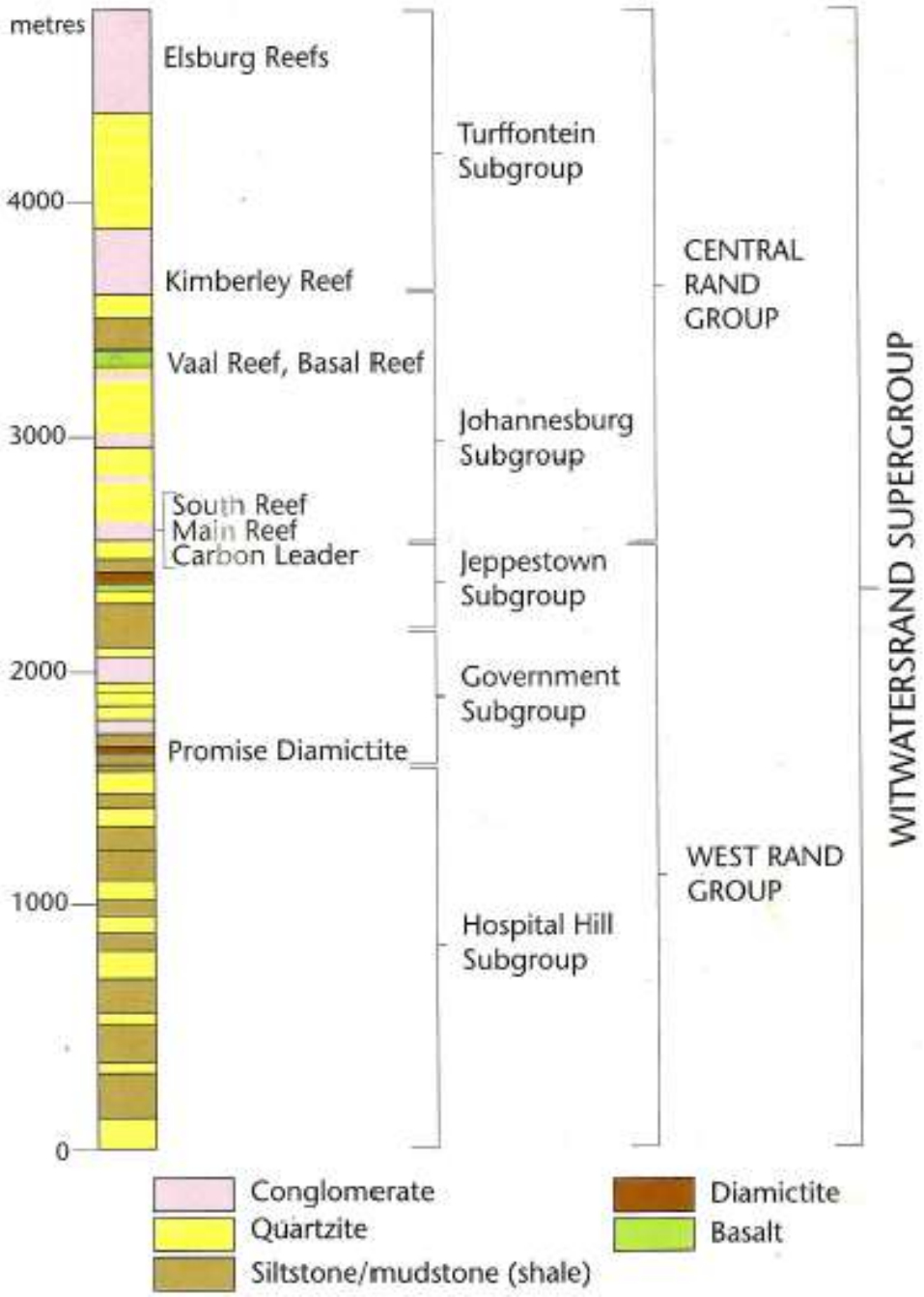


Figure 2.3: A composite stratigraphic column of the Witwatersrand Supergroup, a 7 km thick sedimentary rock formation. Illustration taken from McCarthy and Rubidge (2005).

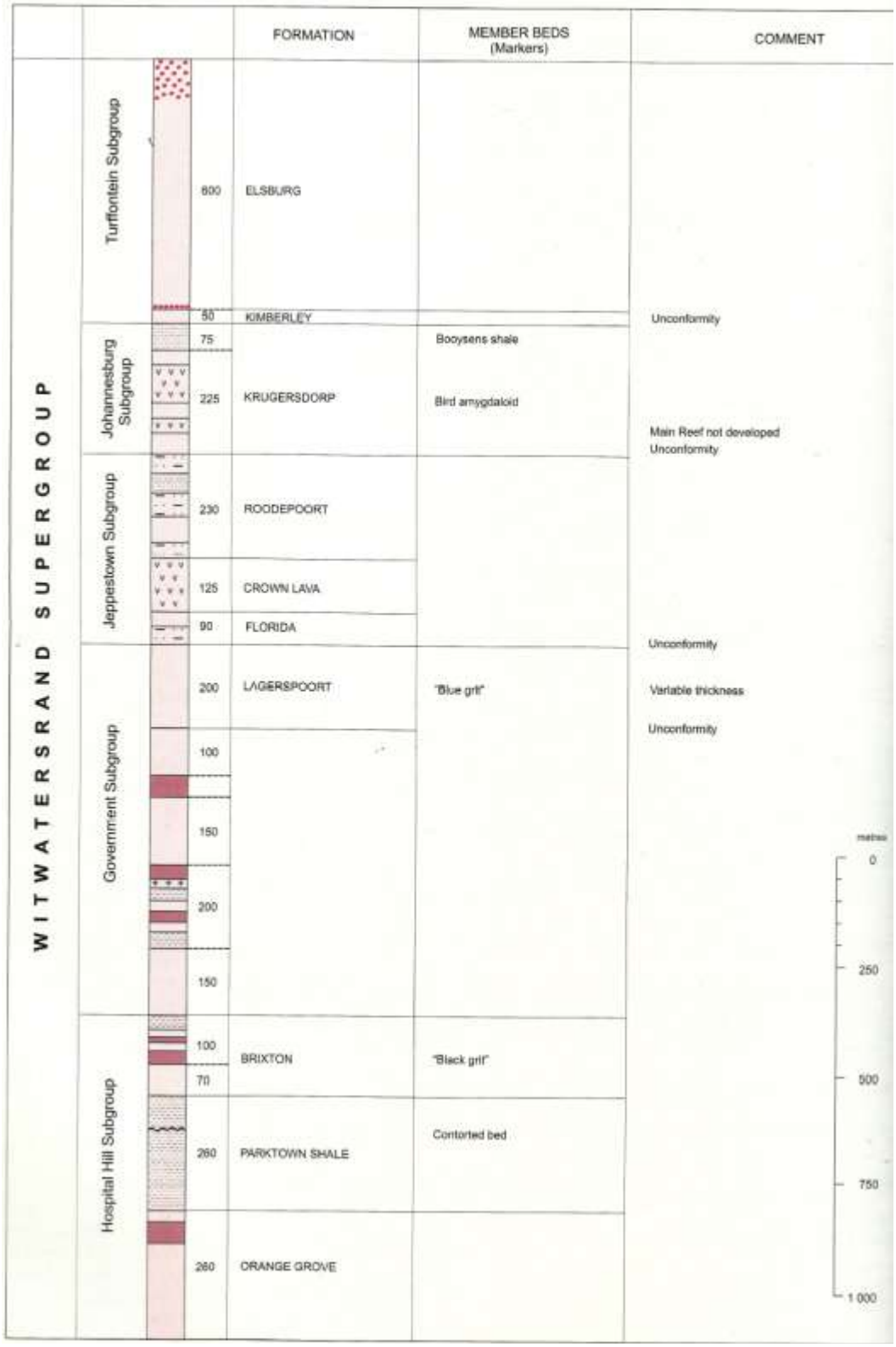


Figure 2.4: A simplified composite stratigraphic column demonstrating the gradual development of West Rand Group (Hospistal Subgroup, Government Subgroup and Jeppestown Subgroup) and Central Rand Group (Johannesburg Subgroupand Turffontein Subgroup) sediments. Illustration taken from Wilson and Anhaeusser (1998).

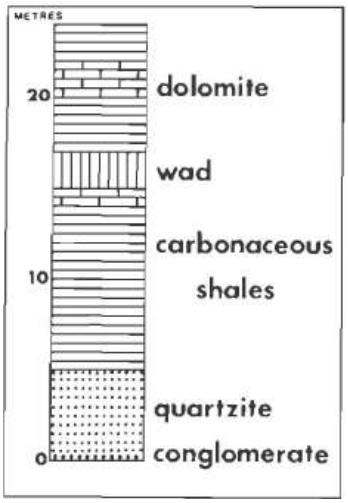


Figure 2.5: A simplified composite stratigraphic column demonstrating the Black Reef Formation. Illustration taken from Eriksson and Trustwell (1974).

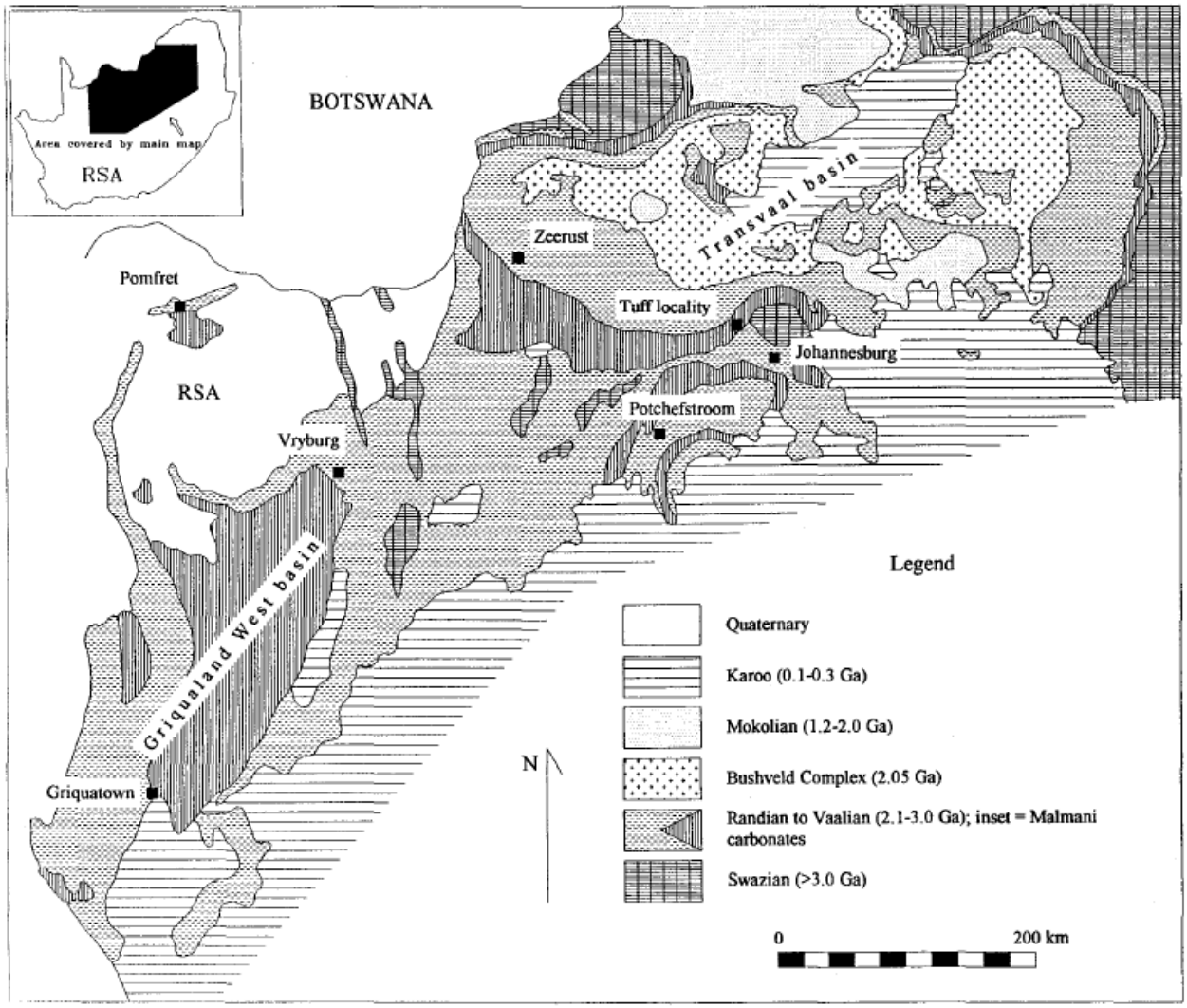


Figure 2.6: A simplified geological sketch map of the regions illustrating the locations of major stratigraphic units and the Malmani Subgroup. Illustration taken from Walraven and Martini (1995).

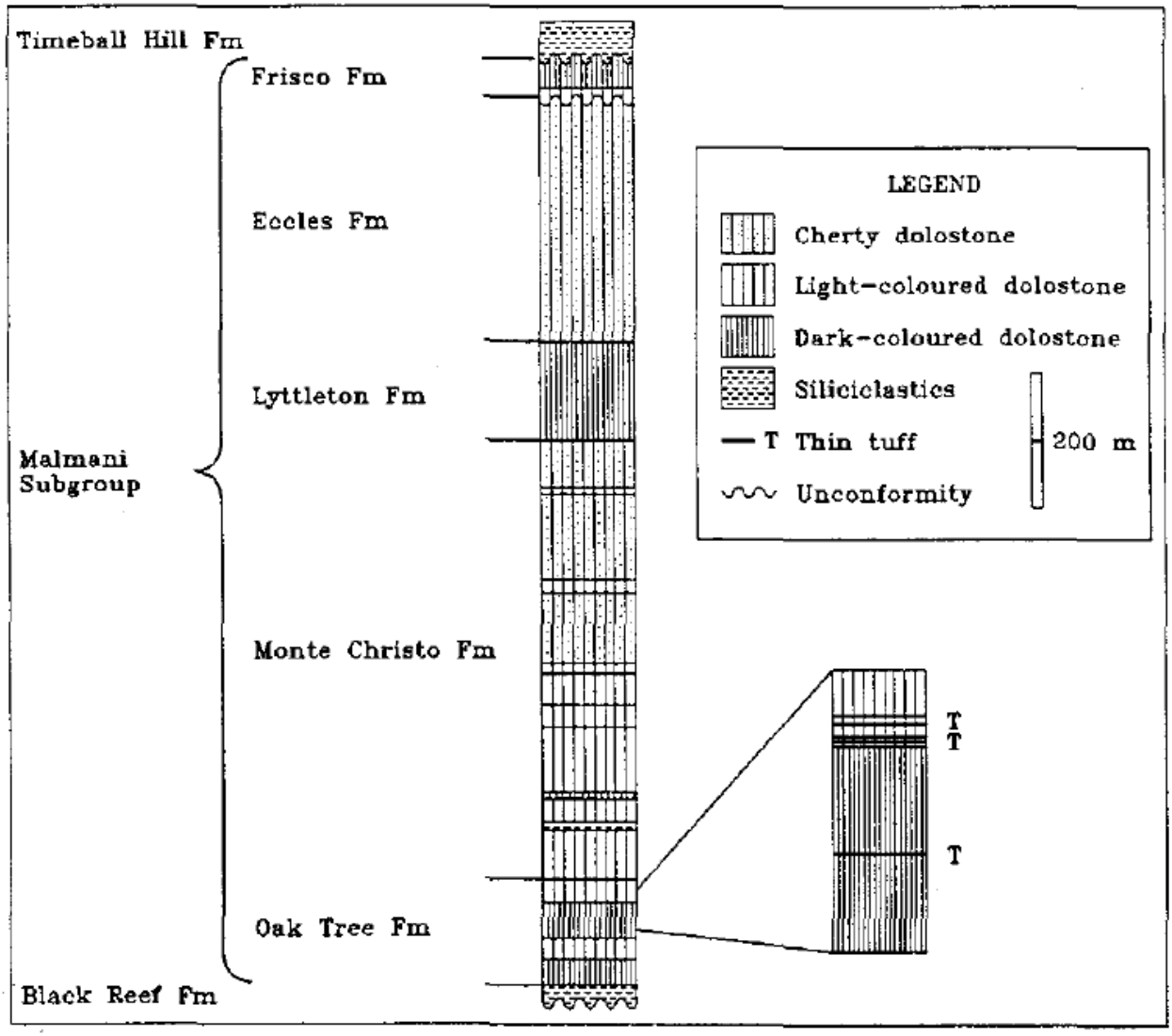
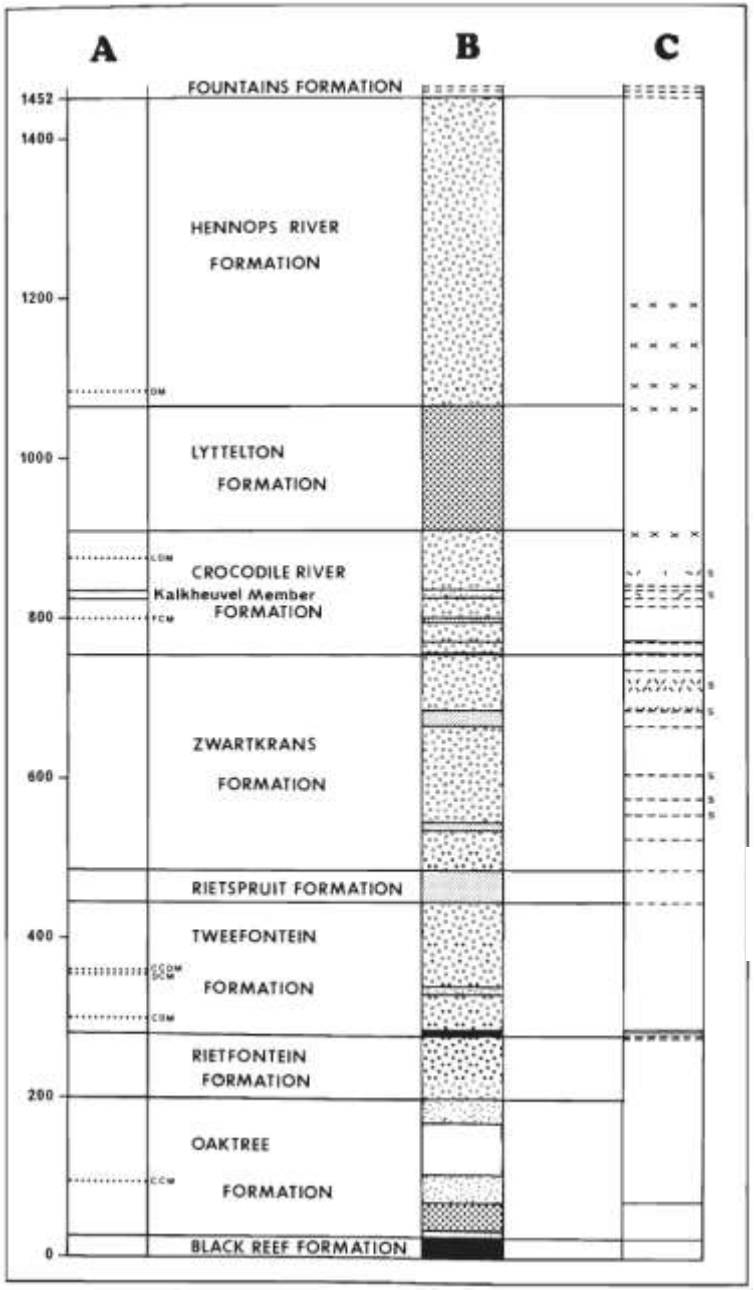


Figure 2.7: A simplified composite stratigraphic column demonstrating the Malmani Subgroup in the area northwest of Johannesburg. Inset demonstrates the tuff layers. Illustration taken from Walraven and Martini (1995).



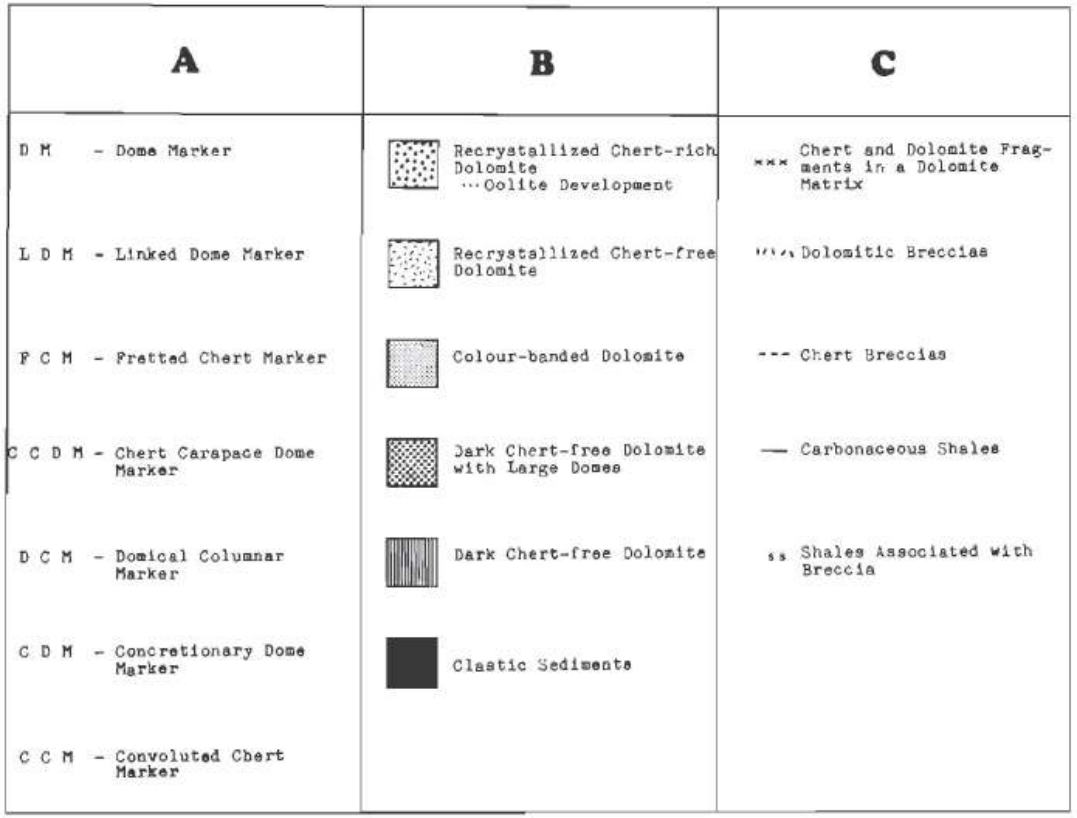


Figure 2.8: A simplified composite stratigraphic column demonstrating the Malmani Subgroup. Illustration taken from Eriksson and Trustwell (1974).

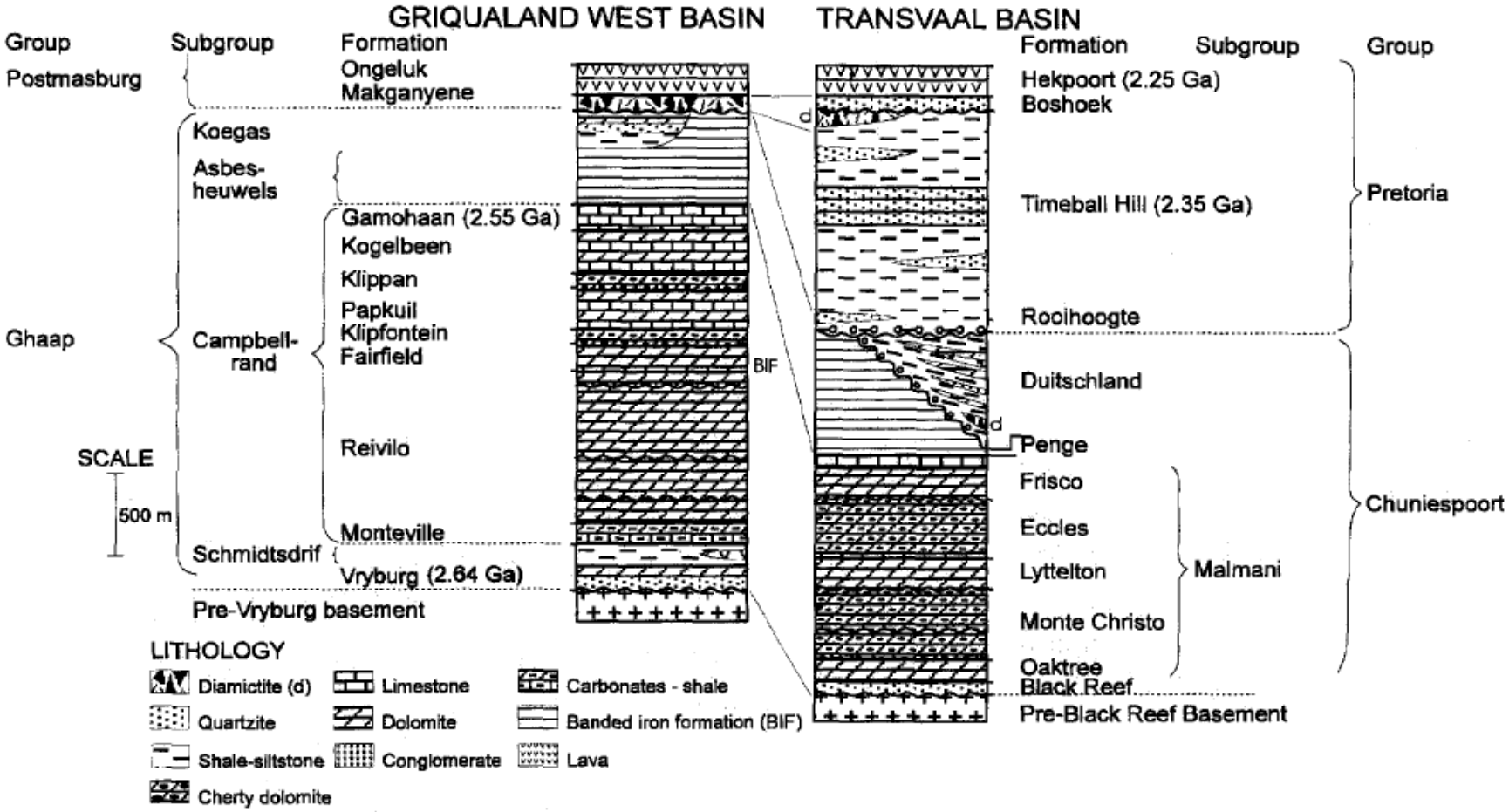


Figure 2.9: A simplified composite stratigraphic column demonstrating the Chuniespoort and Ghaap Groups. Illustration taken from Eriksson *et al.* (1995).

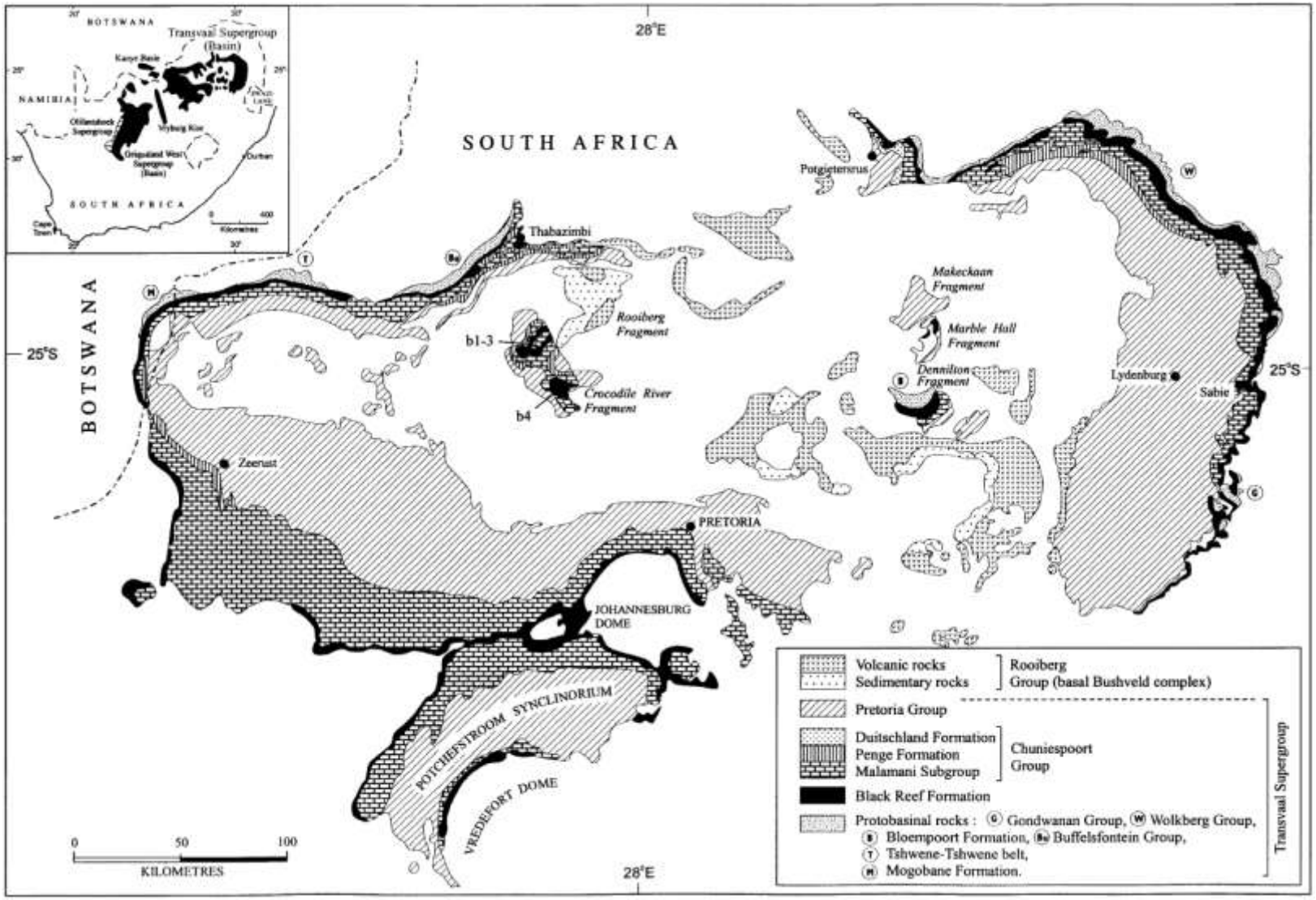


Figure 2.10: A simplified geological sketch map of the regions illustrating the locations of the Transvaal lithostratigraphic units with the Transvaal basin. Illustration taken from Catuneanu and Eriksson (1999).

2.3 Vaal River Operations site

The VR Operations are amidst the Klerksdorp gold field (Figure 2.2) with the predominant geological units being the Witwatersrand (Figures 2.2-2.4), Transvaal (Figures 2.9 and 2.10) and Ventersdorp Supergroups (Figure 2.11) and Swazian basement granites (Figure 2.6).

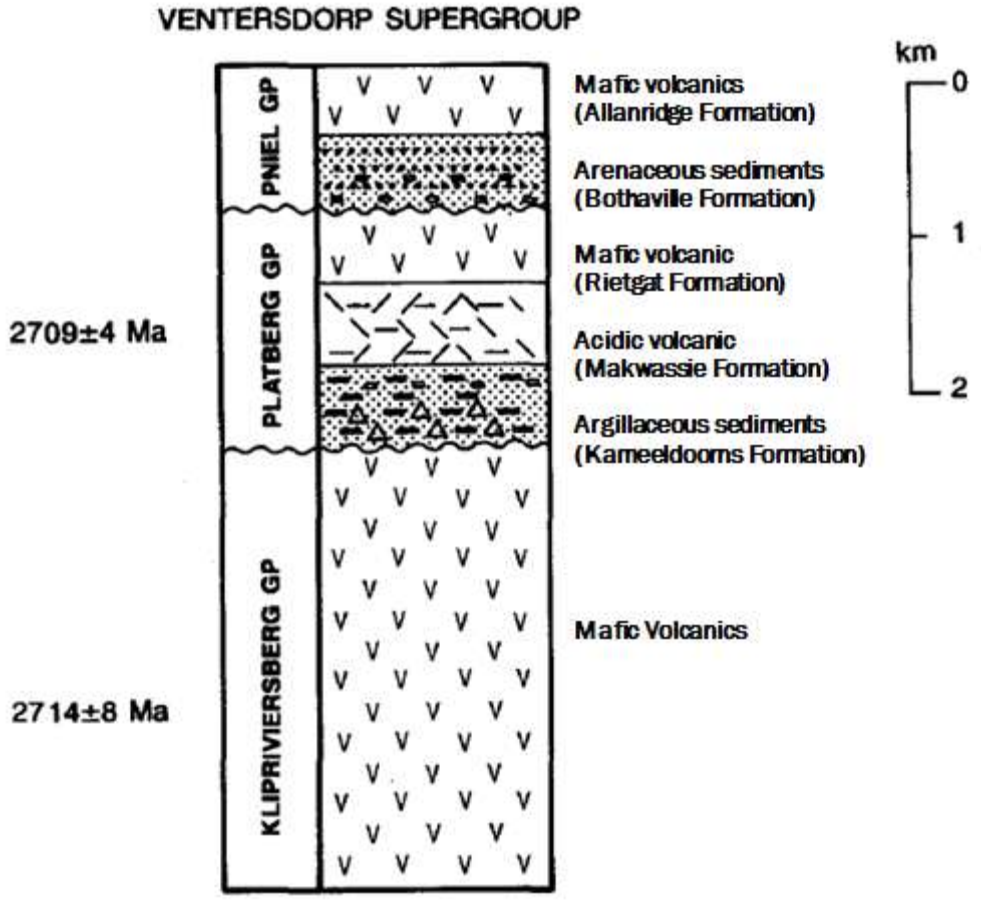


Figure 2.11: A simplified composite stratigraphic subdivision column demonstrating the Ventersdorp Supergroup. Modified illustration taken from White (1997).

The WRG as mentioned previously is composed of alternating shale and quartzite, however, the ratio of quartzite when compared to other parts of the basin is higher (Robb and Robb, 1998). Specifically, the WRG deposition occurred as a result of a shallow sea (McCarthy and Rubidge, 2005). Overall the WRG has a maximum thickness of 4.5 km and is well developed in the gold field.

The CRG, subdivided into the Johannesburg (Figure 2.12) and Turffontein Subgroups (Figure 2.13), acquires a thickness of 2.2 km in the Klerksdorp gold field and is overlain by the prominent volcanic base of the Ventersdorp Supergroup and the intercalated quartzite and conglomerate of the Ventersdorp Contact Reef (VCR). The VCR is exploited at the Tau Lekoa mine (Weiersbye, unpublished). Specifically, the CRG deposition occurred as a result of river deposits and is predominantly terrestrial (McCarthy and Rubidge).

The Vaal Reef, which lies within the Johannesburg Subgroup, is the most economically vital area of this gold field. It is well mineralised, with gold, uraninite, both crystalline and nodular pyrite, and carbonaceous matter concentrated along the base of the conglomerate layer and it is also usually not more than 50 cm thick (Weiersbye, unpublished).

Generally the reef (conglomerate) matrix is comprised of: pyrite (3-4 %); other sulphide compounds (for instance, arsenopyrite, chalcopyrite, cobaltite, galena, gersdorffite, linnacite, pentlandite, pyrrhotite, sphalerite) (1-2 %); micaceous minerals such as chlorite, chloritoid, muscovite, pyrophyllite, sericite (10-30 %); grains of primary minerals, such as chromite, garnet, ilmenite, rutile, tourmaline, zircon, xenotime, alteration products such as goethite and leucoxene, and secondary minerals, such as anatase and skutterudite (1-2 %). Gold and uranium (mainly uraninite (UO_2)) are usually found in this matrix (Liebenberg, 1957).

Malmani Subgroup Dolomites dominate the geology and form significant aquifers. These dolomites are comprised of several chert-rich and chert-poor formations of the Chuniespoort Group, which affects the nature of weathering. The dolomites are surrounded by the Black Reef Formation to the north and the lowest 2 formations include the Oaktree Formation (Figures 2.7-2.9) (lower 200m of dark brown, manganiferous chert-poor dolomite) and the Monte Christo Formation (Figures 2.7 and 2.9) (upper 250 m of light grey, recrystallised chert-rich dolomite).

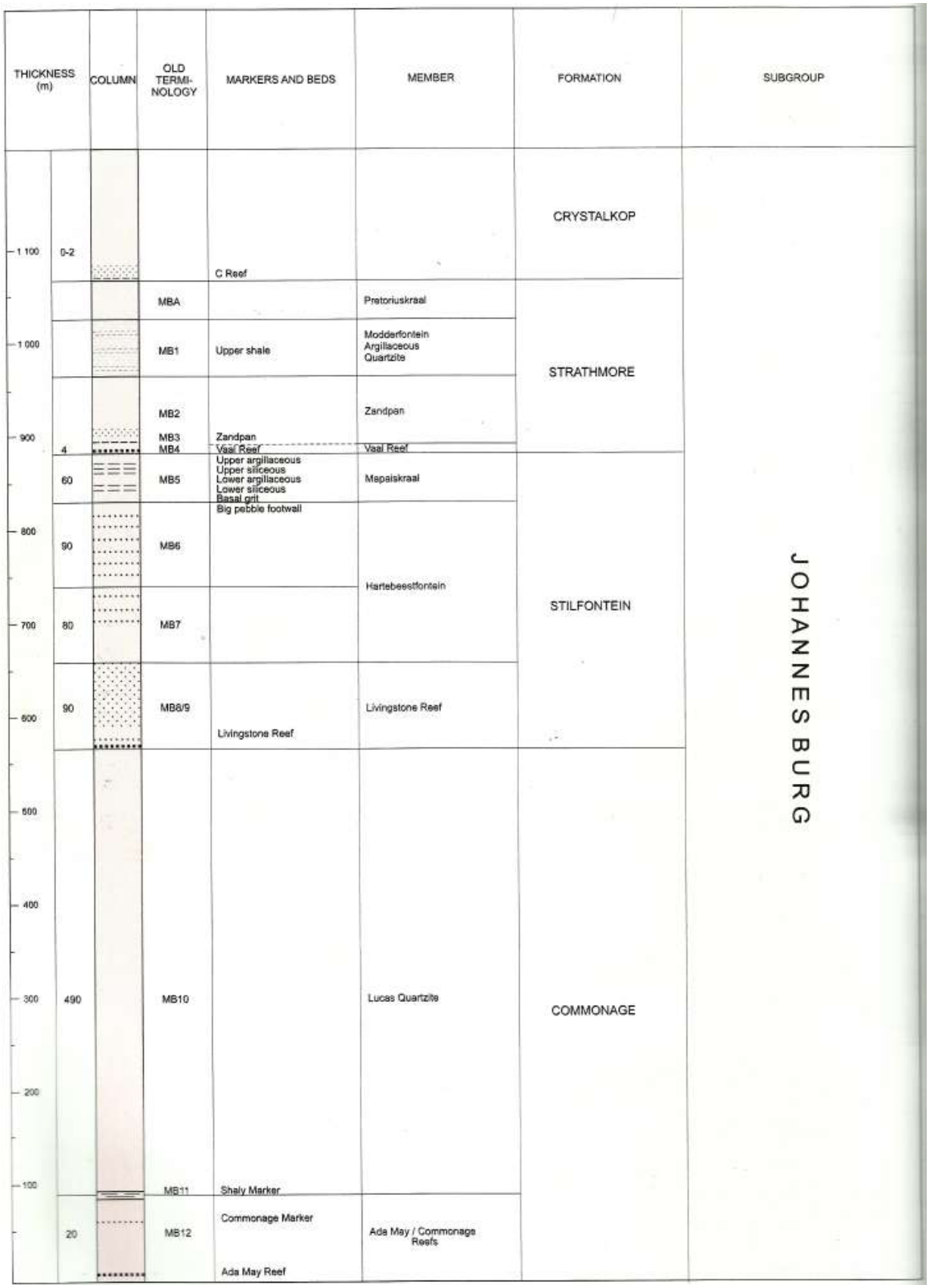


Figure 2.12: A simplified composite stratigraphic column demonstrating the Johannesburg Subgroup in the Klerksdorp goldfield. Illustration taken from Wilson and Anhaeusser (1998).

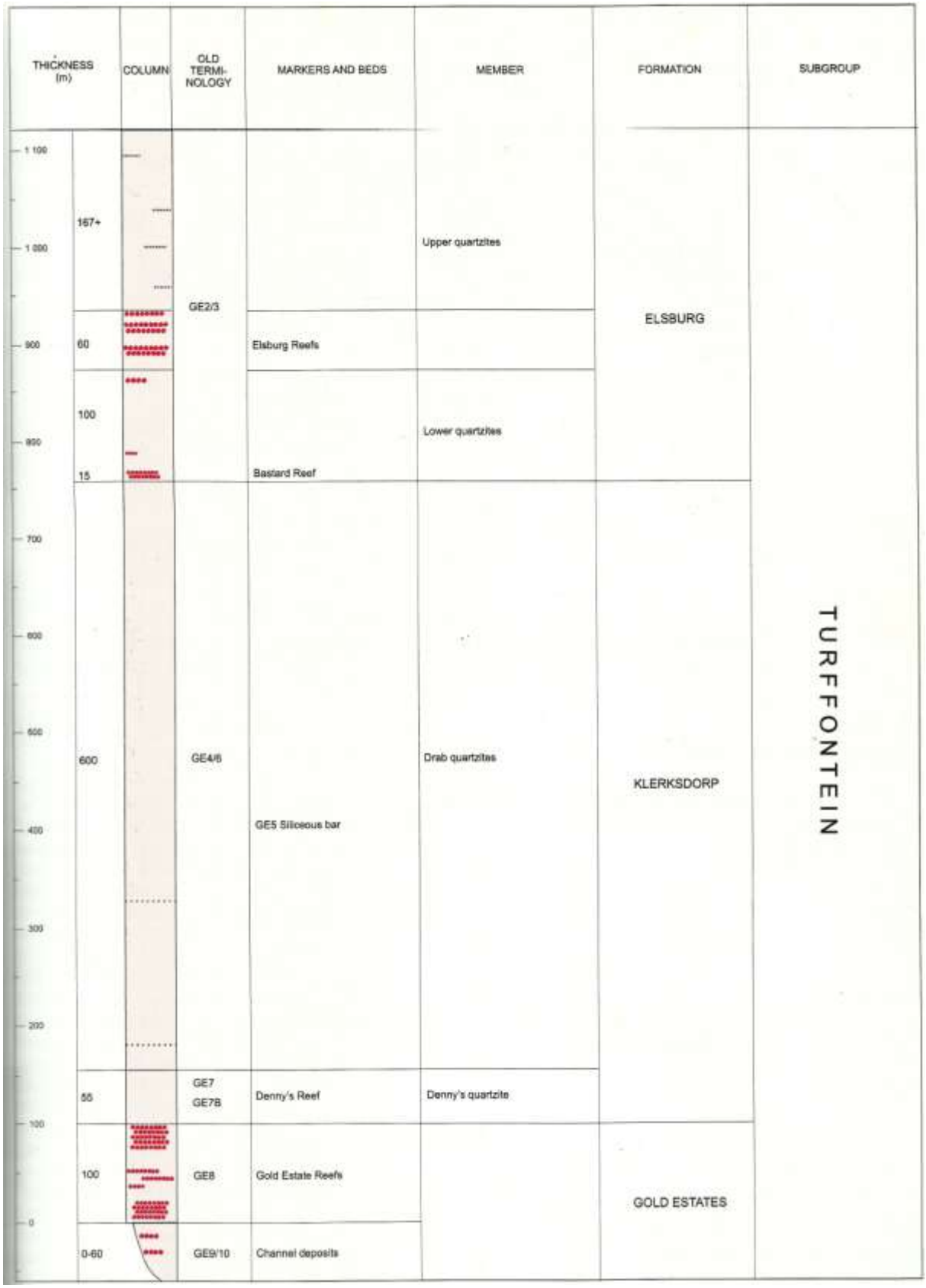


Figure 2.13: A simplified composite stratigraphic column demonstrating the Turfontein Subgroup in the Klerksdorp goldfield. Illustration taken from Wilson and Anhaeusser (1998).

Percolation of groundwater weathers the dolomites and the more impervious chert bands influence the pattern and rate of the weathering. The chert bands in the chert-rich dolomites of the Monte Christo Formation may act as barriers to the percolation of this water down fractures. Deeper weathering and cavities occur where the chert bands are disrupted by faulting. Groundwater infiltration can take place along small faults and fractures in the chert-poor dolomites of the upper Monte Christ and Oaktree Formations. Dolomite pinnacles surrounded by depressions filled in with wad material or transported red sands result from the wreathing of this Formation. The soils are very shallow (Weiersbye, unpublished).

2.3.1 Site topography

The mine lease area of the VR Operations is comprised of flat to gently undulating terrain. This area is situated between 1270 m and 1340 m above sea level. To the northern boundary of this area there is a natural rocky ridge (Black Reef rocks). There are also other manmade structures, for instance, headgears, mine tailings and waste rock dumps that have caused topographical alterations in the area.

2.3.2 Prevailing weather conditions in an area in general

The precipitation records were well documented at Klerksdorp (1950-2000) and Vaal River (1975-2007), demonstrating a mean annual precipitation measured at 598 mm and 657 mm, respectively, although this is prone to variability from year to year. Between May and September an average of only 50 mm rainfall occurs resulting in a well-defined dry season over this period. Between November and February (summer months) roughly 60 % of the annual precipitation occurs, mainly in the form of intense thunder storms (Herbert, 2008). Regarding temperature, the daily minima and maxima are 0 °C (July) and 25 °C (January), respectively (Schulze *et al.*, 2008). Winter occurs from March to August but frost generally occurs from May to August with an estimated 34 frost days per year (Mucina and Rutherford, 2006).

2.3.3 Vaal River Operations flora

The VR Operations area is considered to have a high species richness and diversity containing a total of 412 indigenous plant species and infraspecific taxa which may be due to the study area also being located adjacent to 2 additional vegetation types (Mucina and Rutherford, 2006), specifically the Rand Highveld Grassland and the Klerksdorp Thornveld – although a more recent species count has not been published and 10 years may demonstrate a drastic change/alteration in the floral distribution/status.

Originally the general region of the VR Operations was described as a western variation of Cymbopogon-Themeda grassland (Acocks, 1988), but is currently classified as Dry Highveld Grassland. The Operations fall within vulnerable and endangered vegetation types of high conservation priority in the western Grassland Biome, transitional to Savanna Biome (mean annual precipitation less than 600 mm). Gradients of altitude and moisture provide the basis for the major subdivisions of the Grassland Biome with the higher rainfall regions containing a higher proportion of grasses and the lower rainfall regions containing a higher proportion of deep-rooted shrubs and less grass, as long as groundwater is available.

A grassland is an area dominated by graminoids, usually of the family Poaceae, namely herbaceous vegetation of relatively short and simple structure and although woody plants are not as common as the grasses, they are, however, associated with specific habitats for instance dykes, rocks, hills, kopjies, smaller escarpments and sinkholes. Shrubs and large trees tend to also be associated with run-off and riparian zones where water tends to be more available and/or fire is less common. Although not usually dominant, semi-woody and woody plants (including trees, shrubs, sub-shrubs, forbs and re-sprouters) and geophytes contribute also to the floral diversity of the Grassland Biome than grasses, which means that even though there is a higher density of grasses the major diversity is comprised of non-grasses (Mucina and Rutherford, 2006).

The Dry Highveld Grassland can be subdivided into vegetation units based on environmental and floristic factors. These vegetation units include the Vaal Reef Dolomite Sinkhole Woodland, the

Vaal Vet Sandy Grassland, the Klerksdorp Thornveld (Mucina and Rutherford, 2006), the Black Reef Metallophyte Woodland (Weiersbye and Witkowski, unpublished), the Vaal River Riparian Zone (Hlongwane, 2009) and the tolerant flora of tailings and polluted soils (Weiersbye *et al.*, 2006).

The dominant vegetation unit at the VR Operations is the Vaal Reefs Dolomite Sinkhole Woodland, which is vulnerable and barely conserved, and it is mainly limited to the dolomites of the Malmani Subgroup (Chuniespoort Group and Transvaal Supergroup), where underground dissolution of the rock causes sinkholes. It is located in the North-West and Free State Provinces and small area associated with the sinkholes of the Orkney-Stifontain area, at an altitude of 1280-1380 m. The main vegetation type is the naturally occurring woodland, which aggregate in clumps around sinkholes and dolines and in areas of dolomite outcrops. Important flora taxa include tall shrubs and trees, such as *Vachellia karroo*, *V. erioloba*, *V. robusta* subsp. *clavigera*, *Senegalia caffra*, *Searsia lancea*, *Celtis africana* and *Diospyros lycioides* subsp. *lycioides*. This area was heavily affected by clearing for mining activities and construction of tailings storage facilities (TSFs), however, according to 2008 aerial photographs small regions that were fenced off and have been protected from clearing and fire for years have maintained or increased in woodland biomass (Weiersbye, unpublished; Weiersbye *et al.*, 2006; Witkowski and Weiersbye, 1998a,b).

The Vaal Vet Sandy Grassland is located on sands overlying shales, sandstone and mudstone of the Karroo Supergroup. This grassland occurs in the North-West and Free State Provinces at an altitude 1220-1560 m but mainly at 1260-1360 m. This vegetation unit covers close to half of the VR Operations and is considered to be endangered and a high priority for conservation (Weiersbye, unpublished; Weiersbye *et al.*, 2006; Witkowski and Weiersbye, 1998a,b).

The Klerksdorp Thornveld is located on relatively shallow and rocky soils. The thornveld occurs in the North-West Province at an altitude of 1260-1580 m. This vegetation unit was also heavily affected by clearing for mining activities and construction of TSFs. Woodland dominated by *V. karroo*, *V. erioloba* (to the north) and *Euclea* spp is common on the Ventersdorp lavas bordering

the shallow Black Reef to the west of VR Operations (Weiersbye, unpublished; Weiersbye *et al.*, 2006; Witkowski and Weiersbye, 1998a,b).

With the presence of manganiferous dolomite, wad and shales the native flora is expected to be tolerant to elevated concentrations of manganese (Mn) in the soil and water. A unique community of flora are found on the Black Reef which are characterised by facultative metallophyte species with tolerance to acidic and metal-enriched soil. Due to the uncommon ore deposits on which they grow, metallophyte species can be highly endemic, rare, and vulnerable to mining activities. In contrast facultative metallophyte species are more generally distributed in a region but may conatin locally tolerant ecotypes or varieties. These plants in general are of internationally high conservation and restoration ecology significance and thus the conservation of metallophyte communities is the responsibility of the mining operation (Baker and Whiting, 2008).

2.4 West Wits Operations site

The Far West Rand goldfields stretch from the south of Johannesburg, beyond Carletonville to Orkney in the west and falls within a prominent semi-circular deposit of Transvaal Supergroup rocks. These goldfields represent the southern limb of the asymmetrical Hartbeesfontein-anticline (also known as the Westrand-anticline). Small cracks of Archaean granitoid and Black Reef Formation quartzites outcrop in the crest of the anticline. The southern limb, formed by the Transvaal Supergroup, of the anticline dips 6 degrees to the south. This anticline acts as an important watershed, which separates rivers draining to the north, specifically towards the Limpopo River and finally into the Indian Ocean, from those draining to the south, specifically the Orange and Vaal Rivers finally leading into the Atlantic Ocean. Cross-sectioning the region from north to south shows that the Pretoria Group has been eroded along the edge of the West rand-anitcline, which exposes the Malmani dolomites of the Chunniespoort Group along the Wonderfonteinspruit. Prominent hills (Gantrante) are formed south of the dolomites by the Pretoria Group sediments (Roihoogte and Timeball Hill Formations) and serve as the southern boundary of the Wonderfonteinspruit catchment.

The Ventersdorp Supergroup overlie the Witwatersrand Supergroup quartzites and conglomerates overlie the Archaean granitoids. On the other hand, the Ventersdorp Supergroup is absent locally which results in the Witwatersrand Supergroup rocks being directly overlain by the Black Reef Formation. The Klipriviersberg Group lava is intercalated with the VCR due to the discordances between the Klipriviersberg Group lava and the Witwatersrand Supergroup. West Wits mines exploits the VCR and Carbon Leader Reef (CLR) gold bearing reefs that are present in the Johannesburg and Turffontein Subgroups, respectively.

The Chunniespoort Group dolomites cover a large part of the study area and have a gradational contact with the Black Reef Formation at the base of the Transvaal Supergroup. The irregularity of the palaeo-landscape causes the Black Reef Formation to be absent locally. The Malmani Subgroup dolomite is inter-layered with chert bands (30 cm to several metres), varies in thickness from 1.2 km to 1.45 km and is overlain by the Pretoria Group sedimentary rocks. The different formations vary considerably in the chert content. It is noteworthy to mention that there is a direct relationship between the dolomite-chert content and the sinkhole-formation potential, with sinkhole-formation most likely occurring from more chert-rich units (Department of Minerals and Energy, 2008).

Karoo Supergroup sediments are generally preserved in deeper palaeo-karst troughs and occur as outliers in the dolomite. At the base of the Karoo Supergroup a thin tillite layer occurs with some inter-layered carboniferous shales. Towards the base of the Malmani dolomites, intrusion of the diabase sills related to the intrusion of the Bushveld Complex occurs. Pilanesberg age dykes have intruded the dolomites and are predominantly striking from the north to south. The dykes, being impervious syenite and composite, are roughly 50 m wide and spaced 5-15 km apart, result in the compartmentalising of the dolomitic catchments into several groundwater compartments.

2.4.1 Site topography

The Gatsrand (a prominent rocky ridge) extends across the WW Operation from east to west forming a watershed. Mine headgears and TSFs have been erected with the start of mining and now interrupt/disrupt/cut the horizon. The TSFs also affect local drainage patterns. The mine

lease area of the WW operations is divided into a northern (in the Gauteng province) and southern (in the North-West Province) portion by the Gatsrand. A total of 1460 ha (roughly 34.96-38.57 %) is made up of transformed land presently in use by mining related infrastructure, for instance mining plants, shafts, headgears and related operations TSFs, residential areas and excavations.

The northern portion of the mine lease area is generally comprised of gently sloping midslopes and footslopes which are largely transformed by mining activities (roughly 85 %) and a history of agriculture (croplands). The central and southern portions of the mine lease area are generally comprised of slightly steep to steep rocky crests, midslopes and footslopes. The central portion (plateau), although overgrazed in regions, is mainly (roughly 80 %) still in the natural state. Only one third of the southern portion is still in the natural state with the rest having been transformed by a history of mining activities and a history of agriculture (croplands).

2.4.2 Prevailing weather conditions in an area in general

According (as reported by) to the West Wits Internal Records (1995-2008) the AngloGold Ashanti Limited West Wits Operations is located in a summer rainfall area and demonstrate a mean annual precipitation of 725 mm, which is higher than VR Operations by roughly 97.5 mm. The rainfall over this area, much like Vaal River Operations, also tends to be in the form of thunderstorms as well as showers. This happens during the summer months from October to April (different to being between November and February of VR Operations) and the maximum occurring/happening in January. Between May and September an average of only 64 mm rainfall occurs resulting in a well-defined dry season over this period. As well as being the wettest month January is also the warmest month and July is the coldest month (Herbert, 2008). Winter occurs from March to August but frost generally occurs from May to August with an estimated 37 frost days per year (Mucina and Rutherford, 2006).

2.4.3 West Wits Operations flora

Floral surveys, and their regeneration by seed, were performed for roughly 24 000 ha polluted soils and TSFs (Weiersbye and Witkowski, 2003; Weiersbye *et al.*, 2006; Witkowski and Weiersbye, 1998a,b), plants of the naturally metal-rich environments (exposed reefs) and of phreatophyte trees on polluted groundwater (Weiersbye and Witkowski, 2003 and Weiersbye and Witkowski, 2007). On the TSFs and polluted soils alone, close to 200 plant species were recorded (Weiersbye *et al.*, 2006).

Mucina and Rutherford (2006) reported 3 distinct vegetation regions, namely, Gauteng shale mountain bushveld, Carltonville dolomite grassland and Rand Highveld grassland. The Gauteng shale mountain bushveld is located in the Gauteng and North-West Provinces, at an altitude of 1300-1750 m and is comprised of short (3-6 m), semi-open thicket dominated by a variety of woody plant species with the understorey dominated by a variety of grasses. The Carletonville dolomite grassland is located mainly in the North-West Province and a little in the Gauteng Province, at an altitude of 1360-1620 m, but mainly 1500-1560 m and is comprised of species-rich grasslands forming a complex mosaic pattern. The Rand Highveld Grassland is located in Gauteng, North-West, Free State and Mpumalanga Provinces, at 1300-1635 m but 1760 m in places and is comprised of wiry, sour, species-rich grassland alternating with low, sour shrubland on rocky outcrops and steeper slopes. All vegetation regions are considered to be vulnerable when considering their conservation status.

2.5 Phosphorus and nitrogen cycles

Essentially nutrient cycles involve the cyclic transfer of elements, such as phosphorus, nitrogen, magnesium, calcium and potassium, between non-living and living components of the ecosystem specifically nutrient cycles involve the process whereby nutrients (elements) arising from minerals are absorbed by organisms, for instance plants, to form living tissue, which then die and decompose, returning the nutrients to the ecosystem so that they may be reabsorbed (Eash *et al.*, 2008; Lawrence, 2005). Since mycorrhiza and rhizobia are key organisms in the tripartite

symbiotic association with legumes (in this case *V. karroo*) their key elements (cycles) will be discussed, namely the phosphorus (P) cycle for mycorrhizas and nitrogen (N) for the rhizobia.

2.5.1 Phosphorus (P) cycle

P is a constituent of every living cell and is constantly being transported throughout the entire plant, although it is generally concentrated in the growing points in plants and in seeds (Thompson and Troeh, 1978; Townsend, 1973). P plays important roles in metabolic processes enabling plant growth and is vital for a plant to complete its life cycle (Thompson and Troeh, 1978; Townsend, 1973). P mainly occurs as organic esters in plants where P is bonded to carbon and oxygen (Rowell, 1994). P absorbed by plants tends be in the form of orthophosphate, specifically dihydrogen orthophosphate ion ($H_2PO_4^-$) as it is the most efficient carrier. However, P can be absorbed in other forms especially in a few organic combinations, for instance lecithin and phytic acid may operate as direct P providers as well as nucleic acids (contain P) being able to be decomposed at root hair surfaces enzymatically forming inorganic phosphates (Townsend, 1973).

P supplied in applied fertiliser has very poor recovery by plants and it is likely that more than 85 % of the phosphorus added is not recoverable (Townsend, 1973). Soil has the ability to retain phosphate ions, called phosphate fixation, which is economically problematic. This ability is not limited to any one type of soil and occurs in all soil types at any pH (Townsend, 1978).

Most common form of P in the soil is apatite (mainly as fluorapatite, $Ca_5(PO_4)_3F$, but also as chlorapatite, $Ca_5(PO_4)_3Cl$ and hydroxyapatite, $Ca_5(PO_4)OH$), which is a near insoluble compound made up of calcium and P. Apatite is derived from igneous parent rock. Due to apatite's low solubility it is not readily available to plants, which require P to be in solution, hence it is thought to be of little value as an immediate nutrient compound (Rowell, 1994; Thomspson and Troeh, 1978; Townsend, 1973).

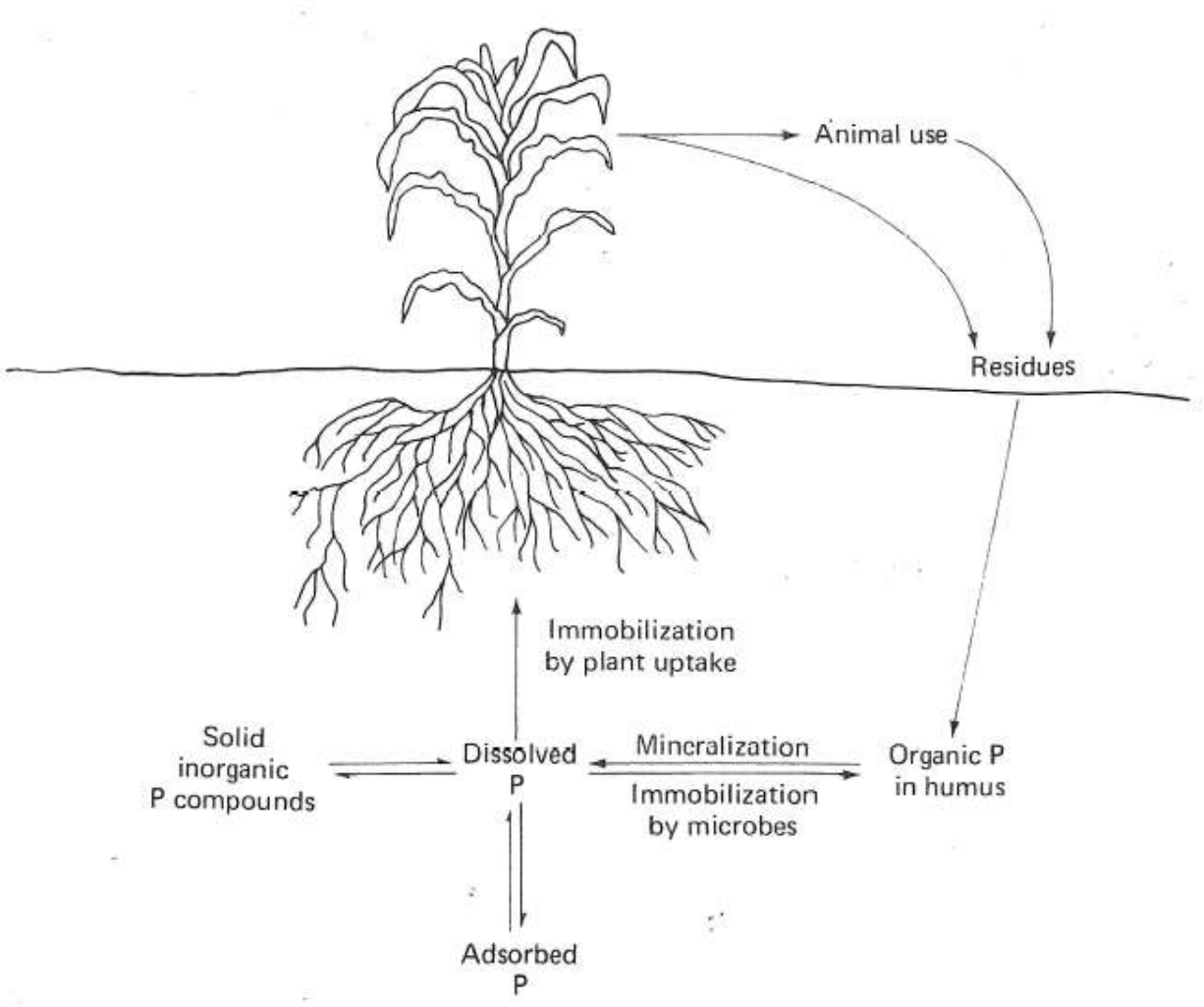


Figure 2.14: The phosphorus cycle (Thompson and Troeh, 1978).

The P cycle (Figure 2.14) doesn't involve atmospheric changes and thus appears simpler than the N cycle. Unlike the N cycle, inorganic phosphate ions bind to positively charged sites of soil organic matter and soil clays (Thompson and Troeh, 1978).

The bound P and the additional solid forms of P are in equilibrium with the dissolved P and thus with each other. It is suspected that P in solution is where plants obtain most or all of their P. It is noteworthy to mention that the quantity of dissolved P, occurring mostly as $H_2PO_4^-$ and HPO_4^{2-}, in soil is very low, for instance in most soils there is less than 1 kg in 2 million kg of soil (hectare-furrow slice) and in some soils there is less than 0.1 kg per 2 million kg of soil. It has been reported that crops use from 10 to 30 kg of P per ha per year and from this it is apparent that the P content in the soil solution must be replaced frequently. The Figure illustrates how P

may be replaced either through equilibrium reactions with bound phosphate ions and solid P compounds or mineralisation of organic matter (Thompson and Troeh, 1978).

Sufficient plant growth can be made with a very low concentration of dissolved P as long as that concentration is sustained. Generally the rate of creating available P, through the equilibria and organic matter mineralisation, is sufficiently rapid to equal plant requirements (Olsen and Watanabe, 1966), however, transfer and/or transport of the P can be a constraint (Thompson and Troeh, 1978), although mycorrhiza assist in this regard (Rowell, 1994). In order for the plants to absorb the P the $H_2PO_4^-$ and HPO_4^{2-} must be transported from the site where they are introduced into the solution to a plant root. Albeit some P is absorbed directly from the soil by growing roots, and a minimal quantity of this is transferred to the roots by mass flow as the plant absorbs water, while the majority of the flow is through ion diffusion. However, decreasing soil moisture content or distances from the root surface between 5-10 mm are factors which limit P uptake through diffusion. Less soluble P compounds have a slower effective transport rate even at short distances; therefore in order for these compounds to be effective sources of P they must be well distributed throughout the soil (Thompson and Troeh, 1978).

It is important to note that plant uptake of P is effected by the availability of N. It was determined that including even a small amount of N in a P fertiliser causes it to be more effective (Miller, 1965). The improved effectiveness may be due to a physiological process in the plant but the mechanism is not known. P absorption by the plant may be stimulated with as low a ratio as 1 in 6 parts of N to P added (Thompson and Troeh, 1978).

Microbial enzymes hydrolise P from various organic P-esters, from phytic acid and also from reactive inorganic P. However, most of soil P cannot ionize away from the organic compounds in the initial steps of decomposition as it is built into the structure of these compounds, and its mineralisation follows the microbial decomposition of organic matter, which frequently occurs at a slow rate (Thompson and Troeh, 1978).

Two types of indirect influence are imposed on P availability by organic matter. Organic matter complexes and adsorbs iron and aluminium ions, which results in less of these being in solution

to precipitate out insoluble P compounds, especially in acidic soils (this is problematic with acid mine drainage). Alternatively, calcium phosphates' solubility is improved when acids are released through organic matter decomposition. From this it can be seen that the amount of P in soils increases with the presence of large amounts of organic matter. It is therefore advised that application of P fertilisers to soil is done with organic matter in order to improve P availability (Rowell, 1994; Thompson and Troeh, 1978; Townsend, 1973).

2.5.2 Nitrogen (N) cycle

N acts as a vital macronutrient as it is a constituent in amino acids and nucleic acids (Lawrence, 2005) and in the case of plants, cyanobacteria and algae, chlorophyll (Thompson and Troeh, 1978). Lack of N leads to physiological changes, such as plants appearing stunted and spindly and having pale leaves and stems as well as the cessation of plant growth and reproduction (Thompson and Troeh, 1978; Townsend, 1973). Rate of N absorption by plants is dependent on their metabolic activity with younger plants generally having a higher N uptake than older plants. This is due to the fact that N must be available to the plant prior to growth as growth is unable to be advanced over N uptake as N must be available to make new cells (Thompson and Troeh, 1978).

N in the soil mainly originates from the biological fixation of atmospheric N_2, that convert gaseous N into NH4-N that can be utilised by plants for forming amino acids and N-containing essential biological molecules (Eash *et al.*, 2008; Rowell, 1994). N in soil organic matter is mainly of proteinaceous nature and is decomposed and mineralised by soil microorganisms by the action of hydrolytic extracellular enzymes (Mylona *et al.*, 1995).

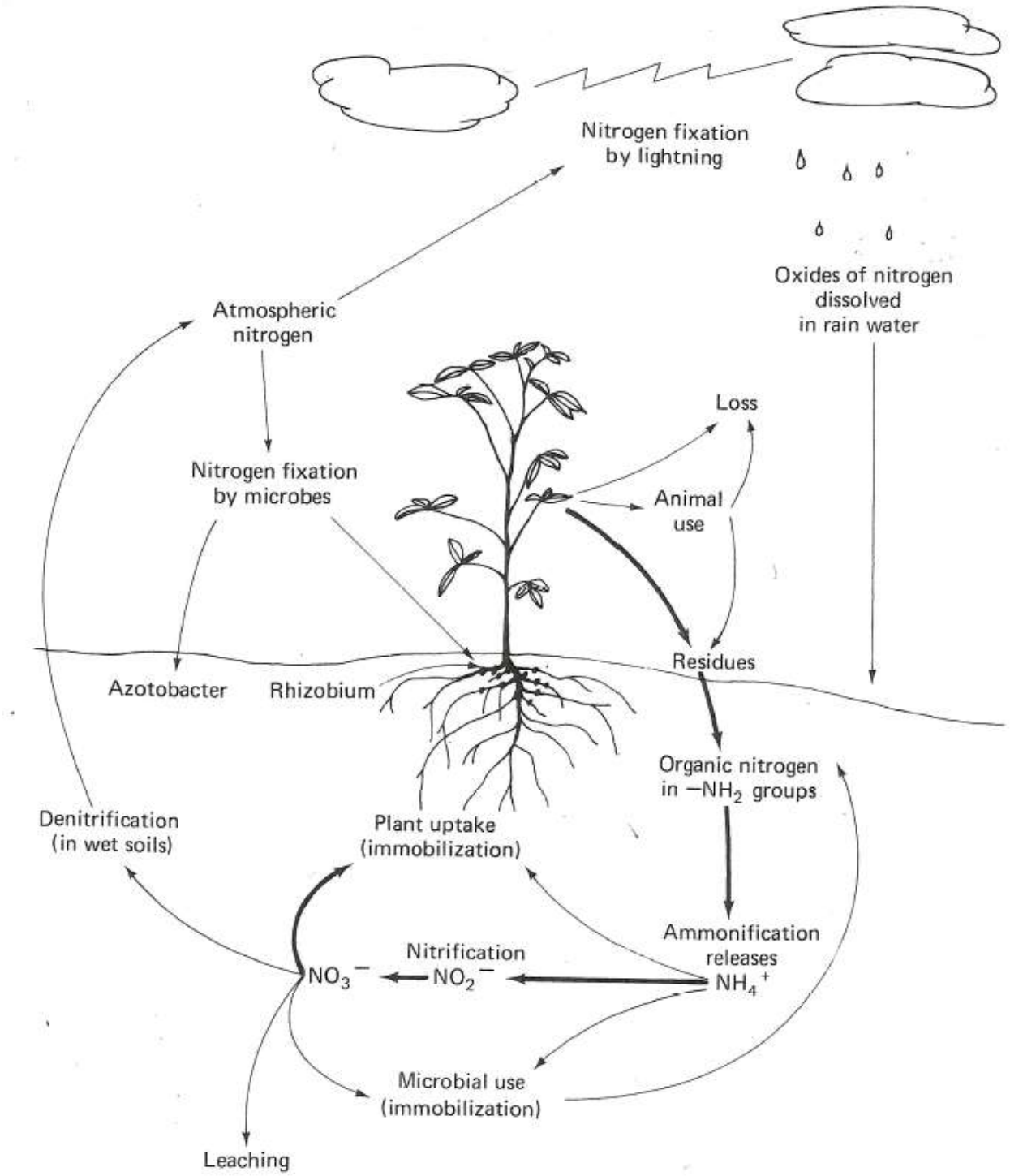


Figure 2.15: The nitrogen cycle with the darker lines illustrating the primary cycle of mineralisation and immobilisation (Thompson and Troeh, 1978).

N fixation is the process whereby N is combined with another element and is naturally achieved by lightning and specific microorganisms (Eash *et al.*, 2008; Rowell, 1994). However, generally the N quantity fixed is rarely sufficient for plant use (Eash *et al.*, 2008). Biological N fixation is

performed by diazotrophs (organisms that have the ability to convert (reduce) elemental N into ammonia (NH_4^+) using the nitrogenase enzyme system) (Lawrence, 2005; Mylone *et al.*, 1995; Oldroyd *et al.*, 2011; Sulieman and Tran, 2014; Udvardi and Poole, 2013). The nitrogenase enzyme complex is a metalloenzyme with the most common complex containing molybdenum, although other nitrogenase enzymes may contain vanadium or iron (Lawrence, 2005). The N reduction reaction is an extremely oxygen-sensitive process and the most effective N fixers involve symbiotic relationships with higher plants as the plant host provides energy requirements for N fixation as well as in some cases providing the oxygen protection system (Mylone *et al.*, 1995; Sulieman and Tran, 2014). Biological N fixation may also be performed by non-symbiotic (by free living prokaryotes) or associative prokaryotes (Eash *et al.*, 2008).

Prokaryotes in the symbiotic relationships include rhizobial bacteria with legumes, *Frankia* bacteria with actinorhizal plants and *Nostoc* (cyanobacterium) with *Gunnera* (Mylona *et al.*, 1995). At the time of the symbionts infection, root nodules (new organs), are created by the host plant in the case of legumes and actinorhizol plants and in the case of *Gunnera* the symbionts inhabit already existing stem glands. Another instance where symbiotic N fixation takes place is the association of actinomycetes with some woody plants such as sweet fern, alder and Russian olive (Eash *et al.*, 2008). In all cases the bacteria reduce N within the cells of their respective hosts, however, host derived membranes separate the bacteria from the plant plasmalemma (Mylona *et al.*, 1995).

Non-symbiotic N fixation is carried out by a variety of prokaryotes including those from the genera *Azotobacter* and *Clostridium* (Delwiche and Wijler, 1956; Eash *et al.*, 2008; Jensen, 1940, 1941; Jensen and Swaby, 1941; Schlegel, 1992; Townsend, 1973). Other taxa, including anaerobic and aerobic heterotrophic bacteria, were identified in a study by Dart and Wani (1982). More specifically, isolates identified include *Azospirillum brasilense*, *Azospirillum lipoferum*, *Azospirillum vinelandii*, *Azospirillum* spp., *Bacillus polymyxa*, *Bacillus* spp., *Enterobacter cloacae*, *Enterobacter* spp., *Klebsiella pneumonia*, and *Erwinia herbicola* (generally considered a plant pathogen). Other organisms with N fixation abilities have been revealed to include anaerobic phototrophic bacteria, facultative anaerobes, cyanobacteria, methanogenic and desulphurising bacteria, and methylotrophic and autotrophic bacteria (Schlegel, 1992).

Regarding N fixation by *Azotobacter* the N only becomes available to plants once the bacterium dies (Eash *et al.*, 2008). *Azobacter* also fixes 4-5-fold more N than *Clostridium* (Townsend, 1973).

Mineralisation, specifically ammonification, involves the microbiological break down/decomposition of organic materials, such as plant residue, into inorganic N (Eash *et al.*, 2008). The decomposition product, ammonia (NH_3), is ionised to form ammonium ions (NH_4^+), which can be taken up by plants but generally undergoes nitrification in the soil when moist and warm (Eash *et al.*, 2008; Townsend, 1973).

Nitrification occurs only if the soil is warm and moisture and oxygen is well provided. Nitrification involves the oxidation of ammonium ions into nitrite (NO_2^-) by the *Nitrosomonas* and *Nitrococcus* bacteria. Nitrite is then oxidised further by *Nitrobacter* into nitrate (NO_3^-), which is N's highest oxidation state. In this state N is highly soluble and is thus prone to leaching, which can lead to groundwater contamination (Eash *et al.*, 2008; Townsend, 1973). It is noteworthy to mention that nitrite levels in soils are generally much lower than the levels of ammonia with N oxidation by *Nitrobacter* being more rapid than initial oxidation by *Nitrosomonas* and *Nitrococcus* and finally the end product, nitrate, has the highest concentration (Townsend, 1973).

The transformation of inorganic N, mainly from ammonium and some from nitrate, into organic N, into amino acids and proteins in microbes and plants, is referred to as N immobilisation. Post nitrification, microorganisms, decomposing organic residues, or higher plants may take up the nitrate. Nitrification is responsible for supplying inorganic N in a form suitable (nitrate or ammonium) for plant nutrition. Unfortunately, after nitrification microorganisms may become overabundant and thus outcompete vegetation for any available nitrate or ammonium. This could result in vegetation becoming N deficient if the soil inorganic N residue becomes too low (Eash *et al.*, 2008).

Under anaerobic conditions nitrate may undergo reduction returning N to the atmosphere through the process of denitrification whereby N is volatilized (Eash *et al.*, 2008). Under anaerobic

conditions and in saturated soils, microorganisms thrive through this process by nitrate ions as an alternative terminal electron acceptor in the process of oxidative phosphorylation (or similar oxides) (Eash *et al.*, 2008; Townsend, 1973). This can be avoided by improving soil drainage and by carefully timing N fertiliser application (Eash *et al.*, 2008).

2.6 Trace elements

Trace elements (TEs) are elements that occur as minute quantities in the environment and tissues and as nutrients for organisms they are only required in small quantities. Generally TEs are utilised as prosthetic groups in certain proteins, including iron, zinc, copper, manganese, molybdenum, nickel, cobalt, boron, chromium, selenium, vanadium, tungsten; however, not all of these elements are needed by all organisms (Hooda, 2010; Lawerence, 2005; Townsend, 1973). "TE" is a convenient term since it includes non-metals, metalloids, metals and other elements present in the soil-plant-animal system, however, it may be too vague due to it including any element whilst ignoring its function (Hooda, 2010).

In some cases TEs have been referred to as trace metals, toxic metals or heavy metals, however, not all TEs are metals (Hooda, 2010). Similarly the term "toxic metals" is also incorrect as the elements only become biologically toxic once they reach the excess threshold level. In order to improve clarity some researchers use the term "potentially toxic trace elements" (PTEs), which is more inclusive than toxic metals or heavy metals (Hooda, 2010).

Current TEs in soils are derived from multiple sources (Kabata-Pendias, 2011). TEs derived directly from parent materials (the lithosphere) are considered to be lithogenic elements. TEs derived from the lithosphere and then undergo distribution and concentration alterations in the soil layers and particles through pedogenic process are considered to be pedogenic elements. TEs resulting from human (anthropogenic) activities are considered to be anthropogenic elements. Studies have ascertained that anthropogenic metals in the soil are significantly more phytoavailable than metals arising from pedogenic processes (Chlopecka, 1993; Kabata-Pendias and Pendias, 1999). From this it can be suggested that soils under similar conditions, both the

lithogenic and pedogenic TEs will have a lower mobility and bioavailability than anthropogenic elements (Kabata-Pendias, 2011).

PTEs in soil is a major concern as soil acts as a geochemical sink for contaminants and as a natural buffer that influences and controls the transfer of chemical elements and substances to biota, the atmosphere and hydrosphere (Kabata-Pendias, 2011). Soil pollution and soil contamination may have different definitions depending on the research and publication. Soil pollution, in accordance with Knox *et al.* (1999), occurs when a substance or an element is present at larger concentrations than natural/normal concentrations due to anthropogenic activities and the resulting effects are detrimental to the environment and its ecosystem. Soil contamination is when the chemical state of the soil deviates from the natural/normal composition but does not result in detrimental effects of the environment and its ecosystem (Knox *et al.*, 1999). Hence from a biological perspective, soils are only considered to be polluted once a threshold concentration arises so that biological processes are affected (Kabata-Pendias, 2011).

The final outcome of PTEs in the soil greatly depends on the soil physical and chemical properties as well as on the PTEs speciation (Kabata-Pendias, 2011). For this reason it is extremely important that soil characteristics are examined.

2.7 Methods of soil analysis

First step in soil analysis is the collection of samples, although initial analyses may be performed at the site, for instance in the case of soil water studies (Rowell, 1994). After soil samples are collected the rest of the analysis may be performed in the laboratory. The analyses following this depend on the specifics of the research outcome, which allows for specific analyses to be irrelevant in certain studies. An example of this is attempting to determine and follow the process of soil formation and its parent material origin, which may be trivial when attempting to analyse the specific microbial content of a soil sample. In this case knowing the soil properties and its elemental content could be considered more appropriate.

Physical properties of soil are those that can be observed visually (structure and colour) or through touch (its texture) and are difficult to alter (Eash *et al.*, 2008). Chemical properties of soil are neither seen nor felt and are analysed with the use of equipment that can monitor electrical charge (positive and negative charges in the case of cations and anions, respectively) (Eash *et al.*, 2008). Specific chemical analyses include cation exchange capacity, conductivity/resistance and pH (Carter, 1993; Eash *et al.*, 2008; Rowell, 1994). The method of soil pH and the reliability of the results depend on the soil type and use (Carter, 1993); however, pH is consistently measured in solution as all reactions in soil occur in an aqueous environment (Townsend, 1973). Soil pH measured with water has the highest similarity to pH of the soil in the field (this being true in unfertilised soils and soils with low electrical conductivity); however, this is susceptible to change with the degree of dilution. Increasing the volume of water added to soil increases the pH and should be kept as low and as consistent as possible. Other pH analyses include 1M KCl and 0.01 M $CaCl_2$ (Carter, 1993).

Besides analysing the physical properties of soil it is important to ascertain the TEs. Techniques for the analysis of TEs in soils may either be destructive or non-destructive. Destructive analyses involve a preliminary dissolution step whereby elements are discharged from the solid soil phase usually with the use of strong acids and/or an external heat sources that are able to decompose the soil matrix. Following this the elements are determined using instrumental analytical techniques (Hooda, 2010). In some cases the decomposed matrix is passed through a filter to remove residues. The choice of acid and whether or not to include heating is dependent on the matrix nature, the elements to be analysed and on the acid characteristics. Destructive elemental analyses include inductively coupled plasma-atomic emission spectrometry (ICP-AES), inductively coupled plasma-mass spectrometry (ICP-MS), flame atomic absorption spectrometry (F-AAS), graphite furnace or thermoelectric atomic absorption spectrometry (GF-AAS, ET-AAS), hydride generation-AAS (HG-AAS), cold vapour-AAS (CV-AAS), anodic stripping voltammetry (ASV) and neutron activation analysis (NAA) (Hooda, 2010).

Non-destructive analyses involve direct measurements being made on the soil sample. These analyses are uncomplicated with no difficulties with such things as preparation/digestion of the soil sample and separation (contamination, losses and dissolution) is avoided (Hooda, 2010).

Specific non-destructive analyses include X-ray fluorescence (XRF) spectroscopy, particle-induced X-ray emission (PIXE) spectroscopy and instrumental neutron activation analysis (INAA) (Hooda, 2010). INAA is an alternative to NAA where no decomposition of the soil sample is required, however, this method is not sensitive enough for low concentrations when attempting to analyse certain environmentally vital elements, such as cadmium (Cd), copper, (Cu), nickel (Ni) and lead (Pb) and it is also time consuming.

The objective of performing soil analyses is to determine accurate and reliable data about a specific soil (Carter, 1993). Although samples collected are analysed in order to reveal information about a large area/site not all samples reliably represent the whole area (Carter, 1993). For this reason multiple samples are collected randomly in order to reduce error and bias.

2.7.1 Phosphorus soil analysis

Various methods have been developed to quantify total P (Olsen and Sommers, 1982). Among them, the most commonly used are those based on the Na_2CO_3 fusion (Sommers and Nelson, 1972) and acid (HF, $HClO_4$, H_2SO_4) digestion (Syers *et al*., 1967, 1968), with the alkaline fusion method being generally more efficient. Various methods have also been established for analysing the soil phytoavailable P content, as no single technique being appropriate in all cases. Leaching out available P is challenging and in the case of acidic soils a dilute-acid is used as an extracting solution and in the case of alkaline soils an alkaline solution is used (Thompson and Troeh, 1978). The most commonly used extraction method is the Bray no. 1, based on soil extraction iwht the use of 0.025N HCl and 0.03N NH_4F, where HCl solubilise P into soil solution NH_4F complexes the soluble Fe and Al preventing their precipitation (Thompson and Troeh, 1978). The Bray no. 1 is efficient for young acid or neutral soils, and the the Bray no. 2 (0.1 N HCl plus NH_4F) is more efficient on mature acidic soils but produces high P values for calcareous soils or in soils amended with rock phosphate. For alkaline soils, the most efficient methods of analysis is that of Olsen (Olsen and Sommers, 1982), based on the use of 0.5 M $NaHCO_3$ at a pH of 8.5, as this extractant decrease Ca in solution and enhances the dissolution of P by Ca-phosphate Fe oxidises. Other extractable P pools can be quantified by the Mehlich 1 and 3 methods based on more complex acidic mixtures (Mehlich, 1984; Nelson and Mehlich; Sims, 1989).

2.7.2 Cation exchange capacity (CEC) analysis

CEC is an important characteristic in humus, silicate clay minerals and allophone with each of these material binding cations due to their negative charges. The cations are considered exchangeable if they can be swapped with other dissolved cations. This is only possible when the bonds are not excessively strong and if the bonding sites are available to the soil solution.

Soil clay CEC is partially dependent on pH, with the CEC being lower in acidic soils and increasing as pH increases. This change is likely due to the large supply of H^+ ions in acids changing several exposed O^{2-} ions to OH^- ions which reduces the CEC (Thompson and Troeh, 1978).

CEC of soil plays an important part in the potential soil fertility (Carter, 1993; Thompson and Troeh, 1978). Measuring CEC is based on replacing the cations present with another cation thereby demonstrating the exchangeable cations. Possible exchangeable cations include potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^+) and sometimes aluminium (Al^{3+}). An examples of the procedure involves leaching a soil sample of known weight with a surplus solution of ammonium (NH_4^+) acetate (NH_4OAc) resulting in ammonium ions occupying all the negative sites. The ammonia ions are then replaced with another ion (rinsed with alcohol) and the released ions are then measured as the representative CEC (Eash *et al.*, 2008; Thompson and Troeh, 1978). When using neutral 1 M ammonium acetate solution several cations are extracted from the soil solution, however, K, Mg and Ca are considered to be the "plant-available" cations (Carter, 1993).

2.7.3 X-ray fluorescence (XRF) analysis

XRF spectroscopy uses a technique whereby an inner shell electron of an atom is ejected and the gap is filled by an electron from a higher shell. An X-ray photon (energy) is released from this which equals the energy difference between the 2 shells. The radiations with intensities and wavelengths makes up an X-ray spectrum with characteristics that match the atoms and their concentrations present in the sample. The state of the elements or the chemical combination in

the sample only slightly affects the spectrum. In the case of XRF the inner-shell electrons are ejected when high-energy X-ray photons are directed at the sample (Hooda, 2010).

XRF is superior to PIXE in certain aspects due to it lacking the requirement for an expensive particle accelerator and not having interference and background issues (Pillay, 2000). XRF has a greater ultimate sensitivity than PIXE (Hooda, 2010).

No major difficulties are presented when XRF is used for semiquantitative analysis, however, the quantitative analysis is not entirely simple as a large fraction of the radiation emitted from the outer surface of the sample is reabsorbed before being released from the sample (Rouessae and Rouessae, 2007). Not to mention the measured concentration may not be a representative of the overall sample if the surface is not a true representative and/or is heterogenous to the rest of the sample. This may also be true due to the sample not being prepared prior analysis by either pellet formation or fusion (Hooda, 2010). Another influence on the XRF results is the water content of the soil with the presence of water decreasing the XRF intensity, particularly regarding low-energy X-rays (Imanishi *et al.*, 2010). Various versions of XRF exist and are currently thought of as a robust, mature and well established technique for environmental analysis. An additional advantage of the XRF is that the technique has been adapted for portable use (Hooda, 2010).

2.8 Methods and materials

2.8.1 Sample sites and *Vachellia* trees sampled

The two mine tailing sites utilised in the study (Figures 2.16-2.18) were VR and WW Operations. From each niche site 3 trees were sampled in the vicinity of the mine tailings at varying distances and for each control 2 trees were sampled. Briefly the revegetation strategies and construction of each niche site will be outlined, however, it is noteworthy to mention that all sites at VR and WW operations have received vast quantities of tailings dust over the last 80 years and are thus being progressively acidified and salinised by the acid mine drainage and the tailings/tailings dust (I. W. Weiersbye, *pers. comm.*).

2.8.1.1 Vaal River Operations

Madala site (approximately 12044-16516 m from controls): comprised of acid mine drainage polluted soil which resulted from old tailings spills that were scraped off (from the adjacent West Complex TSF) and was developed through the planting of vegetation. Figure 2.17, points 1-3.
Black Reef site (approximately 11759-16231 m from controls): comprised of acid mine drainage polluted soil (from the adjacent old mined reef) although no tailings spills occurred and was developed through the natural establishment and growth of vegetation. Figure 2.17, points 4-6.
East Pay Dam site (approximately 9116-13588 m from controls): land/soil that was originally covered by the East Pay Dam TSF that was scraped off and is thus acid mine drainage polluted soil and was developed through the planting of vegetation. Figure 2.17, points 7-9.
R. G. Williams site (approximately 13298-17770 m from controls): comprised of acid mine drainage polluted soil (from the up-gradient TSFs) although no tailings spills occurred and was developed through the natural establishment and growth of vegetation (I. W. Weiersbye, *pers. comm.*). Figure 2.17, points 10-12.

2.8.1.2 West Wits Operations

Tailing Storage Facilities lower slope, over soil, retaining wall (approximately 2652-2860 m from controls): comprised of acid mine drainage polluted soil (from the adjacent TSF) mixed with old tailings and was developed through the planting of vegetation. Figure 2.18, points 1-3.
Toe-Paddock West Wits Old North Tailings Storage Facilities North Toe Paddock – Eastern Boundary (approximately 4974-5182 m from controls): comprised of acid mine drainage polluted soil which resulted from old tailings spills that have not yet been scraped off (from the adjacent TSF) and was developed through the natural establishment and growth of vegetation. Figure 2.18, points 4-6.
Toe-Paddock West Wits Old North Tailings Storage Facilities North Toe Paddock North-Eastern Boundary (approximately 5194-5402 m from controls): comprised of acid mine drainage polluted soil which resulted from old tailings spills that have not yet been scraped off (from the adjacent TSF) and was developed through the natural establishment and growth of vegetation. Figure 2.18, points 7-9.

Varkenslaagte Woodlands Blocks site (approximately 3791-3999 m from controls): comprised of acid mine drainage polluted soil which resulted from old tailings spills that were scraped off (from the adjacent West Complex TSF) and was developed through the planting of vegetation (I. W. Weiersbye, *pers. Comm.*). Figure 2.18, points 10-12.

2.8.2 Sampling strategy

Root and soil samples were collected from areas around TSFs found in the VR and WW Operations (Figure 2.16). Sampling was performed by digging up complete root systems as well as sections of root systems that provided a representative of the complete root system (fine and course roots) of *Vachellia karroo* with immediately surrounding soil. Three plants were sampled from each niche site from around MTFs and Figures 2.17 and 2.18 show the exact sites of sampling. The GPS co-ordinates were recorded for each tree (Appendix 1).

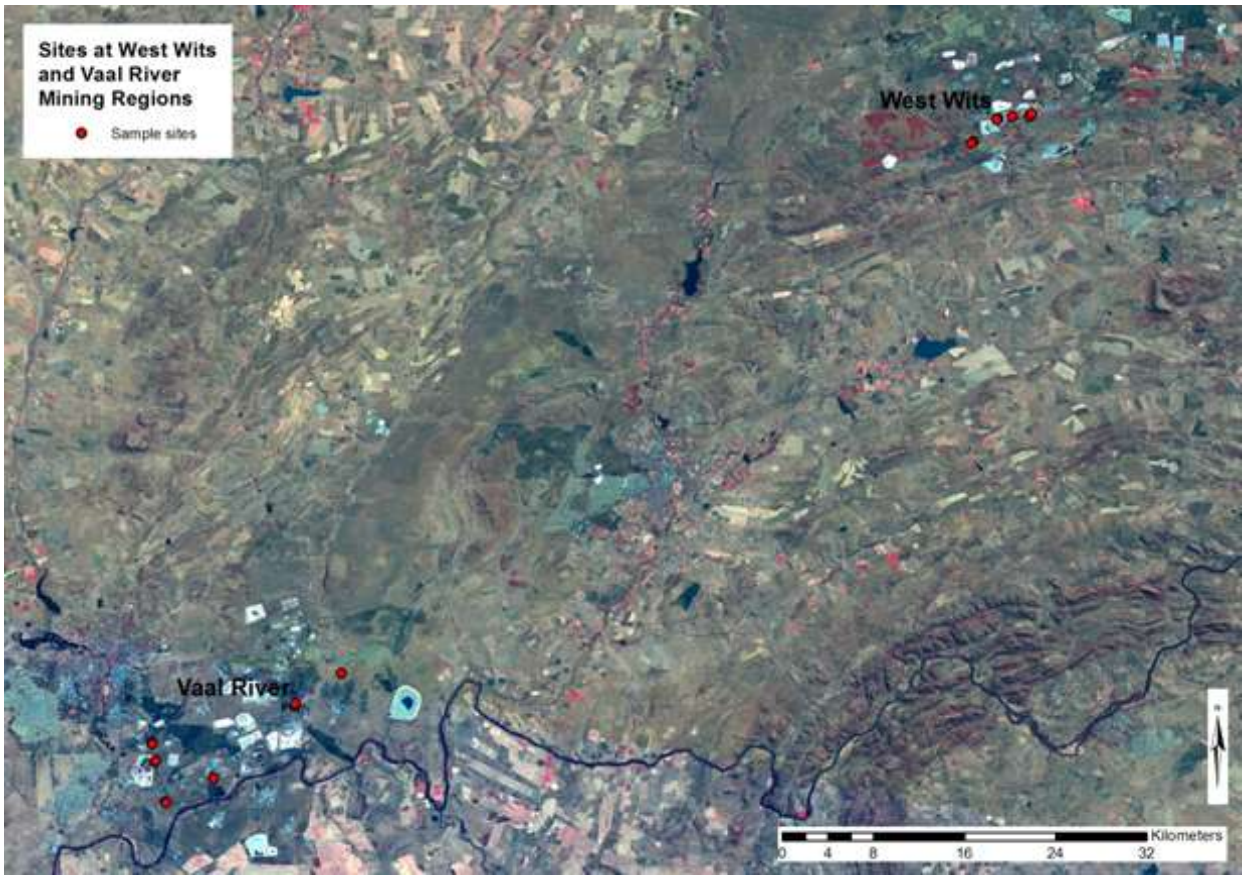


Figure 2.16: A Google image compiled to illustrate locations sampled for Vaal River and West Wits Operations.

Figure 2.17: Aerial view of Vaal River Operations. Dots indicate the trees that were sampled.

Figure 2.18: Aerial view of West Wits Operations. Dots indicate the trees that were sampled.

2.8.3 Soil analyses

2.8.3.1 pH value

Water: The procedure used was a modified version of that outlined by Anderson and Ingram (1989). The modifications included the use of 10 ± 0.1 g of soil instead of 20 ± 0.1 g soil and the addition of 25 mℓ distilled water as opposed to 50 mℓ. All water-soil mixtures were stirred for 10 min on a rotor shaker at 250 rpm. The mixtures were then left to stand for 30 min and stirred again for 2 min before measuring the pH (pH-meter, type 540 GLP, WTW, Germany). The pH of the supernatant was measured and noted when the reading became stable (does not change more than 0.1 unit per 30 s or 0.02 units per 5 s; van Reeuwjik, 2002).

Potassium chloride: The procedure used was that of van Reeuwjik (2002) whereby 10 ± 0.1 g of soil samples were combined with 25 mℓ 1 M KCl. The combinations were stirred on a rotor shaker at 250 rpm for 2 h. The samples were immediately analysed using a pH meter (type 540

GLP, WTW, Germany). Just before readings were taken the samples were briefly shaken by hand, the electrode was submerged in the upper part of the suspension and the reading was recorded when the reading became stable.

2.8.3.2 Conductivity

The conductivity method used was a modified version by Anderson and Ingram (1989). This was performed using the water-soil mixtures from pH screening. Once the pH measurements were completed the mixtures were left to stand for 1 h before the conductivity (Thermo Scientific™ Orion™ Star A222 Conductivity Portable Meter with 0130101MD DuraProbe) of each mixture was measured.

2.8.3.3 Chemical elements

2.8.3.3.1 X-ray fluorescence (XRF) spectroscopy

Soil samples were placed in prepared sample cups up to the indicated mark on the inside of the cup. For every 10 samples placed in the XRF spectrometer (SPECTRO XEPOS) a certified reference material (CRM) sample was used as regulatory control. Prior the analysis run the samples were gas flushed using helium. Specific CRMs used were NCS 87103, NCS 73315 and GBW 07312, and acquired from the China National Analysis Center for Iron and Steel.

2.8.3.3.2 Analysis of soil CEC and P availability

Available P content and cation exchange capacity of soils were analysed by the Agricultural Research Council Institute for Soil, Climate and Water (Hatfield Pretoria, South Africa), with the methods described below.

Available phosphorus content

Summarising the process used by the ARC the available P content of each sample was done by combining 6 g soil with 45 mℓ Bray 1 solution following which 2 drops of flocculant was added.

The combination was then stirred on a shaker for 60 s, filtered and the concentration of P was determined on a flow within 24 hr.

Cation exchange capacity

Summarising the process used by the ARC the CEC of each sample was done by combining 5 g soil with 50 mℓ ammonium acetate (pH 7) solution. The combination was shaken, filtered and the available cations were determined using inductively coupled plasma (ICP) mass spectroscopy.

2.8.4 Statistical analysis

The soil characteristics pH (water and KCl) and conductivity values (n = 4 for niche sites and n = 2 for controls) were subjected to a Two-Way ANOVA; followed by a Tukey-Kramer analysis (IBM SPSS Statistics 24). The mean of each chemical analysis of the soil samples from each niche site (n = 4 for niche sites and n = 2 for controls) was determined and subjected to a Two-Way ANOVA; followed by a Tukey-Kramer analysis (IBM SPSS Statistics 24). Since the n-sizes were different between sample sites and controls the Tukey-Kramer analysis was used as it was designed for situations in which n-sizes are not equal.

2.9 Results

2.9.1 pH and conductivity

The pH and conductivity were analysed for each soil sample and the mean for each niche site was determined (Table 2.1). It was determined that there were only significant differences between niche sites for pH measured with water. The pH for all soil samples was acidic with the lowest pH being determined to be from the WW control (4.13 ± 0.032) from pH (KCl) and the highest being determined from VR control (6.35 ± 0.25) from pH (water). The overall trend showed that the pH measured with water was higher than that measured with KCl except in the case of East Pay Dam which demonstrated the opposite. The highest conductivity value was determined to be from Toe-paddock WW Old North TSF N TP north-eastern boundary (826.33 ± 518.74) and the lowest value was from Vaal River Control (67.55 ± 24.69) (an overall a 12.23-fold difference). Both control soil samples had the lowest conductivity values.

Table 2.1: The pH and conductivity readings of areas surrounding Vaal Reef and West Wits mine residue deposits.

Analysis	Vaal River					West Wits				
	Madala Site	**Black Reef**	**East Pay Dam**	**R. G. Williams**	**Vaal River Control**	**TSF lower slope, over soil, retaining wall**	**Toe-Paddock WW Old North TSF N TP – Eastern Boundary**	**Toe-Paddock WW Old North TSF N TP North-Eastern Boundary**	**Varkenslaagte Woodlands Blocks**	**West Wits Control**
pH (Water)	5.13 ± 0.22^{ab}	5.13 ± 0.093^{ab}	5.28 ± 0.10^{ab}	4.67 ± 0.24^{a}	6.35 ± 0.25^{b}	5.27 ± 0.36^{ab}	5.88 ± 0.26^{ab}	4.75 ± 0.63^{a}	4.93 ± 0.23^{a}	4.92 ± 0.060^{a}
pH (1M KCl)	4.78 ± 0.13	4.94 ± 0.39	5.39 ± 0.14	4.45 ± 0.46	5.18 ± 0.34	4.63 ± 0.50	5.64 ± 0.33	4.58 ± 0.44	4.20 ± 0.25	4.13 ± 0.032
Conductivity (µSiemens/cm)	106 ± 26.16	90.67 ± 5.81	236.67 ± 25.89	121 ± 41.73	67.55 ± 24.69	369.67 ± 153.64	316.67 ± 78.15	826.33 ± 518.74	208.67 ± 51.06	101.93 ± 14.04

The means with different superscripts are significantly different ($p<0.050$) between sites according to the Tukey Kramer analysis.

2.9.2 Soil elemental analysis

2.9.2.1 X-ray fluorescence (XRF) spectroscopy

The total concentration of various elements was determined for each niche site and using XRF spectrophotometer (Table 2.2 and 2.3). Specific elements included in the analysis were selected based the CRM percentage agreement and all those included in Table 2.2 had a CRM percentage agreement above 75 % (with the exception of phosphorus and uranium, which have a percentage agreement of 65 % and 54 %, respectively). Table 2.3 reports the elements considered important in the study but didn't have a CRM agreement high enough to be analysed statistically. From Table 2.2 it was determined that silicon (Si) was present in the highest concentration, followed by aluminium (Al) and iron (Fe) and niobium (Nb) and thorium (Th) were overall present in the lowest concentrations for all niche sites. Chemical concentrations between niche sites were not significantly different for vanadium (V), Fe and yttrium (Y), meaning that almost all elements in Table 2.2 had at least one site that was significantly different to the other sites. From Table 2.3 it may be determined that nickel (Ni) was the highest concentration in this case and thallium (Tl) was the lowest concentration in this case. Elements in Table 2.3 did not undergo statistical analysis as the results would not be reliable as indicated by a lower CRM percentage agreement.

During the XRF analysis a few chemicals were not detected, either due to not being present or they were present in concentrations that were too low to be detected and an alternative method of analysis may be better suited. The undetected elements include sodium (Na), magnesium (Mg) phosphorus (P), chlorine (Cl), germanium (Ge), selenium (Se), silver (Ag), tin (Sn), tellurium (Te), iodine (I), caesium (Cs), lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), ytterbium (Yb), hafnium (Hf), tantalum (Ta), tungsten (W) and mercury (Hg).

Table 2.2: Total concentrations (in $\mu g.g^{-1}$) of selected elements, using X-ray fluorescence, of areas surrounding Vaal Reef and West Wits mine residue deposits.

Element	Vaal River					West Wits				
	Madala Site	**Black Reef**	**East Pay Dam**	**R. G. Williams**	**Vaal River control**	**Tailings storage facility lower slope, over soil, retaining wall**	**Toe-Paddock West Wits Old North Tailings storage facilities North TP – Eastern Boundary**	**Toe-Paddock West Wits Old North Tailings storage facilities North TP North-Eastern Boundary**	**Varkenslaagte Woodlands Blocks**	**West Wits Control**
Aluminum	44793.33 ± 3778.20^{a}	44586.67 ± 1898.92^{a}	70230.00 ± 6011.50^{ab}	59986.67 ± 3039.79^{ab}	69782.50 ± 4271.54^{ab}	69050.00 ± 17647.81^{ab}	68253.33 ± 4030.95^{ab}	48066.67 ± 10465.63^{ab}	82920.00 ± 4391.97^{ab}	82332.50 ± 8798.54^{b}
Silicon	224033.33 ± 4446.85^{ac}	198233.33 ± 6370.33^{a}	251300.00 ± 2478.57^{ab}	222566.67 ± 2790.66^{ac}	299350.00 ± 13718.14^{a}	306133.33 ± 62036.56^{a}	349166.67 ± 2776.29^{b}	323900.00 ± 36173.10^{c}	267822.22 ± 13539.88^{a}	291200.00 ± 2217.36^{a}
Calcium	1769.33 ± 371.88^{ac}	461.20 ±35.76^{a}	4191.67 ± 292.34^{bd}	1186.00 ± 201.96^{ae}	2357.25 ± 408.20^{ad}	2436.67 ± 724.30^{ad}	3673.67 ± 358.61^{cd}	3107.33 ± 488.34^{cde}	728.84 ± 96.82^{a}	1810.50 ± 456.40^{ad}
Potassium	6082.67 ± 474.70^{a}	7271.00 ± 1081.53^{ac}	12061.00 ± 1659.18^{bc}	10034.67 ± 412.75^{a}	8680.50 ± 653.31^{a}	8337.67 ± 652.52^{a}	13626.67 ± 479.11^{b}	8854.67 ± 1990.67^{a}	10630.44 ± 638.07^{a}	11442.50 ± 1197.38^{a}
Sulphur	1092.00 ± 22.19^{ab}	1005.40 ± 21.43^{ab}	1452.33 ± 65.02^{ab}	1170.67 ± 72.45^{ab}	445.83 ± 29.76^{b}	2251.00 ± 263.25^{ab}	1227.33 ± 18.84^{ab}	2601.00 ± 1356.78^{a}	1010.12 ± 278.91^{ab}	799.10 ± 236.83$_{ab}$
Titanium	2452.00 ±	2644.33 ±	2891.00 ±	3332.33 ±	4101.50 ±	3371.33 ±	2286.67 ±	1995.67 ±	4543.11 ±	5008.25 ±

	273.55^{ad}	184.96^{ad}	109.49^{abd}	155.97^{abcd}	135.14^{de}	986.54^{abcd}	310.90^{a}	286.85^{a}	350.47^{bce}	140.63^{c}
Vanadium	41.53 ± 9.66	63.80 ± 7.87	79.17 ± 5.27	67.07 ± 8.33	67.50 ± 8.67	142.67 ± 76.48	88.23 ± 10.69	54.93 ± 9.68	108.41 ± 10.40	119.43 ± 6.13
Iron	25403.33 ± 1471.44	37050.00 ± 3113.72	29550.00 ± 1877.51	29076.67 ± 178.92	24232.50 ± 1992.22	59186.67 ± 24257.88	35853.33 ± 4193.59	27643.33 ± 1507.50	36205.56 ± 3139.82	40130.00 ± 1508.96
Manganese	2798.67 ± 354.62^{abc}	525.23 ± 56.99^{a}	5051.67 ± 1613.43^{b}	8497.67 ± 508.33^{c}	3581.75 ± 517.33^{bd}	937.70 ± 280.23^{ad}	568.50 ± 44.54^{a}	442.23 ± 139.64^{a}	268.88 ± 42.41^{a}	785.33 ± 113.68^{a}
Copper	20.17 ± 1.56^{a}	42.47 ± 5.55^{ab}	59.93 ± 6.69^{ab}	48.27 ± 8.46^{ab}	26.08 ± 2.15^{a}	123.23 ± 58.29^{b}	70.20 ± 7.66^{ab}	47.47 ± 3.38^{ab}	40.12 ± 0.74^{ab}	51.35 ± 2.99^{ab}
Chromium	111.67 ± 15.30^{ad}	81.93 ± 5.10^{a}	132.30 ± 1.61^{ae}	118.80 ± 5.31^{af}	111.98 ± 6.46^{a}	196.00 ± 8.47^{be}	284.83 ± 11.04^{g}	201.30 ± 36.04^{ce}	170.86 ± 19.50^{def}	184.18 ± 9.12^{bef}
Arsenic	2.50 ± 0.17^{a}	5.50 ± 0.71^{ab}	19.57 ± 4.39^{bde}	4.07 ± 0.24^{ad}	4.50 ± 0.60^{ab}	32.17 ± 5.59^{ce}	41.33 ± 0.58^{c}	42.10 ± 9.26^{c}	12.67 ± 1.75^{ab}	13.98 ± 1.12^{ab}
Bromine	14.27 ± 0.60^{a}	6.27 ± 0.62^{bc}	8.50 ± 2.08^{b}	4.93 ± 0.52^{bc}	4.55 ± 0.55^{bde}	2.50 ± 0.67^{cd}	3.93 ± 1.49^{bde}	0.57 ± 0.42^{ce}	15.23 ± 1.73^{a}	14.48 ± 0.56^{a}
Gallium	8.03 ± 0.75^{a}	9.37 ± 0.92^{ac}	13.27 ± 0.03^{ab}	8.67 ± 0.64^{ab}	9.65 ± 0.36^{a}	20.40 ± 6.90^{bcd}	16.50 ± 1.12^{ad}	10.97 ± 0.54^{ad}	16.63 ± 1.47^{ad}	18.35 ± 1.64^{ad}
Zinc	30.00 ± 8.81^{a}	26.10 ± 1.33^{a}	121.73 ± 21.08^{ab}	48.33 ± 11.81^{ab}	112.40 ± 47.41^{ab}	117.70 ± 56.04^{ab}	182.37 ± 16.68^{b}	65.10 ± 8.94^{ab}	38.07 ± 2.86^{a}	67.23 ± 11.86^{ab}
Rubidium	29.93 ± 2.57^{a}	28.17 ± 1.66^{a}	45.00 ± 5.80^{ab}	32.20 ± 0.92^{a}	36.18 ± 2.34^{a}	50.27 ± 15.21^{ab}	52.73 ± 1.80^{ab}	34.17 ± 2.66^{a}	70.97 ± 3.81^{b}	75.73 ± 10.92^{b}
Strontium	22.37 ± 2.62^{abd}	16.30 ± 1.31^{ad}	36.80 ± 3.51^{b}	16.67 ± 0.58^{ae}	11.98 ± 0.79^{a}	35.53 ± 7.73^{bcd}	44.17 ± 2.08^{c}	41.10 ± 2.35^{c}	22.31 ± 0.91^{ad}	26.73 ± 1.40^{de}
Yttrium	10.63 ± 1.05	11.60 ± 0.69	11.77 ± 0.38	10.23 ± 0.41	11.20 ± 0.46	20.30 ± 6.96	12.20 ± 0.60	10.87 ± 0.73	16.61 ± 0.39	18.35 ± 1.21
Zirconium	281.17 ± 13.60^{ade}	278.27 ± 6.51^{a}	288.97 ± 15.29^{a}	296.60 ± 1.19^{a}	348.78 ± 29.45^{e}	241.00 ± 29.56^{acd}	141.00 ± 13.58^{b}	155.07 ± 5.11^{bc}	309.68 ± 8.74^{de}	312.05 ± 14.11^{ae}

Niobium	4.53 ± 0.20[a]	4.93 ± 0.44[a]	6.07 ± 0.47[ac]	4.73 ± 0.80[a]	5.60 ± 0.45[a]	6.70 ± 1.56[ac]	5.27 ± 0.33[a]	4.13 ± 0.24[a]	10.20 ± 0.51[bc]	11.05 ± 1.53[b]
Barium	1264.33 ± 179.91[a]	117.33 ± 15.45[bc]	461.67 ± 136.66[bc]	610.33 ± 117.93[b]	484.75 ± 110.99[bc]	182.67 ± 48.55[bc]	194.33 ± 17.46[bc]	158.33 ± 13.74[bc]	146.44 ± 4.84[c]	216.75 ± 18.58[bc]
Thorium	4.37 ± 0.23[ac]	2.83 ± 0.22[a]	9.23 ± 1.42[bcd]	4.27 ± 0.62[ac]	4.98 ± 0.38[ace]	9.60 ± 1.55[bd]	9.77 ± 0.09[bde]	8.50 ± 1.97[cdf]	10.54 ± 0.67[bf]	11.20 ± 1.14[bf]
Lead	8.40 ± 0.59[a]	12.10 ± 1.15[a]	36.33 ± 5.93[bc]	13.00 ± 1.11[a]	15.00 ± 0.89[a]	45.93 ± 1.79[be]	50.43 ± 2.88[b]	49.23 ± 12.75[b]	18.76 ± 1.12[ac]	27.13 ± 2.39[ace]
Uranium	0.30 ± 0.00[a]	0.30 ± 0.00[a]	19.37 ± 7.65[bd]	0.30 ± 0.00[a]	0.33 ± 0.00[a]	23.10 ± 5.40[b]	49.90 ± 3.39[c]	29.60 ± 3.77[b]	4.68 ± 3.07[ad]	0.33 ± 0.00[a]

The means with different superscripts are significantly different ($p<0.050$) between sites according to the Tukey Kramer analysis.

Table 2.3: Total concentrations (in $\mu g.g^{-1}$) of selected elements, using X-ray fluorescence, of areas surrounding Vaal Reef and West Wits mine residue deposits.

Element	Vaal River					West Wits				
	Madala Site	Black Reef	East Pay Dam	R. G. Williams	Vaal River Control	TSF lower slope, over soil, retaining wall	Toe-Paddock WW Old North TSF N TP – Eastern Boundary	Toe-Paddock WW Old North TSF N TP North-Eastern Boundary	Varkenslaagte Woodlands Blocks	West Wits Control
Nickel	35.93 ± 1.92	57.30 ± 5.14	63.87 ± 2.40	72.03 ± 5.79	45.10 ± 4.81	97.50 ± 40.00	95.60 ± 5.04	70.97 ± 22.59	61.77 ± 2.06	55.28 ± 3.21
Cobalt	1.77 ± 0.77	1.77 ± 0.77	7.17 ± 3.87	1.77 ± 0.77	9.14 ± 1.91	13.23 ± 7.72	11.71 ± 8.04	13.97 ± 7.56	3.91 ± 0.61	6.23 ± 2.79
Molybdenum	0.79 ± 0.46	3.93 ± 0.34	4.63 ± 0.91	3.43 ± 0.65	4.10 ± 0.31	5.83 ± 0.73	4.80 ± 0.26	0.94 ± 0.78	5.66 ± 0.45	5.63 ± 0.34
Cadmium	0.67 ± 0.00	0.67 ± 0.00	0.67 ± 0.00	0.57 ± 0.10	0.67 ± 0.00	0.91 ± 0.24	0.54 ± 0.12	0.50 ± 0.17	0.25 ± 0.05	0.67 ± 0.00
Antimony	1.33 ± 0.83	5.70 ± 2.36	6.60 ± 2.81	6.07 ± 3.55	9.90 ± 1.14	2.07 ± 1.07	2.67 ± 0.84	8.60 ± 2.90	6.34 ± 1.16	8.03 ± 1.94
Thallium	0.24 ± 0.09	0.33 ± 0.00	0.42 ± 0.09	0.33 ± 0.00	0.33 ± 0.00	0.24 ± 0.09	0.42 ± 0.09	0.29 ± 0.04	0.30 ± 0.03	0.23 ± 0.07

2.9.3 Soil P availability and CEC values

Two analyses were performed by the Agricultural Research Council Institute for Soil, Climate and Water, namely, Bray no. 1 for P and cation exchange capacity for K, Ca, Mg and Na. The results are reported in Table 2.4.

Significant differences between niche sites were only observed for P and K with the highest P and K concentrations occurring in soil from the East Pay Dam (71.43 ± 37.49 and 268 ± 41.33, respectively), which were significantly different to every niche site as well as controls with the exception of the Madala site and the lowest concentrations of both elements were from the Toe-paddock WW old north TSF N TP north-eastern boundary and the Varkenslaagte woodlands blocks. From the table it can be seen that the East Pay Dam site had the highest concentration for all cations with the exception of Na which had the greatest concentration at TSF lower slope, over soil, retaining wall. Both control soil samples had the lowest Na concentrations.

Table 2.4: The exchangeable cations (in µg.g^{-1}), phosphorus, potassium, calcium, magnesium and sodium of areas surrounding Vaal Reef and West Wits mine residue deposits.

Analysis	Vaal River					West Wits				
	Madala Site	**Black Reef**	**East Pay Dam**	**R. G. Williams**	**Vaal River Control**	**TSF lower slope, over soil, retaining wall**	**Toe-Paddock WW Old North TSF N TP – Eastern Boundary**	**Toe-Paddock WW Old North TSF N TP North-Eastern Boundary**	**Varkenslaagte Woodlands Blocks**	**West Wits Control**
Phosphorus	19.07 ± 3.38^{ab}	3.5 ± 0.74^{a}	71.43 ± 37.49^{b}	2.63 ± 1.46^{a}	6.6 ± 0.16^{a}	1.03 ± 0.24^{a}	1.66 ± 0.81^{a}	0.55 ± 0.19^{a}	0.55 ± 0.09^{a}	10.85 ± 3.57^{a}
Potassium	162.67 ± 26.30^{ab}	122.67 ± 13.98^{a}	268.00 ± 41.33^{b}	153.67 ± 8.41^{a}	94.00 ± 5.60^{a}	72.33 ± 4.33^{a}	107.67 ± 32.46^{a}	55.33 ± 10.48^{a}	65.00 ± 10.41^{a}	141.25 ± 11.43^{a}
Calcium	525.67 ± 166.30	385.33 ± 31.80	1163.67 ± 123.01	267.00 ± 28.00	542.00 ± 92.06	569.67 ± 171.81	840.00 ± 263.12	1018.33 ± 587.77	249.67 ± 19.06	518.00 ± 117.44
Magnesium	130.67 ± 32.20	106.00 ± 17.21	158.00 ± 22.03	85.33 ± 2.33	73.25 ± 7.87	102.00 ± 31.79	95.00 ± 29.55	64.00 ± 24.21	63.33 ± 13.33	129.75 ± 10.91
Sodium	8.20 ± 1.00	2.97 ± 0.46	5.40 ± 0.20	2.27 ± 0.30	1.05 ± 0.39	52.83 ± 35.06	6.63 ± 1.58	5.80 ± 2.57	27.43 ± 13.68	1.03 ± 0.04

The means with different superscripts are significantly different ($p<0.050$) between sites according to the Tukey Kramer analysis.

2.10 Discussion

2.10.1 pH and conductivity

Soil pH directly and indirectly influences chemical processes, which in turn influence the soil TEs (Hooda, 2010). Previous analyses have ascertained that soil pH values vary between 4 and 8 with the majority of soil pH values below containing sulphuric acid and soils with a pH above mainly have a percentage of sodium ions (Thompson and Troeh, 1978). The pH of the niche sites and the controls did not exceed either of these values. Soil pH may also indicate that there may be a problem in the soil (Eash *et al.*, 2008). Soil analsysis showed that the pH values of the niche sites and the controls were more acidic than the typical range (5.5-8.25) of agricultural soils (Eash *et al.*, 2008), with the exceptions being Vaal River control and Toe-paddock WW Old North TF N TP- eastern boundary.

Since pH is influenced by a vast selection of factors, namely, season, soil-forming factors, land use, water content at the time of sampling, level of soil (soil horizon) sampled, and method of pH analysis; it may also be used as an indicator that certain elements/nutrients are absent from the soil (Eash *et al.*, 2008; Rowell, 1994). Soils with a pH below 5 usually have limited available P and Fe (as well as certain nutrients) and levels of K, Mg and Ca are also generally low (Eash *et al.*, 2008).

The study performed by Straker *et al.* (2008) found that there was a positive response of AM fungi to increasing pH. This may be a concern since several niche sites were measured at below pH 5 with water and only 2 sites were above pH 5 with KCl. The plant species diversity is affected by pH and grasses grow less prominently on acidic soils but the diversity of woody species is greater on acidic soils (Weiersbye *et al.*, 2006). Although plants are not usually directly influenced by soil pH ranges below 5 or above 9 the indirect influences may be severe (Eash *et al.*, 2008). AM fungi vary in their response to pH (Entry *et al.*, 2002) and may distinguish between different ecotypes (Straker *et al.*, 2008). Some AM fungi do poorly in limed acidic soils whilst others do poorly in acidic soils (Mosse, 1972a,b) and some AM fungi promote plant growth in limed soils, while other AM fungi perform equally well in acidic soils. It has not been ascertained definitively whether AM fungi protect host plants from harmful effects of

unfavourable pH conditions (Sylvia and Williams, 1992). It is important to note that several AM fungi are capable at tolerating low pH conditions (Entry *et al.*, 2002).

The conductivity of tailings solution extracts tend to be extremely high (Weiersbye *et al.*, 2006), which was observed from a study performed on TSF in Welkom (Buck, 2014). However, the conductivity of these sites, when comparing the highest values and lowest values, were up to 5.17 and 7.55-fold less than the conductivity measured on the TSF (Buck, 2014). Straker *et al.* (2008) found that there was a weak, but significant, negative correlation between slime/soil conductivity and percentage infectivity of roots by AM fungi and spore levels in the soil, which suggests that there is a harmful effect of conductivity on AM fungal inoculum potential.

2.10.2 Soil chemical elements

2.10.2.1 X-ray fluorescence (XRF) spectroscopy

XRF analysis does not determine chemical speciation but only total elemental concentrations and those relating to the soils/slimes of this study are discussed in order reported in the table. The consideration of the concentrations of these elements is important because at sufficiently high concentrations they present a risk to both human and animal health in the regions they are found.

Aluminium (Al), a HM, can affect roots by increasing cell wall rigidity, impairing cell division, altering root respiration, interfering with the uptake and transport of calcium (Ca), magnesium (Mg), phosphorous (P) and iron (Fe) and precipitating nucleic acids (Foy, 1983). The total average concentration of Al globally is 80 000 $\mu g.g^{-1}$ (Koljonen, 1992). Varkenslaagte Woodlands Block and West Wits Control were above this average but the rest of the sites were lower than the average with the Madala site and Black Reef site almost being half the average.

The most plentiful element present in soil, silicon (Si), a non-metallic element, is the most stable element (preferred oxidation state Si^{4+} as SiO_2) and is a TE due to its biochemical roles (Kabata-Pendias, 2011). Si is generally more mobile in soils with a higher pH (Kabata-Pendias, 2011). Plants readily absorb Si as dissolved silicate (H_2SiO_4) but its absorption is dependent on soil pH and its concentration in soil solution (Kabata-Pendias, 2011). Although Si is not considered to be

essential for plants, it is still a common constituent in plants with grass species having a greater percentage (0.3-1.2 %) than leguminous species (0.05-0.2 %) (Kabata-Pendias, 2011). Studies have suggested that Si may benefit plants in a variety of ways. It improves plant tolerance to environmental stresses (Savant *et al.*, 1999); it is likely that Si assists in plant development, growth and structural strength (Richmond and Sussman, 2003) and if it is infused into the plant walls of epidermal and vascular tissues it reduces water loss, strengthens the plant tissues and retards fungal infection (Kabata-Pendias, 2011). Si is also known to reduce uptake and/or deleterious effects of certain elements, for instance boron (B), Fe, Mn, Ge, As and Al (Foy *et al.*, 1976; Kabata-Pendias, 2011; Rogall and Römheld, 2002). Si constitutes an average of 28.8 % weight (288 000 $\mu g.g^{-1}$) and can range from 0.52 % weight (5 200 $\mu g.g^{-1}$) to roughly 47 % (470 000 $\mu g.g^{-1}$) weight (McKeague and Cline, 1963; Wedepohl, 1995). All sites contained Si within the range (roughly midway) and the sites TSF lower slope, over soil, retaining wall; Toe-paddock WW Old North TSF North TP – Eastern Boundary; and Toe-paddock WW Old North TSF North TP – North-Eastern Boundary had Si concentrations above the average.

Of all the elements Ca is the fifth most abundant and constitutes about 3 % - 5.29 % by weight of the earth's crust (McLennon and Taylor, 1999). Ca forms several common minerals in the soil for instance anhydrite ($CaSO_4$), calcite ($CaCO_3$), dolomite ($CaMg(CO_3)_2$), fluorite (CaF_2) and gypsum ($CaSO_4$) and distributed widely in other minerals (Zupančič and Pirc, 1999). Ca easily infiltrates a plant's apoplasm and is bound to the cell walls and on the exterior plasma membrane surface in an exchangeable form (Marschner, 1986). However, the uptake rate of Ca into the cytoplasm is extremely restricted and appears to be only loosely connected to metabolic processes (Marschner, 1986). The mobility of Ca^{2+} within the plant is also slow (Marshner, 1986). Ca is non-toxic even at high concentrations and can be decidedly effective in the detoxification of high concentrations of mineral elements in plants (Marschner, 1986). The concentration of Ca present in soil ranges from 5100-76000 $\mu g.g^{-1}$ (Mielke, 1979) with an average of 24 000 $\mu g.g^{-1}$ (Shacklette and Boerngen, 1984). The soil samples from all sites contained amounts of Ca that fell below the lowest value in the concentration range with Black Reef site being 11-fold less than the lowest value in the concentration range. These low are levels are important to consider as they may impair the ability of phytoremediation agents to persist in these environments.

Potassium (K) has one oxidation state, K^+, and belongs to the periodic table group 1 along with lithium (Li), sodium (Na), rubidium (Rb) and caesium (Cs) (Canney, 1953). Potassium plays a key role in the phloem transport of sugars. Excessive amounts of K influences the uptake of Mg, Ca, Mn and Fe by plants and may cause nitrogen deficiency (Evans, 2003). The average concentration of K present in soil is 15 000 $\mu g.g^{-1}$ (Shacklette and Boerngen, 1984) and the sampling sites at most had only two thirds of the average amount (East Pay Dam).

Sulphur (S), much like N, is bound in organic compounds by covalent bonds with carbon (Thompson and Troeh, 1978). S has many properties in common with N, such as being unavailable to plants in its elemental state, exists in several oxidised forms, is most accessible in its highest oxidised form and S forms soluble anions (Thompson and Troeh, 1978). S solubility is restricted when Ca is plentiful in soil (Thompson and Troeh, 1978). S is an important plant nutrient as it is a component in plant hormones (some) and plant proteins (Thompson and Troeh, 1978; Wang *et al.*, 2001). Several plants are sensitive to S deficiency, for instance, legumes, since S being required for N fixation (Tisdale, 1974). It has been reported that S concentrations in soil can range from 140 $\mu g.g^{-1}$ to 501 $\mu g.g^{-1}$ (Rehm and Caldwell, 1968; Spencer and Frency, 1960; Tabatabai and Bremner, 1972; Wang *et al.*, 2001). Only one site, VR Control, had a S concentration within the range and the rest of the sites had a greater concentration than the maximum value. It is noteworthy to mention that WW control had the lowest value of the rest of the sites. This reduces concerns over whether S deficiency could occur.

Titanium (Ti) is a typical constituent of rocks and it forms a variety of minerals, for instance, oxides, silicates and titanates and three of the most prevalent titanium minerals include titanomagnetite (Fe_2TiO_4), sphene ($CaTiSiO_5$), rutile (TiO_2) and ilmenite ($FeTi_3$) (Goldschmidt, 1954; Kabata-Pendias and Pendias, 1984). Dumon and Ernst (1988) reported that Ti does not appear to be essential for plant growth nor is there any evidence that it is toxic to plants. The concentration of Ti present in soil ranges from 330-14 000 $\mu g.g^{-1}$ (Kabata-Pendias and Pendias, 1984) with an average of 2 400 $\mu g.g^{-1}$ (Shacklette and Boerngen, 1984). All site soils contained Ti amounts that were within the range and only 2 sites, both from WW Operations, fell below the average amount. Even at the highest concentration the concentrations were only double the

average concentration, i.e. much less than 14 000 µg.g^{-1}. The sites containing double the average concentration may exhibit/experience Ti toxicity.

Vanadium (V), a HM, exists as 3 oxidation states, namely, V(III), V(IV) and V(V) when water is present at 25 °C (Hooda, 2010). V may replace, in part, Mo in microorganisms that perform symbiotic (rhizobium bacteria) and non-symbiotic N fixation (Kabata-Pendias, 2011; Thompson and Troeh, 1978). It would appear that V stimulates plant growth at low concentrations, however, is toxic at higher concentrations (Hooda, 2010). In acidic soils plants readily absorb V via the root systems (Kabata-Pendias, 2011). On average the V concentration in soil is 108 µg.g^{-1} but can range from trace amounts to 400 µg.g^{-1} (Ure and Berrow, 1982). According Kabata-Pendias (2011) the average soil V concentration is roughly 129 µg.g^{-1} and ranges from 69 µg.g^{-1} to 320 µg.g^{-1}. All site soils contained V amounts that were within both ranges reported and 2 sites, both from WW Operations, were above the lower average reported. This indicates that V toxicity may not be a concern for any of these sites.

Of the HMs, Fe, is the only macro-element (Nies, 1999). Fe^{2+} is not toxic to aerobic bacteria due to its low solubility (Nies, 1999). Fe toxicity displays itself as bronzing; brown spots developing on the tips of older leaves and progressing to younger leaves, where the leaves eventually turn grey or white; foliar injury (leaf damage); inhibited root and shoot growth and sometimes thickened roots (Foy *et al.*, 1978). Generally Fe concentrations in soil range from 3300 to 550 000 µg.g^{-1} (Ure and Berrow, 1982; Bodek *et al.*, 1988) with an average of 21 000 µg.g^{-1} (Rose *et al.*, 1979). All site samples contained Fe within the range reported and all sample concentrations were above the average with 2 sites from WW Operations around double the average.

Of the TEs, manganese (Mn), is the most abundant with the concentration being 350-2000 µg.g^{-1} in rocks and from <7 to >9000 µg.g^{-1} (highly variable) in soils (Hooda, 2010; Kabata-Pendias, 2011). The mean average concentration of Mn in soils is 270-530 µg.g^{-1} (Hooda, 2010) or 411-550 µg.g^{-1} (Kabata-Pendias, 2011). Mn, a free element in nature, predominantly occurs as Mn(II) (forms an equilibrium with the exchange sites on soil surfaces) but also occurs as Mn(III) and Mn(IV) (as oxides, phosphate or carbonates associated with solid phase) (Hooda, 2010). Plants use Mn in many physiological and biochemical processes and plant growth can be severely

inhibited when there is a Mn deficiency, however, Mn becomes toxic when in excessive quantities (Hooda, 2010). The amount of Mn required by plants is around 20-40 $\mu g.g^{-1}$ dry weight and for sensitive plants the amount of Mn within the plant can range from 20 to 100 $\mu g.g^{-1}$; however, deficiency occurs when plant Mn is below 15-20 $\mu g.g^{-1}$ (He, 1998; Hooda, 2010). Acidic soils (pH of around 5.5) can lead to excessive Mn in plants (>1000 $\mu g.g^{-1}$) and causes issues with toxicity (He, 1998). All site samples contained Mn within the range reported and the R. G. Williams sample concentration was on the high side of the range. There is a possibility that Mn toxicity in plants could occur.

Copper (Cu), a HM, has an electrochemical potential of -268 mV for Cu^{2+}/Cu^{+}, which is within the physiological range (Nies, 1999). Cu^{2+} (divalent Cu ion) strongly binds to humic and fulvic acids in soils and forms Cu-organic matter complexes (Stevenson and Fitch, 1981). Cu readily interacts with radicals, ideally with molecular oxygen (Nies, 1999). Due to the radical character of Cu it is very toxic with many organisms being more sensitive (Gordon *et al.*, 1994) to Cu than *Escherichia coli* (Nies, 1999). The toxicity depends on hydroperoxide radical production (Rodriguez Montelongo *et al.*, 1993) and on interaction with cell membranes (Suwalsky *et al.*, 1998). Cu toxicity in plants may cause Fe deficiency (Woolhouse, 1983); however, this depends on the iron supply source (Rahimi and Bussler, 1973). High Cu concentrations act on lipid peroxidation, destroying the thylakoid membranes and causing chlorosis (Sandmann and Böger, 1983). Roots are generally affected by high Cu concentrations with the inhibition of elongation and development (Marschner, 1986). The concentration of Cu present in soil ranges from 12-120 $\mu g.g^{-1}$ (Wedepohl, 1978; Ure and Berrow, 1982) with an average of 68 $\mu g.g^{-1}$ (Mielke, 1979). All sites, with the exception of 1 site, contained Cu within the range. The TSF lower slope, over soil, retaining wall site exceeded the range for Cu concentration. The East Pay Dam site had a lower than average amount of Cu. Cu is an essential micronutrient for plants and thus low concentrations may affect downstream metabolic processes thus causing developmental problems, although this is not a concern as Cu appeared within the range.

Chromium (Cr), a trace HM, generally occurs as the oxyanion chromate, Cr(VI), and the trivalent cation, Cr(III) (Nies, 1999). Cr(VI) is more toxic than Cr(III), thus only Cr(III) can perform favourable functions (Nies, 1999). However, Cr has no beneficial effect on

microorganisms (Nies, 1999). Cr is extremely toxic to plants and severly effects plant development and growth (Shanker *et al.*, 2005). However, the degree of toxicity of Cr on plants varies at different concentrations of Cr and at different stages of the plant's development and growth (Shanker *et al.*, 2005). Cr(VI) toxicity is due to it acting as an oxidising agent and the fact that it forms free radicals when it is reduced to Cr(III) inside the cell (Shanker *et al.*, 2005). Cr(III) may be toxic through its coordination of various organic compounds which results in the inhibition of some metalloenzyme systems, when Cr(III) is present in high concentrations (Shanker *et al.*, 2005). Total Cr concentrations in United States soils average 37 $\mu g.g^{-1}$ (Shacklett and Boerngen, 1984) but globally soil Cr content averages 60 $\mu g.g^{-1}$ (Kabata-Pendias, 2011). The samples from VR Operations were 1.37-4.75-fold more Cr and the samples from WW Operations contained 2.85-3.36-fold more Cr than the average. The higher concentrations may impair the ability of phytoremediation agents to persist in these environments as Cr toxicity is an established cause for the impairment of plant health (Shanker *et al.*, 2005).

Arsenic (As) sometimes acts as a metal but is generally a heavy metalloid and it predominantly occurs as As (V) in AsO_4^{3-} (arsenate) and as As (III) in AsO_2^{-} (arsenite) (Nies, 1999). Arsenate is related to phosphate (PO_4^{3-}) structurally; hence its major toxicity arises from its interference with the vital bioelement P (Nies, 1999). However, bacteria can use it in anaerobic respiration as an electron acceptor (Laverman *et al.*, 1995). Organic arsenicals are microbially degraded, however, it is often done slowly, which complicates analyses of food-chain transfer and plant availability (McLaughlin *et al.*, 1999). As is not required by plants and tends to be toxic (Finnegan and Chen, 2012). As inhibits root development and proliferation; it also inhibits plant growth and reduces the plant's capacity to reproduce (Finnegan and Chen, 1012). If the concentration of As is high enough critical metabolic processes are interfered with and the plant may die (Finnegan and Chen, 2012). The average concentration range of As in the earth's crust is 1.0 $\mu g.g^{-1}$ to 1.8 $\mu g.g^{-1}$ (Mielke, 1979; McLennon and Taylor, 1999) but can be as high as 20 $ug.g^{-1}$ depending on the parent material and on anthropogenic activities (Tremearne and Jacob, 1941; Wedepohl, 1978; Davies, 1980; Ure and Berrow, 1982). Contradicting these values, Kabata-Pendias (2011) reports the average value as 6.83 $ug.g^{-1}$ and that quantity of As can reach as high as 150 $ug.g^{-1}$ in uncontaminated soils (Wang and Mulligan, 2006). Sample As concentrations from the VR Operations were below 20 $ug.g^{-1}$ but East Pay Dam concentration was above the average

concentration reported by Kabata-Pendia (2011). Of the samples from WW Operations, 3 of the 5 sites had As concentrations above 20 $ug.g^{-1}$, 2 of which were double the value, this indicates that the sites have an excessive amount of As.

Chemically, bromine (Br), is highly reactive with the possibility of having several valence states. Br has been reported in all plant tissues but its necessity has not yet been determined, however, Br^- may partly substitute for the Cl^- plant requirements and can thus become toxic when present in excess. Br toxicity symptoms include chlorosis followed by leaf tip necrosis (Kabata-Pendias, 2011). Soil Br content averages 10 $\mu g.g^{-1}$ but can range from 7.9 $\mu g.g^{-1}$ to above 100 $\mu g.g^{-1}$ but this in extreme cases (volcanic ash) (Kabata-Pendias, 2011). Of the samples analysed 3 sites, namely the Madala site, Varkenslaagte Woodlands Blocks and WW control, were above the average Br concentration but remained within range and 6 sites contained less Br than the lowest value in the range.

Gallium (Ga) has a similar geochemistry to Al but is not as reactive as Al. Too little evidence has been reported to determine whether Ga is required or toxic to plants; however, it has been reported that Ga serves a beneficial role in the growth of microorganisms and some fungi, such as *Aspergillus*. The soil content of Ga averages roughly 15.2 $\mu g.g^{-1}$ but can vary between <3 $\mu g.g^{-1}$ and 70 $\mu g.g^{-1}$ (Kabata-Pendias, 2011). Of the samples analysed, 4 sites, all from WW Operations, contained Ga above the average concentration and the remainder of the sites were below the average concentration.

Zn, a HM, exists solely as the divalent cation Zn^{2+} (Nies, 1999). The Zn cation, with completely filled d orbitals, cannot go through redox changes under biological conditions (Nies, 1999). Zn is used to complex polypeptide chains (Fosmire, 1990) and the toxicity of excess Zn in plants appears as chlorosis of young leaves (Marschner, 1986) and in non-tolerant plants, root growth inhibition (Godbold *et al.*, 1983); however, Zn is less toxic than Cu (Nies, 1999). The average concentration of Zn in the soil is 70 $\mu g.g^{-1}$ (Heinrichs *et al.*, 1980). However, according to Göhre and Paszkowski (2006) and Oliveira and Pampulha (2006) Zn concentration tends to be 80 ppm or 36.6 $mg.kg^{-1}$. So according to Heinrichs *et al.*, (1980) the soil from Toe-paddock WW old north TSF north TP north-eastern boundary contains 2.6-fold more Zn; East Pay Dam and TSF

lower contains 1.7-fold more Zn and the VR Control contains 1.6-fold more Zn. The rest of the soil samples contained less Zn than the average concentration.

Rubidium (Rb), a monovalent cation, does not form minerals of its own and is closely linked to K. Rb is readily absorbed by plants and may act as a substitute for K sites, which can be toxic at high concentrations as K is required in plant metabolism. Rb content in soil ranges from 18 $\mu g.g^{-1}$ to 116 $\mu g.g^{-1}$ (Kabata-Pendias, 2011). All sample soil Rb content was within range but the concentrations from WW Operations samples are higher than samples from VR Operations, which could indicate WW Operation sites are at a greater risk for developing plant toxicity.

Strontium (Sr) generally occurs as a bivalent cation and has both biochemical and geochemical characteristics in common with Ca. Sr does not appear to be required by plants, although it is absorbed through the plant requirements for Ca. Sr plant bioavailability could be inhibited through the application of Mg, Ca, K and Na despite it being readily absorbed by plants. Sr soil concentrations tend to be between 130 $\mu g.g^{-1}$ and 240 $\mu g.g^{-1}$ (Kabata-Pendias, 2011). All soil samples analysed were 2.94-10.85-fold less Sr than the range. This indicates that there is little chance of toxicity from this element.

Yttrium (Y) has one oxidation state, Y^{3+}, and belongs to the periodic table group 3 along with scandium (Sc), lanthanum (La) and actinium (Ac) (McLennon, 1999). Y tends to accumulate within plant roots and leaves but is less concentrated in the seeds, fruit and edible roots but otherwise Y in the soil is strongly bound and the uptake and translocation is restricted (Kastori *et al.*, 2010). The average concentration range of Y present in soil is 21-31 $\mu g.g^{-1}$ (Mielke, 1979; Shacklette and Boerngen, 1984). All samples fell below the range, although 1 sample, TSF lower slope, over soil, retaining wall contained a Y concentration close to the range and the other samples fell below the range by 1.14-2 fold less Y. Since it has not been reported to be a requirement by plants the low concentration of Y at the sites should not have an impact on the vegetation.

Zirconium (Zr), a metal, is not efficiently mobile in plants and is concentrated in plant roots especially in legume roots and the nodules. From this it can be suggested that some vegetation,

such as shrubs, trees and legumes are likely to concentration Zr to a greater degree than other plants. Toxic effects of Zr on plant roots have been reported but yeast growth and microorganism metabolism promotion has been reported as well. Zr content in soils has been reported to occur in a range of 140-305 $\mu g.g^{-1}$ (Kabata-Pendias, 2011). All soil samples had Zr concentrations within the range. This indicates that there is little chance of toxicity from this element.

Niobium (Nb), a HM, tends to be fairly scarce in the biosphere and is strongly associated with Mn, Fe, Ti and Zr. However, in the lithosphere Nb is fairly common and is somewhat motile in humid environments indicating that it could be available to plants. Depending on the parent material and the country, Nb concentration in soil may occur in the range of <4-300 $\mu g.g^{-1}$ (Kabata-Pendias, 2011). All soil samples had Nb concentrations within the range and did not exceed 11.5 $\mu g.g^{-1}$. This indicates that there is little chance of toxicity from this element.

Barium (Ba) is a common, universally located element. Ba has been reported to be a constituent in plants although its requirements in plant tissues have not been elucidated yet (Kabata-Pendias, 2011). Ba is readily absorbed by plants in acidc soils and toxicity has been reported in barley and beans whereby growth was inhibited in soils containing 2000 $\mu g.g^{-1}$ Ba (Chaudry *et al.*, 1977). The soil content of Ba is very similar to the parent material and tends to range from 10 to 1 500 $\mu g.g^{-1}$ with the lowest quantity being found in organic soils and the highest quantity being found in loamy soils. Globally Ba averages 362-580 $\mu g.g^{-1}$ in soils (Kabata-Pendias, 2011). Of the samples analysed 2 sites, Madala site and R. G. Williams, contained Ba concentrations above the range but below the toxic level reported by Chaudry *et al.* (1977). The rest of the samples contained Ba concentrations within range.

Thorium (Th), a radionuclide, concentrations in soils can vary from 2.5×10^{-3} $\mu g.g^{-1}$ to 9.8 $\mu g.g^{-1}$ but generally the average concentration of Th is 7.4 $\mu g.g^{-1}$ (Bojanowski *et al.*, 2001; Tzortzis and Tsertos, 2004). The phytotoxicity of thorium is not well known (Shtangeeva *et al.*, 2005) and no reports indicate that Th is required by plants. Of the samples analysed 2 sites, Varkenslaagte Woodlands Blocks and WW control, contained Th concentrations above the maximum value but 4 sites that were within range had concentrations above the average concentration.

The primary natural form of lead (Pb) is lead (II) sulphide (PbS or galena) and on average the total Pb amount in different soils is roughly 27 $\mu g.g^{-1}$ although different soils and countries allows for the range to be between 3-90 $\mu g.g^{-1}$ (Kabata-Pendias, 2011). Higher concentrations of Pb indicate that the soil is contaminated (Kabata-Pendias, 2011). In South Africa the average concentration of Pb in soil is 56 $\mu g.g^{-1}$ and can range from 2.99 to 65.8 $\mu g.g^{-1}$ (Herselman *et al.*, 2005). It is difficult to evaluate soils that have Pb levels that are toxic to plants due to the fact that assessing the amount of Pb available to the plant is difficult (Kabata-Pendias, 2011). However, it has been suggested that in unpolluted soil the maximum Pb content would be 70 $\mu g.g^{-1}$ (Davies, 1977). All sample concentrations were below the average South African Pb concentration but only 5 sites were below the global average of which 1 site was from WW Operations (Varkenslaagte Woodlands Block) and 4 sites were from VR Operations (excluding East Pay Dam). From this it may be suggested that Pb toxicity is more likely to occur in WW Operations.

Of the natural elements, uranium (U) has the highest atomic number, is an actinide and generally occurs as U(IV) in UO_2^{2-} (Nies, 1999). U(IV) has low toxicity to bacteria (Pavlakis *et al.*, 1996). U causes plant death when present at sufficiently high concentrations in the soil (300-10 000 $\mu g.g^{-1}$) but otherwise stunts plant growth and seed production (Sheppard *et al.*, 1992). The average concentration of U in soil is 2.3 $\mu g.g^{-1}$ (Mielke, 1979; Shacklette and Boerngen, 1984). From VR Operations samples, 1 site, East Pay Dam, contained 8.4-fold more U than the average indicating that the level of U present is excessive and could be the main cause of toxicity of the site; however, from WW Operations only 1 site contained less U than the average, which indicates that the soil from WW Operations is more toxic than VR Operations.

The elements reported in Table 2.3 were included due to their significance in nutrient requirements as well as several being known as soil pollutants despite the CRM agreement being less than that required for reliable statistical analysis. The elements are discussed in detail below.

Nickel (Ni), a HM, is included in the iron family that also includes cobalt (Co) (Kabata-Pendias, 2011). Ni is an important element for plants, specifically legumes, in the urease metabolism (Eskew *et al.*, 1983; Hooda, 2010) and for the biosynthesis of bacteria (it is a component of

several enzymes) (Hooda, 2010; Kabata-Pendias, 2011). Ni toxicity causes reduced plant growth and plant injuries (chlorosis) although the mechanism of this toxicity and the biological effects depend on the chemical species of the Ni (Kabata-Pendias, 2011). Ni in its cationic form (Ni^{2+}) is more easily absorbed by plants (high phytoavailability) and thus more toxic than the complexed forms of Ni (Kabata-Pendias, 2011). Ni mobility increases with a decrease of pH and CEC (McGrath, 1995). Another important factor in Ni toxicity is its solubility in the soil, which tends to rely on the origin of the Ni, for instance, application of Ni is more toxic than from parent material. Tolerance and hyperaccumulation of Ni is observed in several plant species, including those from the families *Leguminosae*, *Cruciferae*, *Caryophyllaceae*, *Myrtaceae* and *Boraginaceae*, which also accumulate Co (Kabata-Pendias, 2011). Ni accumulation most often occurs in the roots (Kabata-Pendias, 2011) and a study by Mirete *et al.* (2007) on the rhizosphere metagenome from plants adapted to acid mine drainage identified Ni resistance genes. Globally the average concentration of Ni in soil is 13-37 $\mu g.g^{-1}$ (Kabata-Pendias, 2011) and in South Africa the Ni concentration range is 3.43-159 $\mu g.g^{-1}$ although the maximum permissible level in agriculture is 50 $\mu g.g^{-1}$ (established in 1997) (Herselman *et al.*, 2005). The highest Ni concentration of unpolluted soils is 3240 $\mu g.g^{-1}$ from Italy (Bini *et al.*, 1988). All soil sample Ni concentrations were within South African range but only Madala site Ni concentrations were within the global range with the rest of the sites being above the global range. Plants tolerant to Ni should not encounter issues growing on the sample sites although more sensitive vegetation may incur Ni toxicity.

Co, a HM that is part of the iron family metals, occurs in several oxidation states of which Co(II) (divalent) and Co(III) (trivalent) (Hooda, 2010; Kabata-Pendias, 2011). The absorption of Co by plants is dependent on the plants ability to absorb Co and soil factors. Co absorption involves active transport in higher plant roots. The plant availability of Co in soil or nutrient solution is high and increasing Co soil concentration results in increased quantities in the plant. Co is an essential element for microorganisms and blue-green algae for N fixation. It has been suggested that Co may be involved in the formation of chlorophyll. A low concentration of Co has been found to promote plant and algal growth; however, by comparison a higher concentration is toxic and results in plant growth and metabolic functions being affected. Co has a role in mitochondrial respiration and is a component of vitamin B_{12} and the cobamide coenzyme, thus

indicating that Co participates in N_2 fixation in the nodules of legume roots. Excessive Co causes chromosome damage, inhibits mitosis and damages root tip endoplasmic reticulum and one toxicity symptom is the white, dead tips and edges of leaves. Grasses accumulate less Co than legumes (Kabata-Pendias, 2011). On average Co concentration in soils is around 10 $\mu g.g^{-1}$ and can be between 3.4-520 $\mu g.g^{-1}$, however, 520 $\mu g.g^{-1}$ occurs in New Zealand and the next highest concentration is only 154 $\mu g.g^{-1}$ in America (Kabata-Pendias, 2011; Lyon *et al.*, 1970). All samples analysed were within range and 3 sites, all from WW Operations, were above the average. It is unlikely that Co toxicity from the sites will occur.

Mo is vital for the development and growth of plants and animals (Hooda, 2010; Kabata-Pendias, 2011). Plants absorbs Mo mainly in the form of molybdate ions and this is directly proportional to its soil solution concentration (Kabata-Pendias, 2011). Plants are able to absorb N with the presence of Mo in certain enzymes, however, Mo deficiency in plants, save legumes, seldom occurs due to requirements for the element being low (Hooda, 2010; Kabata-Pendias, 2011). Mo is found in nitrate reductase and nitrogenase, and is thus an essential elemental requirement for the reduction of nitrate and the fixation of nitrogen. Mo has the potential to be a toxic metal if present in excess and can affect the health of plants and animals (Hooda, 2010). Mo is an essential micronutrient with the enzymes containing Mo being necessary for the health of all animals (Hooda, 2010). There are several enzymes that require Mo as a component, such as, nitrate reductase, sulphide oxidase, aldehyde oxidase, xanthine dehydrogenase and other possible oxidases that catalyze a range of unrelated reactions (Kaiser *et al.*, 2005; Stroumin and Vunkova-Radeva, 2007). Besides being essential for plants and animals Mo is vital for microorganisms and N-fixing microorganisms have a particularly large requirement for Mo. Deficiency symptoms of Mo in plants are similar to those of N deficiency as NO_3 reduction is the most important function of Mo (Kabata-Pendias, 2011). The primary oxidation state of Mo is Mo(VI), however, Mo(IV) also exists in the sulphide, molybdenite (MoS_2) (Hooda, 2010), which is the most common Mo mineral (Kabata-Pendias, 2011). In acidic soils Mo is retained mainly on variable-charge mineral surfaces, such as Fe oxides (Hooda, 2010), meaning that it is least soluble in acidic soils (Kabata-Pendias, 2011). The mobility, hence phytoavailability, is greatly dependent on soil drainage conditions and pH with dry, acidic soils having the lowest mobility and phytoavailability (Kabata-Pendias, 2011). The average concentration of Mo in soils is 1.8

$\mu g.g^{-1}$ but can occur in the range of 0.013 to 17 $\mu g.g^{-1}$ (Kabata-Pendias, 2001). From earlier data, the average soil concentration was calculated to be 1.9 $\mu g.g^{-1}$ (Ure and Berrow, 1982). From areas where Mo toxicity was observed the average Mo concentration was 5.8 $\mu g.g^{-1}$ (Kubota, 1975) and polluted soils from industrial activities can contain up to 180 $\mu g.g^{-1}$ (Neunhäyserer *et al.*, 2001). According to Kubota (1975) the TSF lower slope, over soil, retaining wall had a Mo concentration at toxicity level and 2 sites, Varkenslaagter Woodlands Block and WW Control, were reaching level of toxicity. Only 2 sites, Madala site and Toe-paddock WW old north TSF N TP – eastern boundary, were below the average.

Of the chemicals identified from the soil, cadmium (Cd) is the best known toxic HM (Nies, 1999). Cd is more toxic (Ragan and Mast, 1990) than zinc (Nies, 1999) since the solubility product of cadmium sulphide (CdS) is 1.4×10^{-29} and for zinc sulphide (ZnS) the solubility product is 2.91×10^{-25} (Weast, 1984). There are several effects on biological processes caused by Cd, namely, membrane damage, interaction with Ca metabolism, interaction with Zn metabolism, loss of protective function, thiol-binding and protein denaturation (Nies, 1999). Very rarely does Cd effect a single biological process listed at one time (Nies, 1999). Stunted plant growth, leaf rolling and chlorosis are the predominant symptoms of Cd toxicity (Benavides *et al.*, 2005). These symptoms may be explained by the Cd effect and interference of the plant uptake, transport and metabolism of water and several elements, such as Ca, P, K and Mg (Das *et al.*, 1997). The average concentration of Cd in the soil is 0.098 $\mu g.g^{-1}$ (Heinrichs *et al.*, 1980), or according to Göhre and Paszkowski (2006) and Oliveira and Pampulha (2006) 0.1-0.2 $\mu g.g^{-1}$ or 0.4 $\mu g.g^{-1}$. Only Varkenslaagte Woodlands Block site had Cd levels below the maximum value reported. This would indicate that Cd could be a cause of toxicity of the sites.

Antimony (Sb), a chalcophlic metalloid, was utilised in gold extraction processes (Hooda, 2010). Sb commonly occurs as valentinite (Sb_2O_3) and stibnite (Sb_2S_3) but can also substitute for As in bournonite (Ag_3SbS_3) and pyrargyrite ($PbS.Cu_2S.Sb_2.S_3$). Sb is observed with a higher frequency in areas around industrial activities due to it being associated with over 100 antimony-bearing minerals (Kabata-Pendias, 2011). Sb also tends to occur in soil with other more toxic contaminants such as As and Pb (Tschan *et al.*, 2009). Sb is fixed to particles with Al, Mn and Fe and is fairly motile in soils (Kabata-Pendias, 2011) and when dissolved as oxyanions it is

adsorbed, particularly at low pH (Filella *et al.*, 2002). In its soluble form Sb is absorbed by plants fairly easily although it is not thought of as an essential element (Kabata-Pendias, 2011). Sb decreases the contents of starch, sugar, protein and some essential elements in the aerial part of plants and inhibits the synthesis of chlorophyll (Feng *et al.*, 2013). It has been reported that an increase in Sb leads to an increase in auxin production by rhizobacteria (Picard and Bosco, 2006). The average concentration of Sb in the soil is 0.67 $\mu g.g^{-1}$ and ranges from 0.25 to 1.04 $\mu g.g^{-1}$ (Kabata-Pendias, 2011). All soil samples contained 1.33-9.52-fold more Sb than the maximum and 3 of the highest concentrations were from the control samples and Toe-paddock WW old north TSF N TP north-eastern boundary.

Thallium (Tl), a toxic HM, forms 2 types of compounds, namely, monovalent thallo- and trivalent thalli-compounds (Galván-Arzate and Santamaria, 1998; Zikto, 1975). Tl toxicity is caused by its high affinity for sulfhydril groups from biomolecules and proteins, the interference of the essential K-dependent processes and the substitution of K in the (Na^{+}/K^{+})-ATPase (Aoyama *et al.*, 1988; Galván-Arzate and Santamaria, 1998). As Tl ions mimic the biological action of P ion cell membranes are unable to distinguish between Tl and K (Galván-Arzate and Santamaria, 1998; Zikto, 1975), which results in the inability of the organism to prevent Tl absorption to a degree. Plant toxicological effects include inhibiting seed germination and chlorophyll formation (Scharrer, 1955). The average concentration of Tl in soil is 0.5 $\mu g.g^{-1}$ and ranges from 0.014 to 2.8 $\mu g.g^{-1}$ (Kabata-Pendias, 2011). All sample concentrations were within the range but below the average concentration.

Chemicals analysed in the study due to being toxic to people and animals, are known soil contaminants, and/or because they are necessary for metabolism but were not found during analysis, include mercury (Hg), silver (Ag) and platinum (Pt).

2.10.3 Soil P availability and CEC values

Since pH influences the TEs in the soil it is noteworthy to mention the cation and anion activity with the change in pH (Hooda, 2010; Thompson and Troeh, 1978; Townsend, 1973), this is likely due to the acidic or basic nature of the cations. Basic cations include K, Ca, Mg and Na

(measured during CEC) and acidic cations include Al and H (Thompson and Troeh, 1978). A decrease in pH decreases exchangeable Ca and Mg concentrations and P solubility, and increases exchangeable Al (Rowell, 1994). The concentration of exchangeable cations may also influence pH whereby an increase in basic cations increases the pH and vice versa (Thompson and Troeh, 1978).

Thompson and Troeh (1978) report that the main basic cations are Ca and Mg, with Ca making up 75-85 % and Mg making up 12-18 % of the cations available in soils. The other cations analysed usually make up 1-5 % and 1 % of the soil for K and Na, respectively. The last percent of the soil is made up of other basic cations (Thompson and Troeh, 1978). The ratios were worked out for the sites and the only sites that followed the Ca>Mg>K>Na pattern were TSF lower slope, over soil, retaining wall; Toe-paddock WW old north TSF N TP north-eastern boundary and Varkenslaagte Woodlands Blocks. The sites that fell within 75-85 % for Ca were East Pay Dam; Vaal River Control; TSF lower slope, over soil, retaining wall; Toe-paddock WW old north TSF N TP – eastern boundary and Toe-paddock WW old north TSF N TP north-eastern boundary; the rest of the sites had a Ca concentration below the ratio. The sites that fell within 12-18 % for Mg were Madala; Black Reef; R. G. Williams; TSF lower slope, over soil, retaining wall; Varkenslaagte Woodlands Block and West Wits Control; the rest of the sites were below the ratio. The majority of the sites were 3-4-fold greater than the maximum percentage for K and R. G. Williams site had roughly 6-fold the maximum percentage than normal. The only 2 sites that had a greater than 1 % Na content were TSF lower slope, over soil, retaining wall and Varkenslaagte Woodlands Block and both were at least 6.6-fold the normal amount.

The adsorption-desorption of Mn^{2+} in soils is greatly influenced by pH with the net negative surface charge decreasing rapidly with a decreasing pH, ultimately leading to an increased metal and Mn^{2+} desorption (Basta *et al.*, 2005; Mohan and Chander, 20036; Saeki, 2004; Wu *et al.*, 2003).

2.11 Conclusion

The fertility of the soil could not be completely validated as fertility parameters such as the C:P or C:N ratios were not determined. For future studies it would be advisable that these ratios be determined. The analysis of available Mo, via ammonium oxalate solution, on these acidic soils would also be pertinent to the research of these environments since total concentration does not necessarily indicate its availability and even with liming of the sites, available Mo concentration may even decrease as a result of its adsorption by $CaCO_3$ (Takkar, 1982).

2.12 References

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Chapter 3

3.1 Rational for microbiome analysis

For the majority of Earth's history, life was comprised of only microscopic organisms and currently microorganisms continue to dominate in various ways (Wooley *et al.*, 2010). The microbial communities perform essential roles in the functioning of the environment and organisms they are associated with, for instance human digestive health and plant development and physiology (Mendes *et al.*, 2013; Wooley *et al.*, 2010). Natural habitats are dominated by bacteria, microeukaryotes (including fungi) and archaea, which are the predominant force behind the nutrient biogeochemical cycles and immobilisation, being the "eye of the needle" passed by all elements released by the decomposition of the organic matter (Wooley *et al.*, 2010). Microorganisms have either beneficial, pathogenic or neutral effects on high organisms, depending on the considered species, life cycle stage and the ecosystems, and therefore it is necessary to study the soil microorganisms in projects of soil restoration by phytomanagement.

Research pursuing insight into the uncultured microbial world was driven when researchers realised the extent of the influences exerted by microorganisms on the world (Handelsman, 2004). Through direct environmental DNA and RNA analysis the microorganisms may be analysed through their identification and possible functionality independent of culturing. Several studies have been carried out to begin elucidating different microbial biomes (microbiome) for instance the human gut microbiome.

Previously it was believed that organisms could not be described without being cultured (Society of American Bacteriologists). This premise was disregarded as researchers realised that microorganisms exist whether they are culturable or not. The detailed analyses of the cultured organisms have provided a foundation of knowledge that was used to construct baseline databases upon which further analyses can be built. Studies using molecular techniques have begun to ascertain a variety of unique microorganisms in quantities outstripping the few well investigated varieties of microorganisms (Giovannoni *et al.*, 1990; Hugenholtz *et al.*, 1998a; Rondon *et al.*, 2000; Ward *et al.*, 1990).

Benefits of greater knowledge of microbiomes include human and animal health improvements, advances in agriculture, and environmental rehabilitation (Wooley *et al.*, 2010). Plotting microorganisms and their roles would allow optimum microbiome creation and duplication.

3.1.1 Taxonomic classification

Taxonomic classification and its ranks were dealt with in Chapter 1 and in this case all taxonomic analyses will be focused on the taxon level of bacterial and fungal kingdoms.

3.2 Molecular analysis

Briefly, the Next Generation Sequencers (NGS) vary in their sequencing techniques in that they have differing speed, output, sequence read length and error rate distribution, meaning that different sequencers are applied depending on the specific research purpose and the desired research data. Generally, shorter reads are utilised during metataxonomy, transcriptome analysis and comparing closely related microbial strains or variant strains whilst longer reads are utilised during gene surveys and for genome sequencing (Cardenas and Tiedje, 2008). Interestingly, the different NGS are capable of compensating for their shortcomings in sequence read length and bias created by error rate distribution through generating a massive amount of output data (and thus coverage) whilst still being simple and rapid (Cardenas and Tiedje, 2008). Longer sequence reads allow researchers to target gene regions that permit better classification and reconstruct the metabolic pathways potentially active within complex microbial communities (Wang *et al.*, 2007).

NGS incorporates targeted hypervariable regions of genes with nucleotide barcodes, which allows gene surveys to be more feasible and rapid. This is possible as samples from varying sources tagged with different nucleotide barcodes may be mixed in a single run and separated post sequencing according to the different barcodes (Meyer *et al.*, 2007; Parameswaran *et al.*, 2007). Generally the targeted hypervariable regions are short enough (100-350 bases) to be covered by NGS whilst still long enough for reliable classification through the use of sequence

databases, unfortunately, they cannot be reliably used in phylogenetic comparison, for instance, like the use of full length 16S rRNA gene sequences (Cardenas and Tiedje, 2008).

The microbiome is comprised of all the gene sequences within a microbiota (Marchesi and Ravel, 2015). The microbiota is the assembly of microorganisms existing in a defined environment (Marchesi and Ravel, 2015). The terms “micobiota” and “microbiome” appear to be synonymous. Microorganisms that appear in a microbiome include bacteria, fungi, viruses, archaea and protists (Lawerence, 2005). Thus far it has been determined that bacteria and archaea occur in all environments capable of sustaining other organisms and are often the only occupants of extreme environments, for instance, on rocks within boreholes 6 km underground and deep sea vents that have temperatures of up to 340 °C (Wooley *et al.*, 2010).

The rhizosphere is comprised of a thin area of soil that immediately surrounds and is influenced by plant roots (Lawrence, 2005; Phillippot *et al.*, 2013). It is also thought to be the most active matrix on Earth with an abundance of microorganisms and invertebrates (Philippot *et al.*, 2013). This diverse community is regulated by the complex nutrient feedback system and food web provided by both the microorganisms and plants (Mendes *et al.*, 2013). Plants tend to select the dominant microbial species primarily through the quality or rhizodepositions and based upon their main physiological traits. It is suggested that the primary origin of the microorganism species richness in the rhizosphere is the adjacent bulk soil (root free soil), thus any changes occurring in the communities of the bulk soil, for instance, land-use changes, will also have an effect on the assembly and the final composition of rhizosphere communities (Mendes *et al.*, 2014). This suggestion could also be dependent on the surrounding soil type, for instance dry desert versus moist humic soil, and annual precipitation.

Like the rhizobiome there are different microbiomes depending on the environment and the organism with which the microorganisms are associated. Plant microbiome involves the collective community of plant-associated microorganisms (of roots and aerial structures) and may alternatively be referred to as the plants’ other genome (Mendes *et al.*, 2013).

The metagenome is the collection of genomes of the entire microbiota found in an environment (Handelsman *et al.*, 1998) and currently contains more genetic information than that obtained from cultured organisms (Rondon *et al.*, 2000). Metagenomics is the study which enables research on the genomes of uncultured microorganisms directly from an environment (Rondon *et al.*, 2000; Wooley *et al.*, 2010). The term "meta" is derived from the statistical concept of *meta*-analysis (the process of statistically combining separate analyses) and "genomics" (the comprehensive analysis of an organism's genetic material) (Rondon *et al.*, 2000). This line of study is vital in the aspect that it eliminates the requirement of clonally culturing the organism in order to sequence its entire genome, which is a major issue as a large percentage of microorganisms are unculturable (Wooley *et al.*, 2010). It also allows for a less biased representative of the community as microorganisms rarely occur as single species/isolates in any environment (Wooley *et al.*, 2010). Unfortunately, in spite of its potential, soil metagenomics is still limited by the relatively poor annotation of environmental strains in the databases (Wooley *et al.*, 2010).

It may be thought that an interaction, whether organism-organism (symbiosis) or host-pathogen, may even be considered to be an amalgam of genes (Bäckhed *et al.*, 2005). This is particularly true as very rarely do microorganisms exist in communities of isolated species and generally species interact together with each other and with their environments, which may also extend to their host organisms (Wooley *et al.*, 2010). Hence clonal cultures fail to indicate the actual conditions in nature regarding organism interactions and the development of the biological function and variance in population genomics (Wooley *et al.*, 2010).

The deposition of bacterial, archaeal, fungal and viral genome sequences into GenBank and other such databases has allowed for comparative analyses linking microbes identified in an environment and their possible function and can thus propose/suggest how all the interactions (with each other and possibly the host) work (Wooley *et al.*, 2010). Examples of sequence databases that focus on single targets, for instance the rRNA genes, include Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/index.jsp), Silva and Greengenes (Cardenas and Tiedje, 2008). As of February 2018, 207040555 gene sequences have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank) and 564286852 sequences have been deposited in WGS

(Whole Genome Shotgun) (https://www.ncbi.nlm.nih.gov/genbank/statistics/) thus providing extensive datasets for comparison and analysis (Valverde *et al.*, 2014). Unique databases have been created that focus on specific primer sets, sequences and organisms allowing targeted research to be readily performed. One such database is Maarj*AM*, which is comprised of small subunit (SSU) rRNA gene sequences and available metadata assembled from all relevant taxonomic and ecological publications (Öpik *et al.*, 2010). The data is openly accessible at http://maarjam.botany.ut.ee. Corroboration of studies has allowed for unique 16S rRNA gene sequences, isolated from organisms from diverse environment sources, to be uncovered (Torsvik *et al.*, 1996).

Perhaps as culturing techniques become more advanced more of the originally unculturable organisms will be isolated and maintained in laboratories. Until such time, molecular techniques serve as a reliable alternative for analysis and observation of these organisms. Interestingly, research into methods incorporating specific niche conditions has shown positive results (Cardenas and Tiedje, 2008).

3.3 Introduction

Metataxonomy involves the study of operational taxonomic units (OTUs) in the form of targeted gene or amplicon sequencing and analysis and differs from metagenomic analysis in that it focuses on community structure analysis and not on gene expression or genetic potential of microbial communities (Cardenas and Tiedje, 2008). Metataxonomy utilises PCR amplification which, unfortunately, requires previous information of gene sequences in order for primers to be designed for amplification (Schloss and Handelsman, 2003). With improvements in shotgun sequence processing and genome sequencing, primers are frequently optimised for analyses, allowing for a greater selection for distinct and targeted DNA sequence analyses. Metataxonomic analyses can be comprised of two goals, namely, the estimation of the microbial diversity and highlighting the population dynamics of an entire microbial community (Rajendhran and Gunasekaran, 2008).

Selection of genes for metataxonomic analysis depends/relies on several factors, for instance, the organism that one wishes to identify or classify, the suitability of the gene sequence regarding length, composition (conserved and variable regions) and primer availability as well as the degree of primer degeneration. The primer sets for a gene are required to be broad enough that they match across all members of a large taxonomic group, whilst including mismatches to non-target taxa, and have the capability to produce amplicons that are sufficiently variable so that taxa can be are distinguished reliably at precise, preferably species-level, resolution (Herbert *et al.*, 2003; Justé *et al.*, 2008; Op De Beeck *et al.*, 2014; Seifert *et al.*, 2007; Watanabe *et al.*, 2011). The rRNA gene sequences are important in taxonomic analyses as they are universally present and have the added benefit of being comprised of both highly conserved regions (important for PCR primer design that target all community members) and variable regions (permit different microbial taxa to be distinquished) (Kuczynski *et al.*, 2016; Vos *et al.*, 2012). Research on sequence data has shown that rRNA processing is a conserved process and any variability in gene regions is biologically acceptable provided that they do not disrupt the formation of secondary structures which assist in the rRNA processing (Korabecna, 2007). Hence with the understanding that conserved regions seldom change and that variable regions can be used to distinguish between organisms the rRNA gene sequences serve as favourable targets for molecular based taxonomic analyses.

3.3.1 16S gene sequence components and specific targets for bacterial analysis

Analyses using the 16S rRNA gene techniques (the most prominently used species proxy) to examine bacterial diversity in various soils have been beneficial in that a variety of taxa, shared between geographically distinct soils, have been identified (Ludwig *et al.*, 1997). The gene sequence is preferentially used in analyses as it is one of the few genes that is universal to all known bacteria and archaea and is comprised of roughly 1500 bases (Patel, 2001; Valverde *et al.*, 2014). The gene is also preferably used as it contains both highly variable regions as well as conserved regions (Woese, 1987).

The 16S rRNA gene is comprised of nine hypervariable regions (Figure 3.1) flanked by conserved gene regions (Chakravorty *et al.*, 2007; Van de Peer *et al.*, 1996). The hypervariable

regions, which display substantial sequence diversity between bacterial species, are used for species identification (Van de Peer *et al.*, 1996) whereas the conserved regions allow for primer design and PCR amplification of target gene sequences (Baker *et al.*, 2003; Lu *et al.*, 2000; McCabe *et al.*, 1999; Munson *et al.*, 2004). Interestingly the hypervariable regions demonstrate varying degrees of sequence diversity and no individual hypervariable region has the ability to differentiate between all bacteria (Chakravorty *et al.*, 2007). Thus, with consideration, multiple hypervariable regions should be used in bacterial analyses in order to effectively and reliably identify and distinguish bacterial species. The hypervariable regions vary in length with the shortest regions being the V1 and V9 regions at 30 bp at locations 69-99 and 1435-1465 nucleotides, respectively; and the longest regions being the V2 and V4 regions at 105 and 106 bp at locations 137-242 and 576-682 nucleotides, respectively (Chakravorty *et al.*, 2007). In this study the bacterial gene used was the V1-V3 region (Figure 3.1).

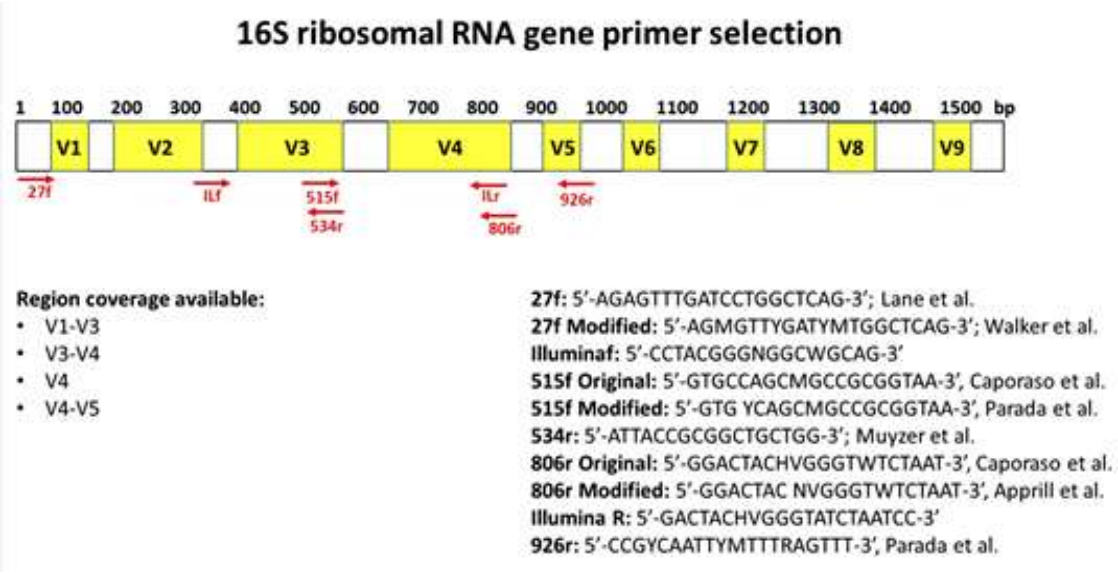


Figure 3.1: Ribosomal RNA gene diagram showing relative positions of primers in relation to variable regions. Primer alignment 27F from Lane *et al.* (1999), 27F Modified from Walker *et al.* (2015), 515F and 806R from Caporaso *et al.* (2011), 515F Modified and 926R from Parada *et al.* (2016), 534R from Muyzer *et al.* (1993), 806R Modified from Apprill *et al.* (2015). Illustration courtesy of http://omegabioservices.com/index.php/16s-reference/.

3.3.2 Internal transcribed spacer (ITS) regions for fungal analysis

A limited taxonomic identification of one kingdom does not permit comprehensive understanding of the microbiome being studied and fortunately the value of the fungal section of the microbiome is improving (Huffnagle and Noverr, 2013).

The internal transcribed spacer (ITS) technique is the official taxonomic barcode for fungi (Schoch *et al.*, 2012), although biases still exist in the amplification process particular taxa may be therefore under-represented (Peay *et al.*, 2016). Additionally, due to the age and genetic diversity of the fungal kingdom the likelihood of a single-marker barcode being sufficient in the identification of each organism to species level is slight (Schoch *et al.*, 2012). Thus often during detailed ecological analyses additional primers and/or gene regions are used, particularly in the case of arbuscular mycorrhizal fungi and basal fungal lineages (Peay *et al.*, 2016). The ITS regions are the best defined barcode gap between inter- and intraspecific variation allowing them to have the greatest probability of successful identification for the broadest range of fungi (Schoch *et al.*, 2012). In fungi the entire ITS region on average is around 600 bp across all fungal lineages and more specifically, 500 and 600 bp for ascomycetes and basidiomycetes, respectively (Porter and Golding, 2011). The most popular eukaryotic model organisms include *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* thus the structures and lengths of their ITS gene regions have been well described (Korabecna, 2007). ITS1 in *S. cerevisiae* is 361 bp and in *S. pompe* it is 412-420 bp and ITS2 in *S. cerevisiae* is 232 bp and in *S. pompe* it is 300 bp (Nazar, 2003).

Specifically the ITS2 gene locus has shown more promise and reliability in fungal community analysis (Ihrmark *et al.*, 2012). Bokulich and Mills (2013) show that ITS1 amplicons tend to be notably shorter than ITS2 or whole-ITS amplicons. The benefit of shorter amplicons is that processing is more rapid; however, resolution of taxa is reduced. Unfortunately, ITS1 and ITS2 both lack the required resolution in certain groups of fungi (Balajee *et al.*, 2009; Blaadlid *et al.*, 2013; Gazis *et al.*, 2011; Lin *et al.*, 2014). In this study the fungal gene used was the ITS2 region.

3.3.3 How are new species introduced into an environment?

The assembly of microbial communities has been hypothesised to be brought about by 2 possibilities, namely, the niche theory and neutral theory (Dumbrell *et al.*, 2010). Each theory predicts the origin of the microbial community and thus possible factors affecting the formation of each community. The niche theory makes the prediction that changes in environmental variables impact changes in the species community composition (Jongman *et al.*, 1995) due to the ability of species being able to uniquely utilise unique niches (Mendes *et al.*, 2014). The neutral theory makes the prediction that geographic distance between samples and dispersal limitation impacts the species community composition due to species having equivalent functional ability to exploit niches (McGill *et al.*, 2006). Interestingly both theories incorporate environmental factors, however, neither theory suggest how the assembly of the major microbial hotspot in soil, that is the rhizobiome, is driven (Mendes *et al.*, 2014). Several studies have highlighted the fundamental role plants species play in shaping of the rhizosphere microbial community (Bulgarelli *et al.*, 2012; DeAngelis *et al.*, 2008; Knief *et al.*, 2011; Lundberg *et al.*, 2012; Rasch *et al.*, 2006; Robin *et al.*, 2006; Uroz *et al.*, 2010; Valverde *et al.*, 2016; Xu *et al.*, 2009). Unfortunately, the understanding of the extent of the plant influence and selection for maintaining the microbial community is limited despite the varied bulk soil reservoirs (Mendes *et al.*, 2014). A model has been proposed whereby microorganism components of the soil microbiome is determined by edaphic factors, after which the microorganisms are selected for through substrate-driven community selection within the rhizosphere, ultimately ending with plant/host genotype-dependent adjustments of endophytic microbes colonising roots and even those colonising leaves and reproductive structures (Bulgarelli *et al.*, 2013; Zarraonaindia *et al.*, 2015).

3.3.4 The importance of rhizobiome mapping

Measuring soil diversity is essential in comprehending the rhizosphere structure and dynamics (Lozupone and Knight, 2008). The interactions in the plant-soil microbe matrix are complex, with both sets of organisms exerting effects on the surrounding soil ultimately playing important roles in soil pathogen suppression (Garbeva *et al.*, 2004). A study by Chanway *et al.* (2000)

showed that plants inoculated with root colonising, growth-promoting bacteria improved plant productivity or provided pathogen resistance. Alternatively, uninoculated plant roots influence the rhizobiome composition through root exudates and root morphology (Berg and Smalla, 2009).

During the process of plant development the soil of the rhizosphere is modified and in turn can affects the rhizobiome (Philippot *et al.*, 2013).

A factor that performs a vital part in the functioning of terrestrial ecosystems is the soil microbial community (van der Heijden *et al.*, 2008; Prober *et al.*, 2015). According to approximations by van der Heijden *et al.* (2008) roughly a minimum of 20 000 plant species require soil microbial symbionts in order to survive and it is suggested that free-living soil microorganisms are capable of promoting plant diversity indirectly through the increase of available nutrient pool variety and they provide evidence that illustrates both symbiotic and pathogenic soil microorganisms can alter plant dominance thereby influencing plant diversity. One of the most diverse and integral groups of organisms is the fungal kingdom (Tedersoo *et al.*, 2014). Their functions range from nutrient cycling, through plant nutrition, to being pathogens (Tedersoo *et al.*, 2014).

In order to produce effective inoculum for plants one must know and understand the microbial communities associated with the target plants in a specific environment and thus effectively translate the knowledge into practical applications (Zarraonaindia *et al.*, 2015).

3.3.5 Techniques and methods of analysis

First step in the analysis is obtaining samples that represent the environment and community/population from which they are extracted (Wooley *et al.*, 2010). Unfortunately, this is problematic to gauge this as one cannot visualise the microbial community that one is trying to analyse (Wooley *et al.*, 2010). Fortunately, rarefaction curves assist in estimating the fraction of species sequenced and thus give an indication of sample coverage (Wooley *et al.*, 2010).

3.3.6 Analysis and interpretive programs; benefits and setbacks

The wealth of knowledge within the metagenome becomes more accessible with development of computational tools that are able to mine the information from sequence data reliably, thereby addressing ecologically driven questions (Schloss *et al.*, 2009). Due to the development of these computational tools, descriptive microbial ecology has evolved into experimental microbial ecology (Schloss *et al.*, 2009). Despite being successful thus far, computational tools require constant development as limitations decreases their effectiveness when sequencing capacity increases and analyses become more complex (Schloss *et al.*, 2009). Improvements in these tools require implementation of more efficient algorithms thereby reducing the generic nature of several analyses (Schloss *et al.*, 2009). Another requirement is that software packages should be comprehensive allowing integration of analysis tools thus creating field-wide analysis standards. This allows for further development to occur and makes it easier to perform meta-analyses (Schloss *et al.*, 2009). Additionally, software packages need to be flexible and frequently maintained so that sophisticated research questions and increased sequence capacity can be processed (Schloss *et al.*, 2009). One such software package is mothur, which screens for sequence quality and is comprised of algorithms that perform Unifrac, TreeClimber, DOTUR, SONS, LIBSHUFF, ∫-LIBSHUFF as well as calculating alpha (α) and beta (β) diversity; produces dendograms, Venn diagrams and heat maps; contains a NAST-based sequence aligner; provides a calculator for pairwise sequence distance, and has the flexibility to allow for individual commands to be recalled from within mothur, from batch files (files with lists of commands) or from the command line directly (Schloss *et al.*, 2009).

mothur was first released in February 2009 and is continually updated allowing bugs in the software to be addressed quickly/efficiently as well as integrating new features and user suggestions (Schloss *et al.*, 2009). Even though the program may not be all encompassing, the files created by the software are compatible with a variety of other analysis tools and can thus be incorporated into other analysis tool pipelines.

3.3.7 Selection of operational taxonomic units (OTUs)

OTUs are comprised of grouped sequences based on DNA sequence or electrophoretic pattern (Schloss and Handelsman, 2005). Generally sequences that contain an identity greater than 80 % are assigned to the same phylum, those that are greater than 95 % are assigned to the same genus and those equal to or greater than 97 % are assigned to the same species; however, the distinctions of these assignments is still debated (Hugenhotz *et al.*, 1998; Schloss and Handelsman, 2005). The inverse of the identity percentage roughly equals the genetic distance (Schloss and Handelsman, 2005). This hierarchy has not been validated thoroughly and the cut-off values in the simplest terms are a best fit of historical taxonomy with modern 16S rRNA gene sequencing (Schloss and Handelsman, 2005).

The tool, DOTUR (Distance-Based OUT and Richness), was developed in order to rapidly and reliably assign sequences into OTUs (Schloss and Handelsman, 2005). DOTUR uses a PHYLIP-generated distance matrix to assign sequences to OTUs for every possible distance, following which DOTUR constructs randomised rarefaction and collector's curves of the OTUs, richness estimators and diversity indices using values it calculates (Schloss and Handelsman, 2005). More specifically three methods of sequence assignment are employed by DOTUR, namely, nearest neighbour, average neighbour and furthest neighbour. The nearest neighbour or single-linkage algorithm creates a connection when one object (individual sequence or sequence group) is similar to any of the sequences in the object to which it is to join (Schloss and Handelsman, 2005). The average neighbour method (otherwise known as the unweighted pair-group method that uses arithmetic average) averages the differences between the objects being joined and all the other objects, and then determines and links the two most similar objects. The furthest neighbour or complete linkage method only assigns a sequence to an object if it is similar to every sequence in the cluster it is to join, thus making it a more constrained method (Schloss and Handelsman, 2005).

3.4 Diversity parameters

A major concern of researchers is whether or not an analysed sample represents a community's actual diversity. In order to resolve this, several diversity statistics (Watve and Gangal, 1996) have been developed including estimating species richness (Kroes *et al.*, 1999), rarefaction curves (Chao *et al.*, 1993; Moyer *et al.*, 1998) and diversity indices (McCaig *et al.*, 1999; Nübel *et al.*, 1999).

3.4.1 Alpha diversity

Alpha (α) diversity measures ecological diversity within a community and/or habitat (Lawrence, 2005; Whittaker, 1972). This is an important measure as there are subtly different ecological niches coexisting, each being inhabited by a unique species and this diversity occurs as a result of competition between species that generally reduces the variation within each species as well as finely tuning species to its niche (Lawrence, 2005).

3.4.1.1 Rarefaction and phylogenetic diversity (PD) measurements

The majority of diversity indices depend on sampling size as well as the extent of sampling and as a consequence they are difficult to utilise when comparing sample collections of varying sizes (Kobayashi, 1982). Rarefaction curves indicate the degree/depth of sampling carried out and extent of the diversity covered through the sampling (whether sampling was satisfactory or required more extensive sampling) and ultimately attempts to demonstrate whether the sampling performed gives a true reflection of the community diversity (Hughes *et al.*, 2001). Rarefaction curves are created through the random drawing of subsamples of *n* individuals from the whole collection and plotting the points that supposedly represent the key features of the diversity of the community onto a graph, however, it is noteworthy to mention that the rarefaction method is affected by the distribution of individuals sampled (depending on the assumption of the equation used) (Kobayashi, 1982). More simply, averaging randomisation of the observed accumulation curve creates a rarefied curve (Heck *et al.*, 1975). It is important to note that rarefaction does not compare communities but compares samples (Hughes *et al.*, 2001).

A measure of precision derived from rarefaction would demonstrate that if a community were to be sampled repeatedly what the observed variation in the number of species would be expected (Hughes *et al.*, 2001). The precision of rarefaction curves can be estimated through bootstrapping (Efron and Tibshirani, 1993). The detection of significant differences in observed richness between communities is shown by error bars calculated from the bootstrapping (Hughes *et al*, 2001).

If the sampling performed captures the community diversity in its entirety the rarefaction curve will reach a point and level off (an asymptote), that is the better sampled the community the more concave-downward the curve, which will also indicate the true community richness due to all communities being comprised of a finite number of taxa (Hughes *et al.*, 2001). This technique makes use of extrapolation, the benefit of which is that once a taxa has been counted it is unnecessary to recount it, thus allowing the researcher to focus on identifying different, often rarer, taxa (Hughes *et al.*, 2001). The disadvantage of extrapolation is that when limited taxa are detected in diverse communities, many curves constructed will frequently fit uniformly "but predict very different asymptotes' (Soberón and Llorente, 1993). This may be due to the fact that most of the extrapolation methods make use of the accumulation curve to "fit an assumed functional form that models the process of observing new species as sampling effort increases" (Hughes *et al.*, 2001)

Difference between rarefaction and phylogenetic diversity (PD) measurements is that rarefaction attempts to estimate taxa richness (expected OTUs) as well suggesting the number of sequences necessary to acquire a measure of taxa richness, whereas PD demonstrates the relative taxa richness (observed OTUs) (Schloss and Handelsman, 2005). Richness estimators evaluate the total richness from a sample of a community using one of 3 techniques, namely, parametric estimators, non-parametric estimators and accumulation curves (Colwell and Coddington, 1994; Gaston, 1996; Palmer, 1990). Determined estimates are then used to compare estimates over several samples (Hughes *et al.*, 2001).

It is assumed that observed community diversity increases with sampling effort until all taxa are observed (depicted by an accumulation or a rank-abundance curve) (Hughes *et al.*, 2001).

PD is a divergence-based qualitative measure that adds and totals the overall branch length in a phylogenetic tree that extends to each member of a community and satisfies the assessment of taxon richness in a community; however, it is influenced by sampling effort due to its assumption that the entire community has been sampled. Additionally, PD is affected and is dependent on the technique used to derive the branch lengths on the phylogenetic tree and may possibly be influenced by errors incurred during phylogenetic tree construction. Considering this, it is advisable that values generated from trees constructed using different techniques is compared, or alternatively, error estimates be calculated (Luzopone and Knight, 2008).

3.4.1.2 Chao1 and Shannon indices

Chao1 is a nonparametric statistic that is adapted from mark-release-recapture (MRR) statistics that estimates the size of a community (Hughes *et al.*, 2001). Chao1 estimates species richness by using something similar to an MRR ratio in order to add a correction factor to the observed number of species (Chao, 1984; Chao *et al.*, 1993). Unfortunately, at low sample sizes Chao1 underestimates the true richness (Hughes *et al.*, 2001).

Shannon index measures both taxon richness and the proportion of each taxon within a community/habitat. Usually in the majority of ecological studies the values generated by the Shannon index lie between 1.5 and 3.5 and the value is seldom above 4 (Margalef, 1972). However, constraining the index so narrowly can make interpretation challenging as the Shannon index increases as both the richness and the evenness of the community increase (Magurran, 2004).

3.4.2 Beta diversity

Beta (β) diversity measures and compares the differences of biological diversity (taxa) between environments/habitats or along a gradient at a local scale (Whittaker, 1972). This measure may

follow qualitative means, which is the presence or absence of taxa to analyse the composition between communities, or quantitative means, which also considers the relative abundance of each taxon (Lozupone *et al.*, 2007).

3.4.2.1 Tree building and Unique Fraction metric (UniFrac) clustering for principal coordinates analysis (PCoA) and non-metric multi-dimensional scaling (nMDS)

Unique Fraction metric (UniFrac) calculates the differences between microbial communities according to phylogenetic information (Lozupone and Knight, 2005). This is performed through measuring the phylogenetic distances between groups of taxa in a phylogenetic tree as the portion of the branch length of the tree that leads to descendants from either one environment or the other, but not both. This may be used to ascertain whether populations are significantly different, to measure the corresponding contributions of various factors, such as geography and chemistry, to similarities between samples, whilst comparing many populations concurrently using clustering and ordination techniques (Lozupone and Knight, 2005). The major benefit of UniFrac is that it utilises the degree of divergence between different sequences through phylogenetic distance measurements (Lozupone and Knight, 2005). When coupled with standard multivariate statistical analyses, such as principle coordinates analysis (PCoA), UniFrac pinpoints factors causing differences between microbial communities (Lozupone *et al.*, 2011). UniFrac may either be weighted or unweighted, with unweighted UniFrac (qualitative) measuring the distance between communities based on the lineages they contain (Lozupone *et al.*, 2005, Lozupone and Knight, 2008) and weighted UniFrac (quantitative) measuring both lineage distance between communities as well as the relative abundance of microbial lineages (Lozupone *et al.*, 2007). Communities are thought to be different if the value calculated using UniFrac is more than would be expected by chance (Lozupone and Knight, 2008).

Importantly, diversity measures based on divergence may potentially illustrate fundamental ecological properties of microbial communities more than has been possible with measures based on species alone (Lozupone and Knight, 2008).

Principal coordinates analysis (PCoA) also refered to as metric dimensional scaling (MDS) depends upon a distance or dissimilarity matrix. Non-metric multi-dimensional scaling (nMDS), is a non-metric variant of PCoA, that maps sample inter-relationships in an ordination (Clarke, 1993). An nMDS plot is a graphical representation of the sample patterns (Clarke, 1993).

When performing an ecosystem census it is crucial that the diversity is quantified using methods so that it can be related to elemental unit of community diversity, namely species, as unambiguously as possible (Vos *et al.*, 2012).

3.5 Methods and materials

3.5.1 Sampling strategy and DNA extraction

Root and soil sample collection was described in Chapter 2 and the GPS co-ordinates were recorded for each tree (Appendix 1). Briefly, root and soil samples were collected from areas around TSFs found in the VR (Vaal River) and WW (West Wits) Operations, with 4 niche sites (3 trees each) being sampled and 2 control trees being sampled from each operation site.

Site descriptions were mentioned previously in Chapter 2; however, for the purposes of this chapter the site names will be abridged so as to simplify graphs and illustrations. Thus the site names are as follows: for Vaal River Operations; Madala site = VR1, Black Reef site = VR2, East Pay Dam site = VR3, R. G. Williams site = VR4, Vaal River Control = VRC, and for the West Wits Operations; Tailing Storage Facilities lower slope, over soil, retaining wall = WW1; Toe-Paddock West Wits Old North Tailings Storage Facilities North Toe Paddock – Eastern Boundary = WW2; Toe-Paddock West Wits Old North Tailings Storage Facilities North Toe Paddock North-Eastern Boundary = WW3; Varkenslaagte Woodlands Blocks site = WW4; West Wits Control = WWC.

Following the sample collection, samples then underwent preparation prior total soil DNA extraction. This preparation involved the following: 5 g of soil (equal proportions), without root material, from each tree was weighed and bulked for each niche site and control; placed in a 50

mℓ centrifuge tube with lid (15 g total soil for niche sites and 20 g for control samples); and placed on a vortex mixer at max speed for 1 hr to ensure thorough dispersal of soil particulates.

From each bulk sample, 2 technical replicates were performed for the DNA extraction. Total soil DNA extraction was performed using the DNeasy® PowerSoil® Kit (Qiagen) with one modification. Instead of using 250 mg soil, 500 mg soil was used for the DNA extraction. It is noteworthy to mention that before bulk sample analysis was performed a trial soil sample was used with the kit to test suitability of the DNA extraction kit with the soil sample types.

The trial analysis comprised of a single, randomly selected, soil sample from VR Operations, also using 500 mg soil. Following the kit DNA extraction the sample was analysed to determine the DNA yield and quality present. DNA yield and preliminary quality was ascertained with the use of a ND-1000 spectrophotometer (NanoDrop, Wilmington, Delaware USA) using 1-2 μℓ of the sample. The DNA quality and integrity was further examined on 1 % agarose/TBE gels containing EtBr and through PCR analysis. The specific PCR reactions and results thereof are presented in Appendix 2. From the trial analysis it was ascertained that the DNeasy® PowerSoil® Kit (Qiagen, USA) was indeed suitable for the soil sample types collected from the sites as well as determining that the DNA integrity isolated from the kits was suitable for downstream analyses.

Once DNA was isolated from the bulked soil samples the DNA yield and preliminary quality was again ascertained with the use of a ND-1000 spectrophotometer (NanoDrop, Wilmington, Delaware USA) using 1-2 μℓ of the sample, the results of which are presented in Appendix 3. The DNA samples were then sent to Molecular Research LP MR DNA (http://www.mrdnalab.com), Texas, in order to be sequenced. Sequencing targeted partial bacterial 16S gene amplicons of the V1-V3 hypervariable region using the primers 27F (5' –AGR GTT TGA TCM TGG CTC AG– 3') and 519R (5' –GTN TTA CNG CGG CKG CTG– 3') (Valverde *et al.*, 2014; Valverde *et al.*, 2016) and partial fungal ITS amplicons of the ITS2 hypervariable region using the primers ITS1F (5' –CTT GGT CAT TTA GAG GAA GTA A– 3') and ITS4 (5' –TCC TCC GCT TAT TGA TAT GC– 3') (Hartman *et al.*, 2012; Valverde *et al.*, 2016).

Total soil DNA was processed at MR DNA whereby the bacterial 16S rRNA V1-V3 hypervariable region (primers 27F/519R) and fungal ITS2 hypervariable region (primers ITS1F/ITS4) with barcode on the forward primer were used in a 28 cycle PCR (5 cycle used on PCR products) using the HotStarTaq Plus Master Mix Kit (Qiagen, USA). PCR products were checked using 2 % agarose gels in order to ascertain the relative intensity of the bands and the success of the amplification. Multiple samples were pooled together (for instance 100 samples) in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were then purified using calibrated Ampure XP beads. Following this the pooled and purified PCR products are used to prepare Illumina DNA libraries. The PCR products were sequenced at MR DNA (www.mrdnalab.com, Shallowater, Texas, USA) using Paired-End Illumina MiSeq according to the manufacturer's guidelines.

3.5.2 Sequence processing

A gene library is comprised of the total number of reads post DNA sequencing and is frequently referred to as the depths of coverage (McMurdie and Holmes, 2014). Library size can vary greatly within sequencing runs and thus methods (generally normalisation methods such as rarefaction; Sanders, 1968) for processing the generated sequence libraries have been developed and are essential for quality purposes (McMurdie and Holmes, 2014). Thus processing of sequences is vital in order to produce reliable unbiased results. Additionally, technical replicates become important due to this issue.

Initial sequence processing (demultiplexing) was performed using a binning program available from MR DNA (http://www.mrdnafreesoftware.com/) on the fastq file provided containing all sequenced and combined contigs with barcodes and primers, thus unlike the study done by Valverde *et al.* (2016) it was unnecessary to process the raw data using *shhh.flows* due to contigs having been provided by MR DNA so that only the primer and barcoding regions need be removed. This follows that the first step in quality processing of the binned gene sequences without barcodes and primers, was performed using the online tool Galaxy Community Hub (https://usegalaxy.org/) whereby the quality of each technical replicate sequence library was

determined and deemed satisfactory. Each technical replicate was then quality processed using the online tool Ribosomal Database Project (RDP; http://pyro.cme.msu.edu/) so that only the sequences within a specific size range were kept.

3.5.2.1 16S region

Following initial quality processing, chimeras were then removed from all technical replicate sequence libraries using the uchime tool on RDP. Following which the total number of sequences for each library was compared and technical replicate libraries were sub-sampled (rarefied) so that all the sequences were equal to the shortest sequence in order to equalise sampling depth and allow for samples to be comparable to one another. This was performed using the *sub.sample* command in mothur (v.1.35.1) (Schloss *et al.*, 2009).

OTU tables were then constructed using the *Classifier* on RDP as well as rarefaction curves (*Rarefaction* under Analysis Tools) and the Chao1 and Shannon indices (*Shannon & Chao1 index* under Analysis Tools) were ascertained.

3.5.2.2 ITS2 region

It was determined previously using mothur with both the *chimera.uchime* and *chimera.vsearch* commands that the ITS2 sequence libraries did not contain chimeras. The total number of sequences for each library was thus compared and technical replicate libraries were sub-sampled (rarefied) to be equal to the size of the smallest library in order to equalise sampling depth and allow for samples to be comparable to one another. This was performed using the *sub.sample* command in mothur.

OTU tables were then constructed using the *Classifier* on RDP but rarefaction curves and Chao1 and Shannon indices were ascertained using mothur. This was done as follows, using mothur, OTUs were constructed from distances calculated following exact pairwise alignment (*pairwise.seqs*) by the Needleman-Wunsch algorithm (Huson *et al.*, 2007) and then clustered using the *cluster.seqs* command. The clustered sequences were then used to ascertain rarefaction

curves, Chao1 and Shannon indices using the *rarefaction.single* and *summary.single* commands, respectively.

3.5.3 Beta diversity analysis

Modified OTU tables were then used to produce nMDS plots with weighted UniFrac distances and heat maps and using R v3.4.3 (R Foundation for Statistical Computing; http://www.R-project.org). Although attempted, differences in community structure could not be assessed by ANOSIM analysis using the *anosim* function in vegan (cran.r-project.org/package = vegan) as no R factor was produced. The compositions of major fungal and bacterial genera were compared using UPGMA clustering on Hellinger-transformed Bray-Curtis or Morisita-Horn distances together with a heatmap of abundance data created with *heatmap.2* in gplots.

3.6 Results

3.6.1 Initial processing

Once initial sequence processing was performed and ambiguous bases and singleton and short sequences were removed, and in the case of 16S, chimeras removed, we were left with the number of sequences illustrated in Figure 3.2.

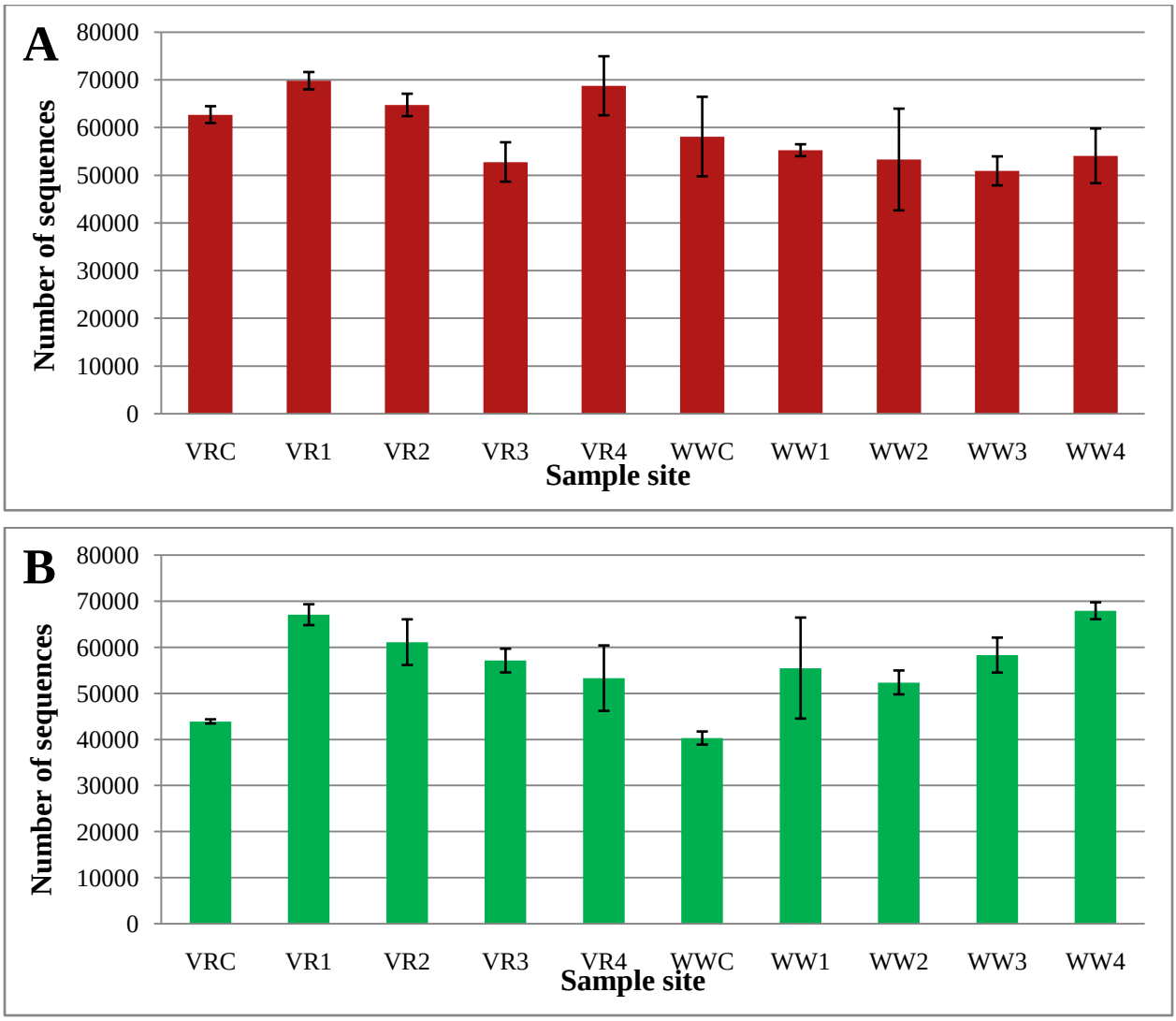
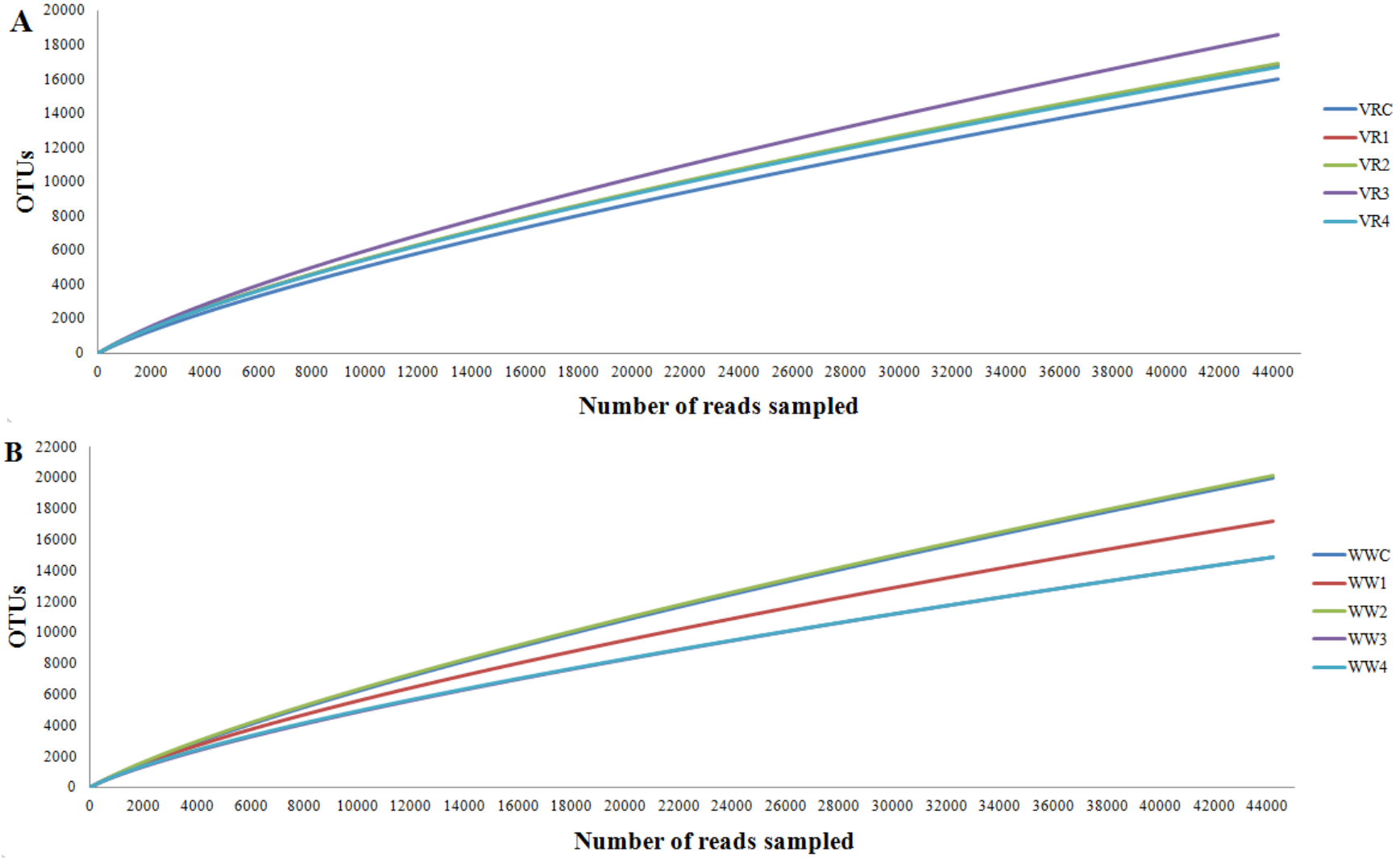


Figure 3.2: Bar graphs illustrating total number of sequences remaining post initial processing for A) 16S V1-V3 and B) ITS2 for DNA isolated from soil from different niche sites.

It would appear that a trend develops in the case of ITS2 sequences whereas for the 16S V1-V3 sequences no trend is apparent. The controls for the ITS2 have the lowest number of remaining sequences when compared to the sample sites for the ITS2 region. Once sub-sampled all sequence libraries for ITS2 region contained 39270 sequences and all the sequence libraries for 16S V1-V3 region contained 44231 sequences.

3.6.2 Alpha diversity

Rarefaction curves were constructed at a 0.03 cut-off in order to compare and ascertain the depth of sampling and are shown in Figure 3.3.



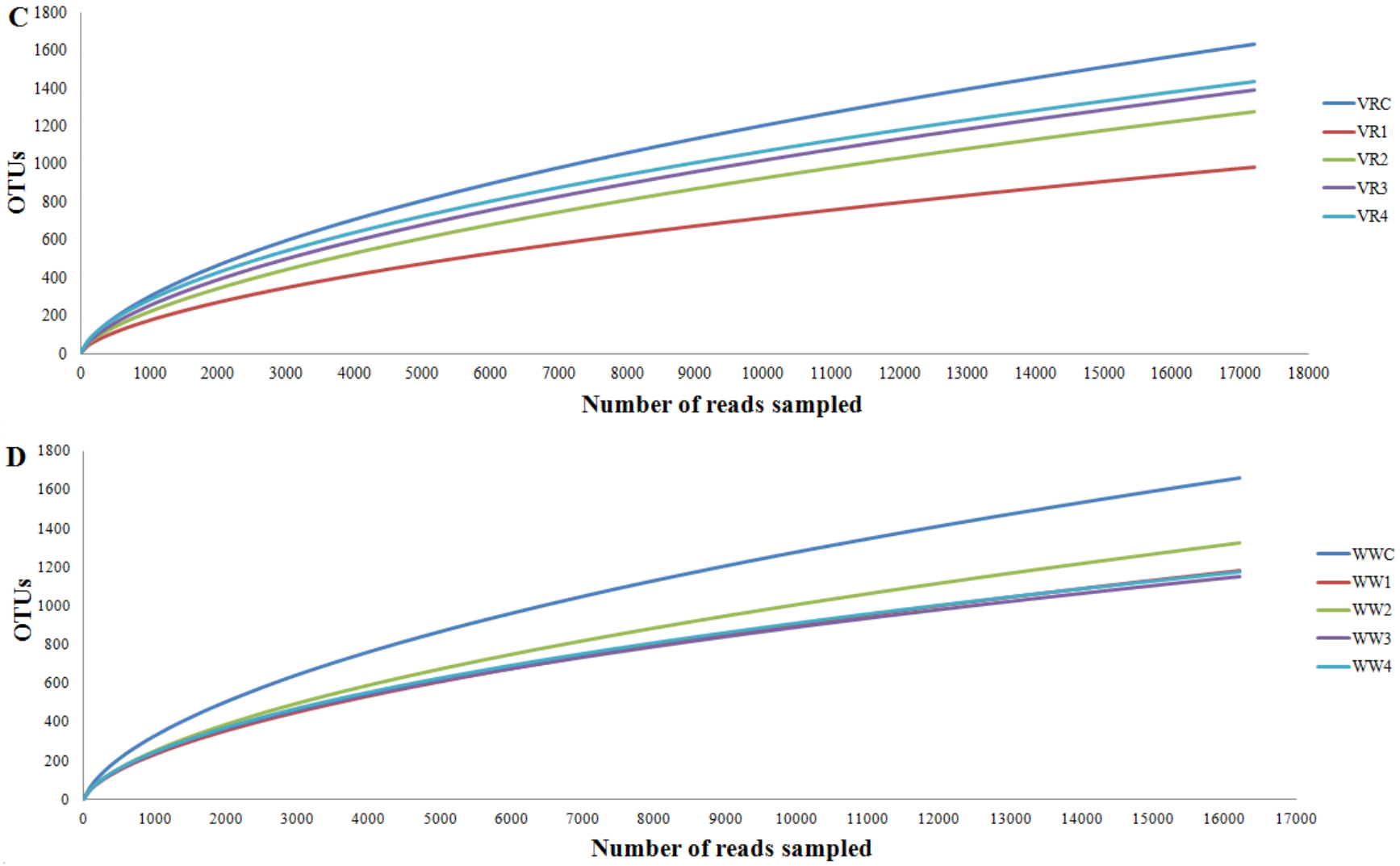


Figure 3.3: Rarefaction curves describing OTUs as a function of sampling effort for OTUs defined at a 0.03 distance cutoff of genes sequenced from soil DNA from different niche sites with A and B) 16S V1-V3 and C and D) ITS2.

As can be seen from the generally shallow curves that although the niche sites were well sampled they did not appear to be completely/fully sampled as the curves did not completely level out and continued to climb with increasing sequencing effort. It would also appear that the 16S sequences had steeper rarefaction curves and were thus not sampled as well as the ITS2 sequences.

Out of all the rarefaction curves, the graph illustrating the 16S sequences from VR operations is the only one that has the curve of the control clustering with sample sequences and the sample from VR3 is the curve that does not cluster with the other curves.

The Chao1 and Shannon indices were determined for both the 16S and ITS2 regions at a 0.03 cutoff and are represented in the tables below. The indices determined using mothur (the ITS2 sequences) followed a method that performed clustering through several iterations and the cluster values (OTU number) as well as the indices were those of the final iteration and were thus lower than those performed by RDP on the 16S V1-V3 region.

Table 3.1: Measures of alpha diversity for the 16 V1-V3 gene region sequenced from soil from different niche sites at 0.03 cut-off for OTUs and/or clustering.

Sample site	Number of sequences	Cluster/OTUs	Chao1	Shannon (H')
VRC	44231	16013	58590.3792	8.084215
VR1	44231	16833.5	58579.15045	8.6882
VR2	44231	16912	58649.41172	8.684595
VR3	44231	18610	70576.34826	8.926365
VR4	44231	16723	57134.97468	8.63155
WWC	44231	20017.5	76768.60345	9.017725
WW1	44231	17229.5	62533.95709	8.76787
WW2	44231	20162	76815.79227	9.122495
WW3	44231	14912	50095.42369	8.27733
WW4	44231	14857	52756.5619	8.397785

Table 3.2: Measures of alpha diversity for the ITS2 gene region sequenced from soil from different niche sites at 0.03 cut-off for OTUs and/or clustering.

Sample site	Number of sequences	Cluster/OTUs	Chao1	Shannon (H')
VRC	39270	1787	3880.796	5.1751235
VR1	39270	986.5	2494.3765	3.6592165
VR2	39270	1356	3295.6792	4.4842245
VR3	39270	1512.5	3511.653	4.727112
VR4	39270	1556	3482.927	5.131101
WWC	39270	1859	3957.1119	5.414581
WW1	39270	1250	2743.2029	4.585419
WW2	39270	1416	3283.4855	4.7484285
WW3	39270	1201.5	2713.833	4.5256455
WW4	39270	1210	2549.1873	4.5461125

According to the Chao1 index for both the 16S and ITS analyses, the number of OTUs is less than that estimated by the Chao1, by 3.36-3.85 and 2.11-2.53 times respectively. The Shannon index for the ITS analysis is above average whereas the index for the 16S analysis is at least double the value expected. It is also noteworthy to mention in the case of ITS2 analysis the Chao1 indices have higher estimates for both controls when compared to the indices from the experiment niche sites.

3.6.3 Beta diversity

Upon review of the OTU tables generated by RDP several taxa were removed in order to create a view of the core biome (taxa that occur over all niches/biomes analysed), thus any taxa that didn’t occur in 1 or more niche sites from the Operation sites (had a value of 0 sequences) were completely removed from the OTU tables. nMDS plots from all niche sites and controls from both Operation sites were constructed and are shown in Figures 3.4 and 3.5.

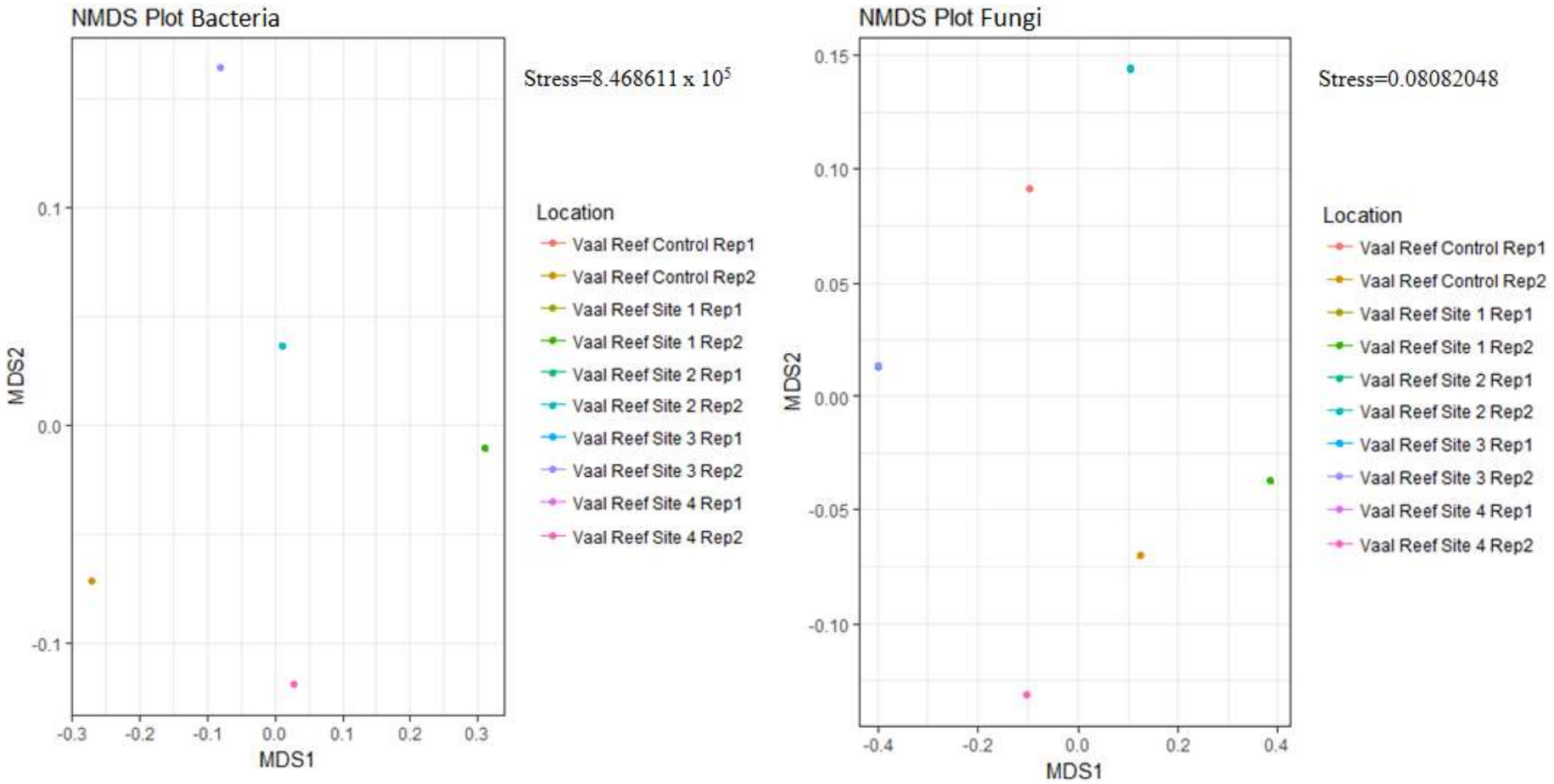


Figure 3.4: nMDS ordination plot (UniFrac dissimilarity matrix). Each point represents either the bacterial or fungal community of an individual sample replicate from Vaal River Operation site.

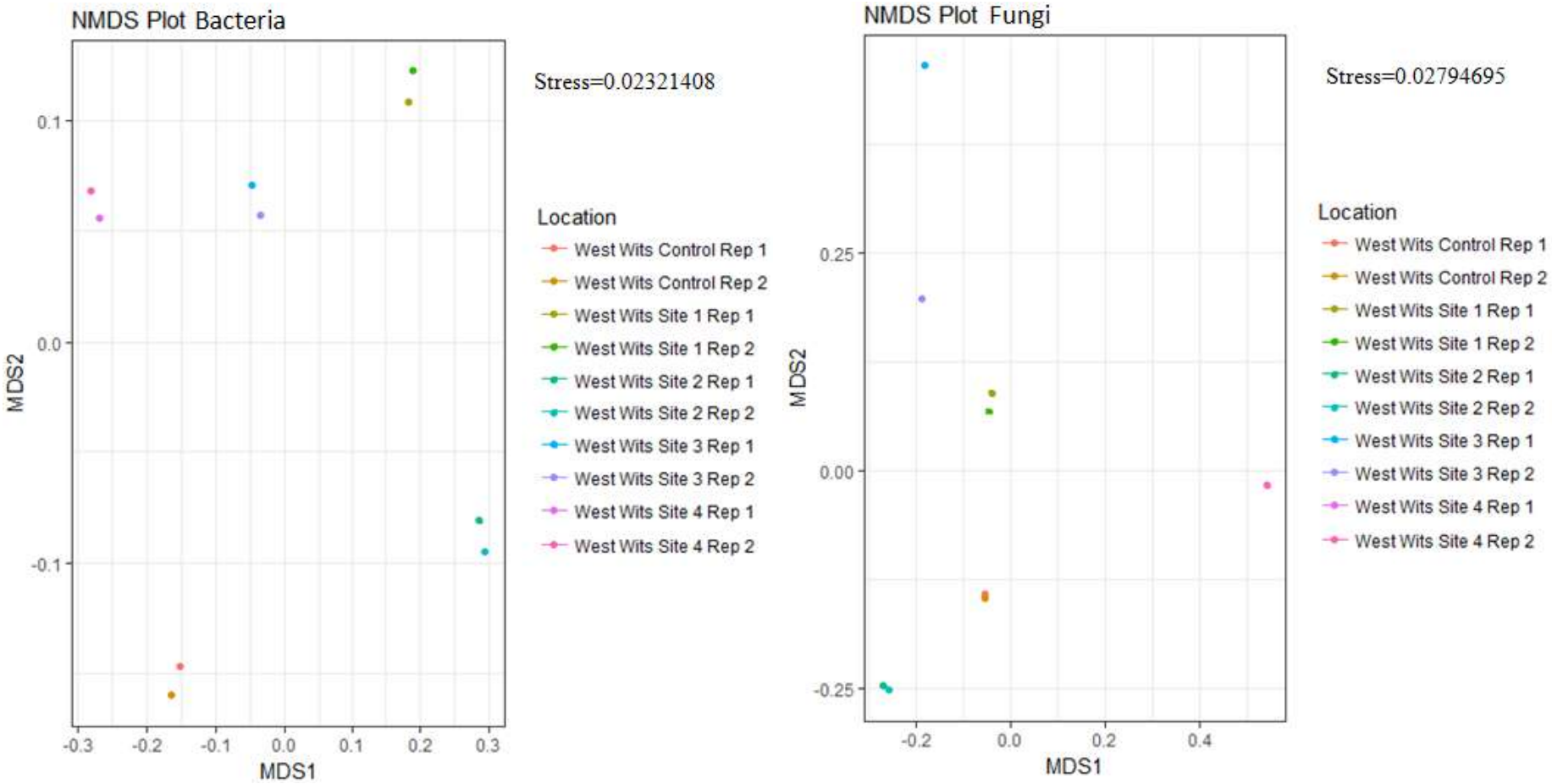
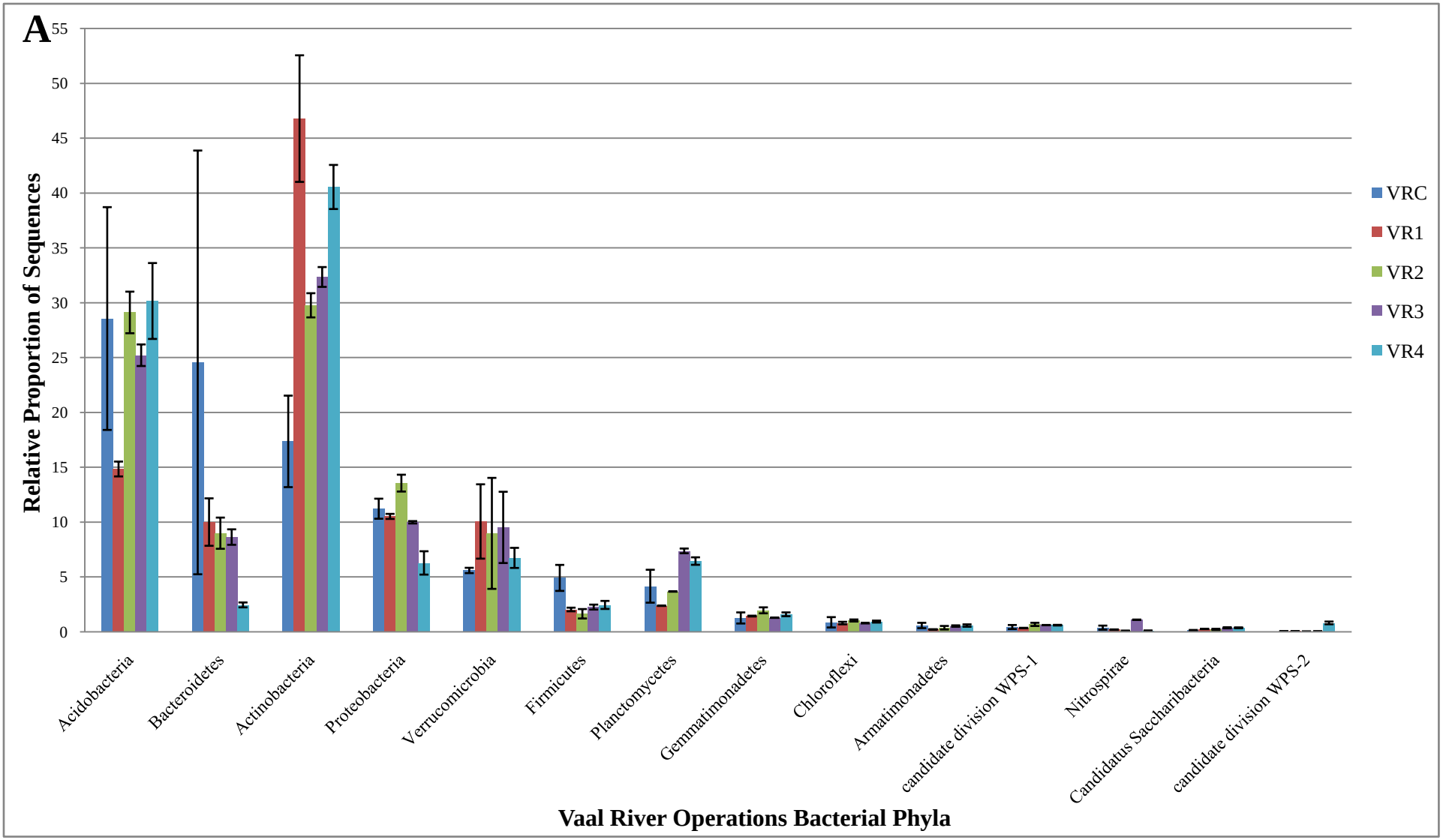


Figure 3.5: nMDS ordination plot (UniFrac dissimilarity matrix). Each point represents either the bacterial or fungal community of an individual sample replicate from West Wits Operation site.

From the nMDS plots no apparent patterns of clustering occurred and as a result ANOSIM could not be determined. From the alpha diversity indices it would have been expected that the control samples would have been separate from the experiment samples and the experiment samples would have clustered together, however, this was not observed. Several technical replicates also did not align with each other in the case of 16S from WW Operations and ITS2 from both VR and WW Operations. The technical replicates for 16S were still closely related unlike some of the replicates from the ITS2 where VRC replicates and WW3 replicates were located in different locations on the nMDS plot in Figure 3.4 and 3.5, respectively. OTU tables containing the core microbiome data and had discrepant taxa removed did not produce reliable nMDS plots due to the fact that data provided by the modified table was insufficient. Taxa were considered to be discrepant when there was an apparent and rather distinct difference between replicates such that the number of sequences between replicates was greater than double.

The overall bacterial communities at the phyla level were analysed, while the overall fungal communities at the class level were analysed and are illustrated in Figures 3.6 and 3.7. Phylum level taxa were used in the case of bacteria and class level taxa were used in the case of fungi as they portrayed the most information in a summarised form. From the graphs the bacteria in the core microbiome are represented by 14 and 15 phyla from VR and WW Operations, respectively. In the case of fungi, 5 phyla were identified in the core microbiome and the phylum that occurred in the lowest relative proportion was Chytridiomycota (below 0.5 % in all niche and control sites) and the most abundant phylum was predominantly Ascomycota (62-91 %) followed by either Basidiomycota or Zygomycota with the exception of VR1 which showed Zygomycota (66 %) as the most abundant phylum and WW4 which showed Fungi-unidentified (45 %) (only 1 % above Ascomycota at 44 %) as the most abundant phylum (data not shown).


A
Relative Proportion of Sequences
55
50
45
40
35
30
25
20
15
10
5
0
VRC
VR1
VR2
VR3
VR4
Acidobacteria
Bacteroidetes
Actinobacteria
Proteobacteria
Verrucomicrobia
Firmicutes
Planctomycetes
Gemmatimonadetes
Chloroflexi
Armatimonadetes
candidate division WPS-1
Nitrospirae
Candidatus Saccharibacteria
candidate division WPS-2
Vaal River Operations Bacterial Phyla

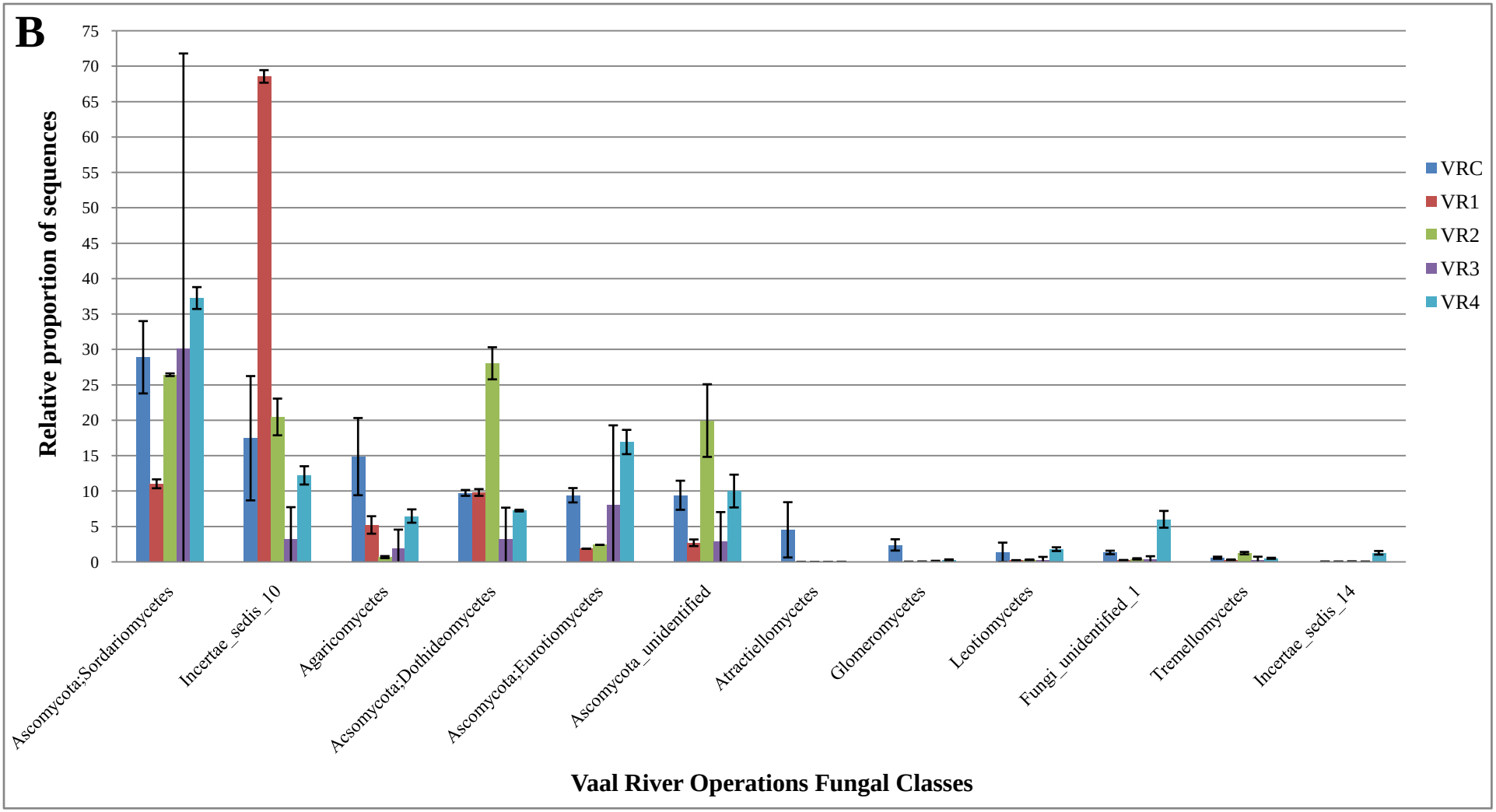
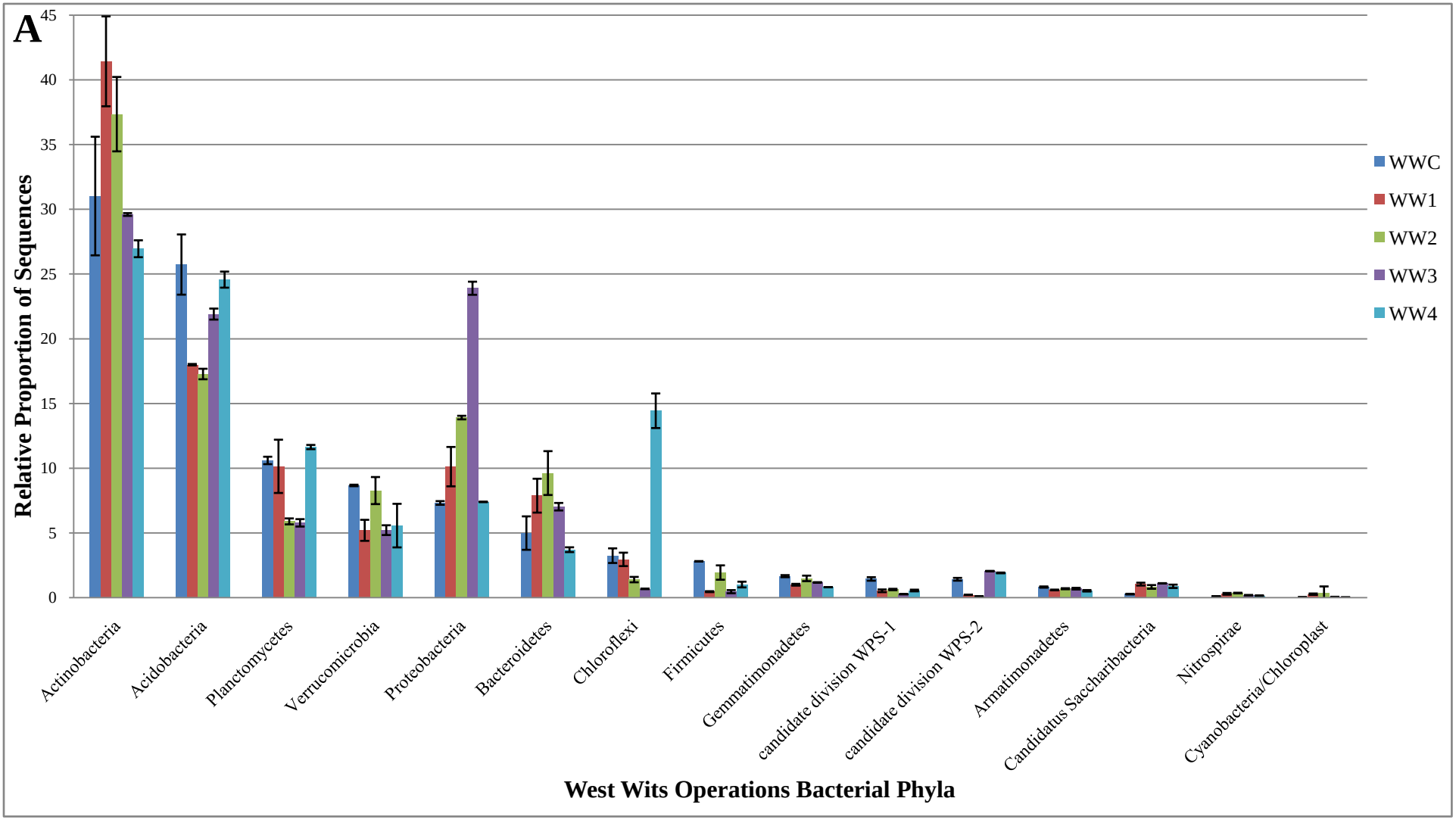


Figure 3.6: Bar graphs illustrating relative proportions of the A) bacteria and B) fungi associated with the *Vachellia karroo* rhizosphere from the Vaal River Operations site.


A
Relative Proportion of Sequences
45
40
35
30
25
20
15
10
5
0
WWC
WW1
WW2
WW3
WW4
Actinobacteria
Acidobacteria
Planctomycetes
Verrucomicrobia
Proteobacteria
Bacteroidetes
Chloroflexi
Firmicutes
Gemmatimonadetes
candidate division WPS-1
candidate division WPS-2
Armatimonadetes
Candidatus Saccharibacteria
Nitrospirae
Cyanobacteria/Chloroplast
West Wits Operations Bacterial Phyla

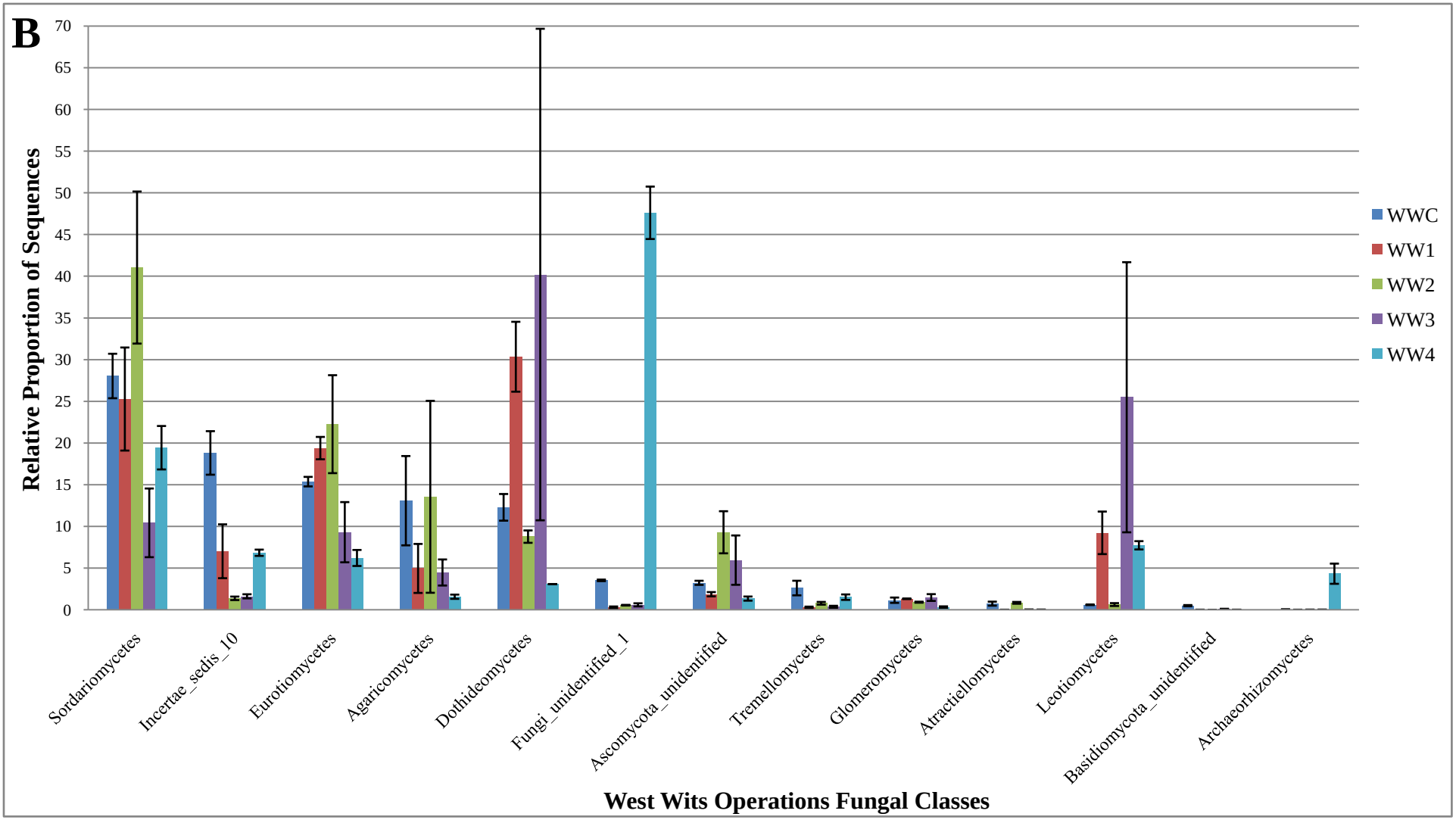


Figure 3.7: Bar graphs illustrating relative proportions of the A) bacteria and B) fungi associated with the *Vachellia karroo* rhizosphere from the West Wits Operations site.

Heat maps were constructed using OTU tables from the core biome of both major bacterial and fungal genera (proportion values of 1 % and above) (Figures 3.8 and 3.9) following which taxa that had large discrepancies between sequence numbers (by at least 2 times difference) between replicates were removed (Figures 3.10 and 3.11). The technical replicates were maintained in the construction of the heat maps so that it may be observed whether the technical replicates paired together and were thus reliable.

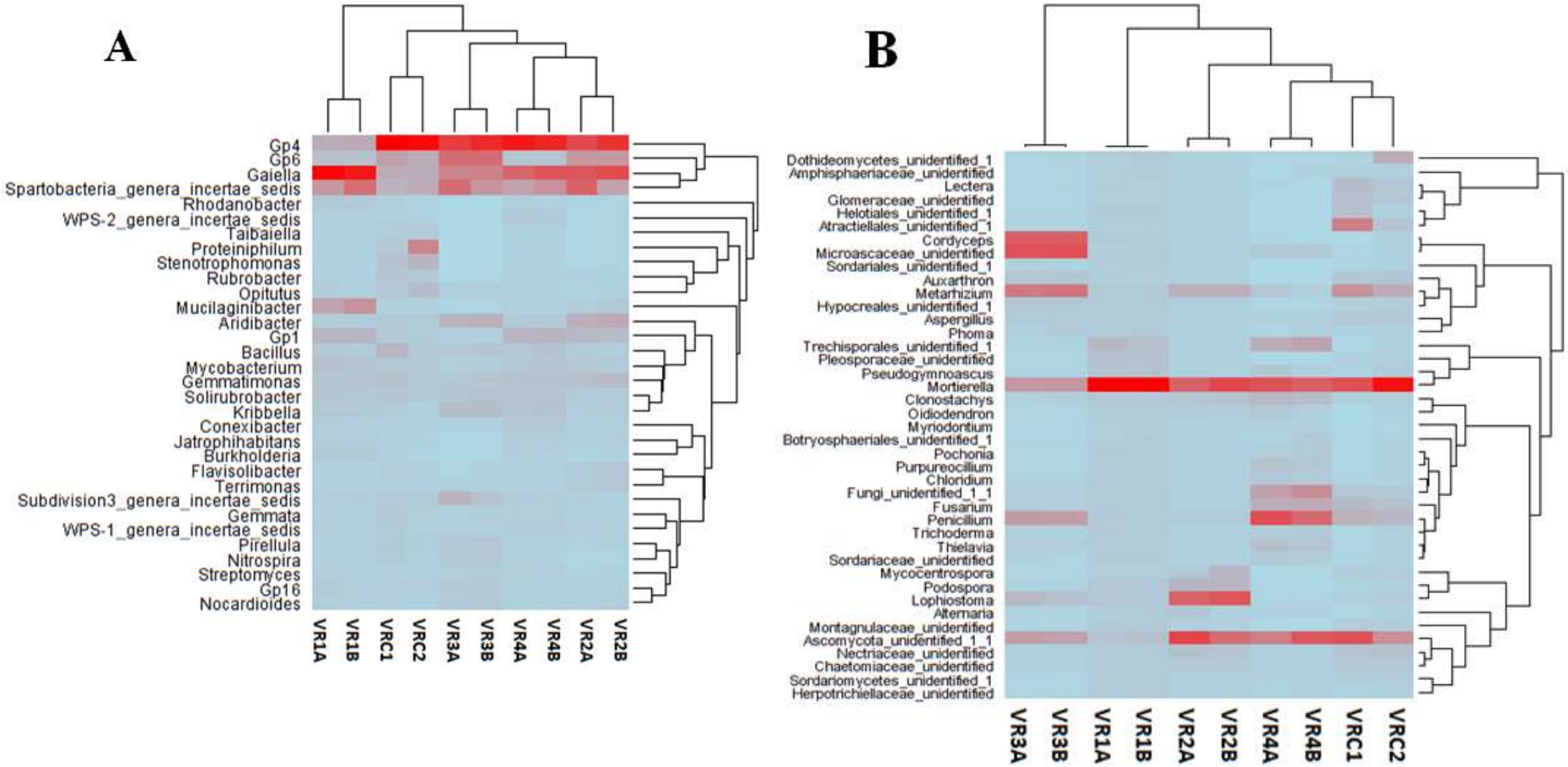


Figure 3.8: Heatmaps displaying the most abundant taxa of A) bacteria and B) fungi of the core microbiome of soil of controls and niche sites from Vaal River Operations. Samples were clustered based on the percent relative abundance of the dominant taxa shown in rows in the figure. Key for colour scale: Increase in red in the increase in abundance and increase in light blue is decrease in abundance.

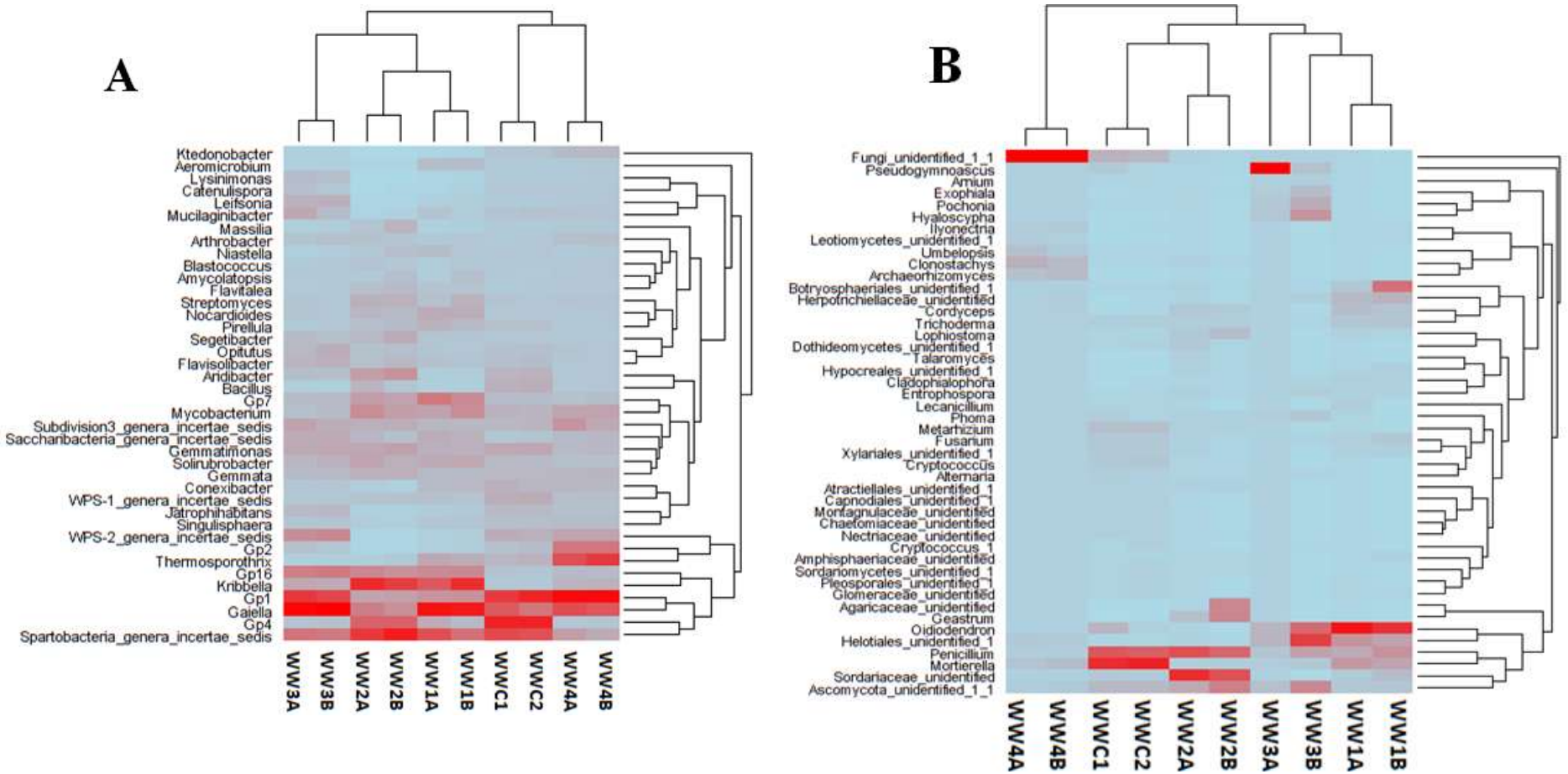


Figure 3.9: Heatmaps displaying the most abundant taxa of A) bacteria and B) fungi of the core microbiome of soil of controls and niche sites from West Wits Operations. Samples were clustered based on the percent relative abundance of the dominant taxa shown in rows in the figure. Key for colour scale: Increase in red in the increase in abundance and increase in light blue is decrease in abundance.

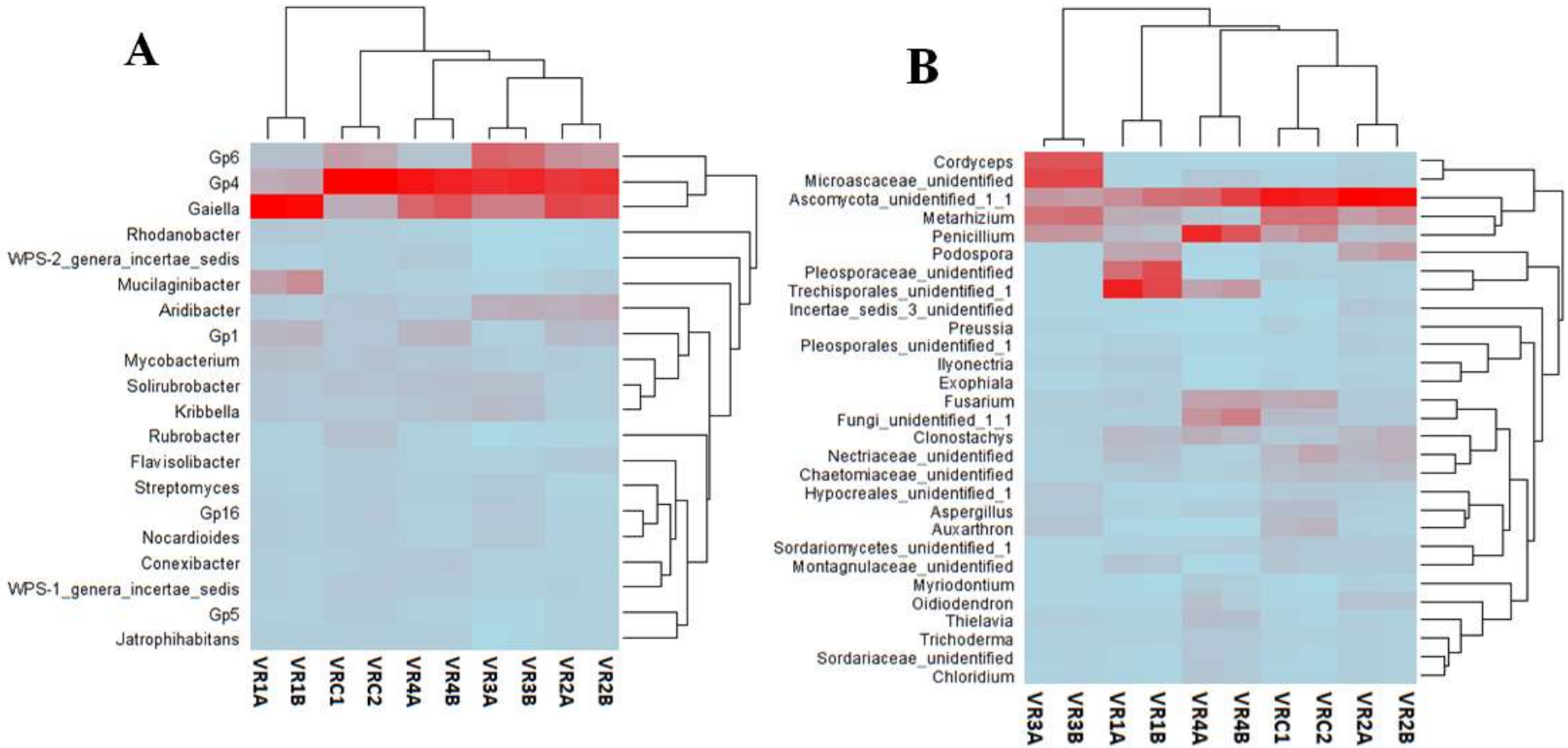


Figure 3.10: Heatmaps displaying the most abundant taxa of A) bacteria and B) fungi of the core microbiome after removal of discrepant taxa of soil of controls and niche sites from Vaal River Operations. Samples were clustered based on the percent relative abundance of the dominant taxa shown in rows in the figure. Key for colour scale: Increase in red in the increase in abundance and increase in light blue is decrease in abundance.

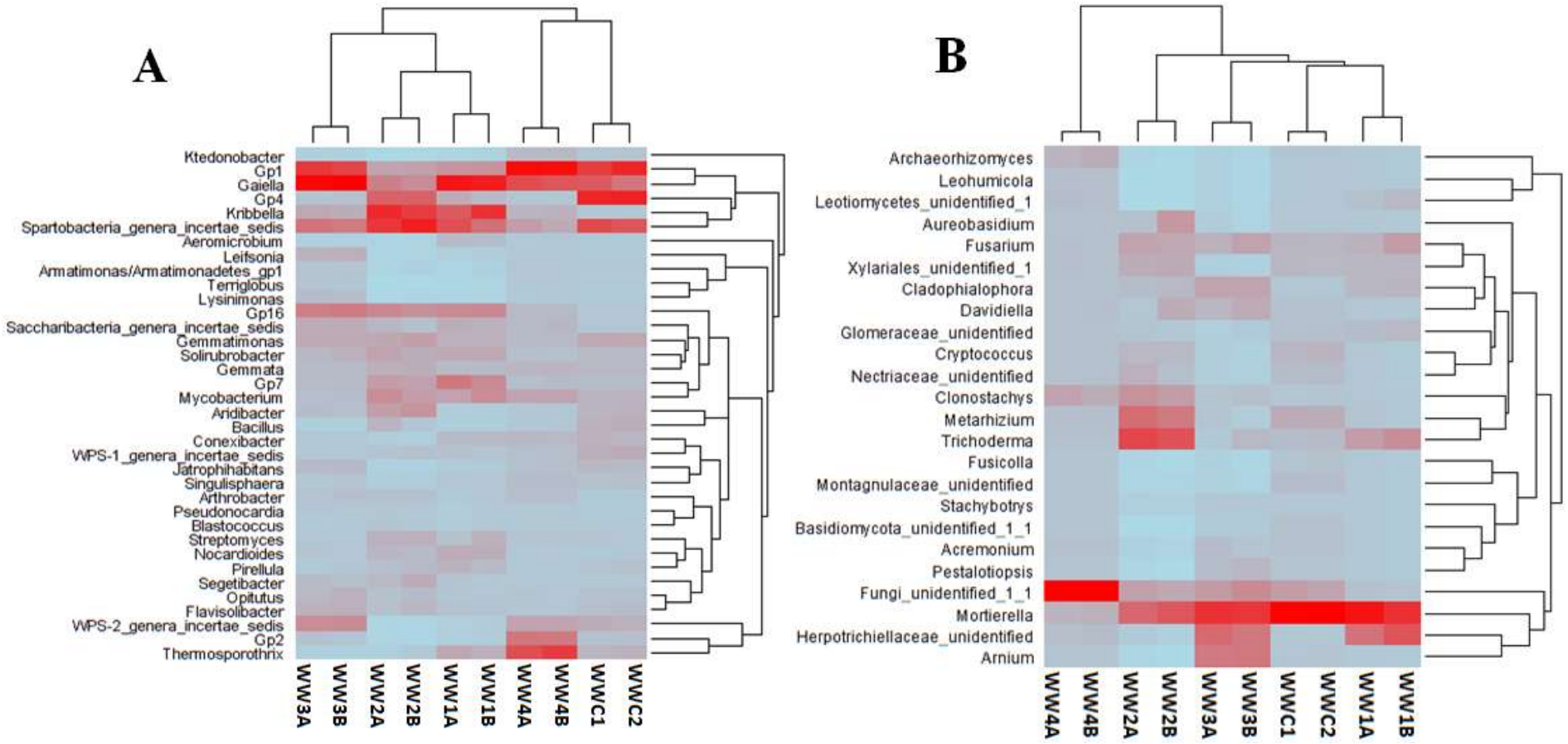


Figure 3.11: Heat maps displaying the most abundant taxa of A) bacteria and B) fungi of the core microbiome after removal of discrepant taxa of soil of controls and niche sites from West Wits Operations. Samples were clustered based on the percent relative abundance of the dominant taxa shown in rows in the figure. Key for colour scale: Increase in red in the increase in abundance and increase in light blue is decrease in abundance.

In all heat maps it was seen that technical replicates were paired together for sample sites; however, although the technical replicates for fungal samples from WW3 prior discrepant taxa removal paired up (Figure 3.9B), they paired up slightly differently to the other replicates with one branch separating before the other branch (i.e. different branch length).

Once discrepant taxa were removed the number of bacterial and fungal genera decreased. Interestingly, there was a greater decrease in fungal genera than bacterial genera, particularly in the case where only one bacterial genus was removed from the WW Operations samples.

From the heat maps only 6 of the 14 bacterial phyla were represented in the dominant genera from VR Operations and 12 of the 15 bacterial phyla were represented in the dominant genera from WW Operations. Regarding the VR Operations, the Actinobacteria were represented by 9 genera; the Acidobacteria were represented by 6 genera; the Bacteroidetes were represented by 2 genera; and the Proteobacteria, Candidate division WPS-1 and Candidate division WPS-2 were each represented by a single genus. With regards to the WW Operations, the Actinobacteria were represented by 14 genera; the Acidobacteria were represented by 7 genera; the Planctomycetes were represented by 3 genera; the Chloroflexi, Bacteroidetes and Verrucomicrobia were represented by 2 genera; and the Armatimonadetes, Candidatus Saccharibacteria, Gemmatimonadetes, Firmicutes, Candidate division WPS-1 and Candidate division WPS-2 were all represented by a single genus.

In the case of the fungal heat maps all 5 phyla were represented by genera before discrepant taxa were removed; however, after removal, 5 phyla were represented in WW Operations and only 3 phyla were represented in VR Operations with both Glomeromycota and Zygomycota were no longer represented in the VR Operations. For the phyla that remained at VR Operations, the Ascomycota were represented by 27 genera and both Basidiomycota and Fungi_unidentified were represented by a single genus each. For the WW Operations, the Ascomycota were represented by 19 genera; the Basidiomycota were represented by 2 genera; and Glomeromycota, Zygomycota and Fungi_unidentified were each represented by a single genus.

The heat maps also portray the phylogenetic relatedness of microbial communities of soils from different operation sites and controls. Interestingly, the microbial community profiles of the control soils did not cluster separately from soils of the operation sites, and in each heat map soil from an experimental site clustered separately from the rest of the replicates.

3.7 Discussion

3.7.1 Initial processing

After the initial sequence processing a pattern was observed from the ITS2 sequences and although a pattern was observed here it was not observed in further analyses. The confidence of the measures changes with sample size (Hughes *et al.,* 2001). It would appear that in this study a large quantity of sequences were produced even after the initial processing and subsampling. Thus possible restrictions in the confidence of measures may be as a result of measures employed themselves. An example of a previous study, by Valverde *et al.* (2016), only utilised 3712 16S rRNA sequences and 27810 ITS2 sequences, which is far lower than each individual replicate sample in this case, especially since the smallest sample size for 16S rRNA (V1-V3 region) was 44231 sequences and smallest sample size for the ITS2 region was 39270 sequences. It is possible that the abundances of genes amplified by PCR are not true reflections of the original template DNA since primers have different binding and elongation efficiency (Raeymaekers, 1995; Reysenbach *et al.*, 1992).

3.7.2 Alpha diversity

Interestingly, despite the ITS2 control samples having the lowest number of sequences after initial processing they had the highest number of clusters/OTUs after subsampling. According to the tables the number of the OTUs clustered for the 16S rRNA and ITS2 genes is less than is possible (according to the Chao1 index), however, the value of the Shannon index implies that both the richness and the evenness of community is better than previous studies. Unfortunately, the Choa1 and Shannon index in this case were ascertained using the sequence data and not the classified data. The benefit of this is that the indices are not limited by sequence databases, however, the disadvantage is that until classifying sequence databases are comprised of all organism data, beta analyses are restricted to what is currently available.

3.7.3 Beta diversity

The fact that initial clustered sequences/OTUs (alpha diversity indices) did not necessarily match the clustering from the classified OTU table (i.e. classification reference database), ultimately suggests that the reference databases are lacking in information and in order for the beta diversity to be comparable to the alpha diversity cluster parameters serious improvements need to be made in the databases. Frequently taxonomy counts are utilised to estimate the relative abundance of organisms (Cox *et al.*, 2017). Metataxonomy addresses richness estimation and does not address how the diversity relates to functional diversity (Hughes *et al.*, 2001).

The ordination of the taxonomic profiles did not reveal a clear separation between control samples and experiment niche samples, which may suggest that the controls may not serve as appropriate taxonomic controls. The lack of differences between controls and experiments may be due to the fact that the plant species was consistent between all samples, and the lack of an ordination pattern may be due to the experiment samples having no consistent parameters. Importantly, this is supported by research by Prober *et al.* (2015), whereby it is reported that the beta soil microbial diversity and not the alpha diversity is influenced by the plant community. Although in this case empirical evidence could not be provided as other plants were not analysed from this environment. Interestingly, a previous study on demonstrated that microbial communities differed between terrestrial biomes (Fierer *et al.*, 2012) and the lack of differences could also indicate that the control and experimental areas were similar environments/biomes.

Since they form a tripartite symbiotic association with *V. karroo*, it was expected to observe rhizobia bacteria (classes within the Proteobacteria) and Glomeromycota in the rhizosphere. This was observed when looking at the overall bacterial phyla and fungal classes on the heat maps, however, once discrepant taxa were removed the representative genera for both the rhizobia bacteria and the Glomeromycota were no longer observed on the heat map. It is noteworthy to mention, however, that the heat maps only show the dominant taxa within the core biome and these bacteria and fungi may still occur as part of the rare taxa.

Microbial taxa that occur in the majority of soil environments are considered to be "cosmopolitan" taxa. It is likely that the microbial species that widely occur significantly contribute to the global chemical cycles. An example of a cosmopolitan taxon is Actinobacteria which represent 20-30 % of the 16S rRNA sequences amplified from total soil DNA (Fierer *et al.*, 2012; Hugenholtz *et al.*, 1998a; Prober *et al.*, 2015).

Bacterial divisions that are poorly represented by cultured organisms and are only characterised by environmental sequences are termed "candidate divisions" in order to indicate their unsubstantiated status as new bacterial divisions (Hugenholtz *et al.*, 1998a,b). Once the candidate division is satisfactorily represented by environmental sequences it can be concluded that it comprises a major bacterial group (Hugenholtz *et al.*, 1998a). Here 2 candidate divisions were observed with the abbreviation WPS and this stands for Wittenberg polluted soil. It is likely that it may become a major bacterial group since it has been identified from soil (in South Africa) that is distant from the original soil (in Germany).

The rhizosphere bacterial communities of *V. karroo* from VR Operations was predominantly comprised of Acidobacteria (15-30 %), Actinobacteria (17.4 % in the case of VRC and 30-47 % for the other samples), Bacteroidetes (24.6 % in the case of VRC and 2.4-10 % for the other samples), Proteobacteria (6.3-13.5 %), Verrucomicrobia (5.6-10.1 %), Firmicutes (1.6-4.9 %), Planctomycetes (2.4-7.4 %), Gemmatimonadetes (1.3-2 %) and the remainder of the phyla (Chloroflexi, Armatimonadetes, Nitrospirae, Candidatus Saccharibacteria, candidate division WPS-1 and candidate division WPS-2) were less than 1 % except for VR3 that had Nitrospirae (1.1 %). The numbers all represent the average percentage of sequences across 14 phyla. For WW Operations the bacterial communities were predominantly comprised of Actinobacteria (26.9-41.4 %), Acidobacteria (17.3-25.7 %), Planctomycetes (5.8-11.6 %), Verrucomicrobia (5.2-8.7 %), Proteobacteria (23.9 % in the case of WW3 and 7.3-13.9 % for the other samples), Bacteroidetes (3.7-9.6 %), Chloroflexi (14.4 % for WW4 and 0.7-3.2 % for the other samples), Firmicutes (0.5-2.8 %), Gemmatimonadetes (0.8-1.7 %), and the remainder of the phyla (candidate division WPS-1, candidate division WPS-2, Armatimonadetes, Candidatus Saccharibacteria, Nitrospirae and Cyanobacteria/Chloroplast) were around 1 %. The numbers all represent the average percentage of sequences across 15 phyla. Other studies do not appear to

have the same level and depth of diversity analysis and only describe 3-6 phyla unlike this study that demonstrates more than double this. The majority of these studies consistently describe either Acidobacteria or Actinobacteria as the dominant bacteria phyla isolated from soil (Bulgarelli *et al.*, 2012; DeAngelis *et al.*, 2008; Gottel *et al.*, 2011; Inceoglu *et al.*, 2011; Knief *et al.*, 2011; Lundberg *et al.*, 2012; Manter *et al.*, 2010; Mendes *et al.*, 2010; Rasch *et al.*, 2006; Robin *et al.*, 2006; Uroz *et al.*, 2010; Valverde *et al.*, 2016; Weinert *et al.*, 2011). Other studies also describe Proteobacteria, Firmicutes, Plancomycetes and Bacteroidetes as predominant soil microbes (Bouffaud, *et al.*, 2012; Bulgarelli *et al.*, 2012; DeAngelis *et al.*, 2008; Gottel *et al.*, 2011; Inceoglu *et al.*, 2011; Knief *et al.*, 2011; Lundberg *et al.*, 2012; Manter *et al.*, 2010; Mendes *et al.*, 2011; Sessitsch *et al.*, 2012; Uroz *et al.*, 2010; Valverde *et al.*, 2016; Weinert *et al.*, 2011).

The rhizosphere fungal communities of *V. karroo* from VR Operations was predominantly comprised of Ascomycota (25.6-77.1 %, Sordariomycetes (11-37.3 %), Dothideomycetes (3.2-28 %), Eurotiomycetes (1.9-17 %), Ascomycota_unidentified (2.7-20 %), Leotiomycetes (0.2-1.8 %), Incertae_sedis_14 (0.02-1.3 %)); Zygomycota (Incertae_sedis_10; 68.6 % for VR1 and 3.2-20.5 % for the other samples); Basidiomycota (1.91-20 %, Agaricomycetes (0.7-14.9 %), Atractiellomycetes (0.01-4.5 %), Tremellomycetes (0.3-1.2 %)); and to a lesser degree Glomeromycota (Glomeromycetes; 0.02-2.4 %) and Fungi_unidentified (0.2-6 %). The numbers all represent the average percentage of sequences across 12 classes. For WW Operations the fungal communities were predominantly comprised of Ascomycota (42-91.42 %, Sordariomycetes (10.4-28 %), Dothideomycetes (3.1-40.2 %), Eurotiomycetes (6.2-22.3 %), Ascomycota_unidentified (1.3-9.3 %), Leotiomycetes (0.6-25.5 %), Archaeorhizomycetes (0.01-4.3 %); Zygomycota (Incertae_sedis_10; 1.4-18.8 %); Basidiomycota (3.21-15.1 %; Agaricomycetes (1.6-13.5 %), Tremellomycetes (0.3-2.6 %), Atractiellomycetes (0.01-0.8 %), Basidiomycota_unidentified (0.001-5 %)); Fungi_unidentified (47.6 % for WW4 and 0.3-3.5 % for the other samples); Glomeromycota (Glomeromycetes; 0.3-1.5 %). The numbers all represent the average percentage of sequences across 13 classes. Other studies have differing views on the fungal phyla found within soil. A review by Peay *et al.* (2016) suggests the predominant fungi within soil are Basidiomycota and mycorrhiza whereas Ascomycota are associated with aerial parts of the plants. This is contradictory to the findings here and in other studies (Gałązka and

Grządziel, 2018; Ma *et al.*, 2013) and it was shown that in fact Ascomycota was the predominant fungal taxa in the soil sampled and Basidiomycota and mycorrhiza were seen as rare taxa, however, this may be due to the choice of gene used for the identification. On the other hand, the study by Valverde *et al.* (2016) showed similar results with the exception that that study isolated Chytridiomycota.

These results are comparable to previous studies performed by Fierer *et al.* (2012) and Prober *et al.* (2015). The most abundant bacterial phyla from VR Operations follow the trend observed in the studies by both Fierer *et al.* (2012) and Prober *et al.* (2015). Interestingly, WR Operations includes Planctomycetes as a predominant phylum. Originally Planctomycetes were associated with being aquatic bacteria, however, they are now considered to be ubiquitous in soil (Buckley *et al.*, 2006; Fuerst and Sagulenko, 2011).

Overall, Actinobacteria, Proteobacteria, Acidobacteria and Verricomicrobia are ubiquitous in soil (Hugenholtz *et al.*, 1998a), they contribute to ecologically important processes in metal contaminated soils (Gremion *et al.*, 2003), and display plant growth promoting activity in revegetated uranium mine spoils (Dimkpa *et al.*, 2008; Dimkpa *et al.*, 2009). Proteobacteria were relatively low in all soils independently on the sampling point and operation area, and including relative controls, which contrasts with the literature on soil microbial diversity where Proteobacteria generally dominate (Spain *et al.*, 2009). This result is also in contrast with the previous findings on the relatively high abundance of Proteobacteria capable of Au oxidation in gold mine tailings (Akcil *et al.*, 2003; Anderson and Cook, 2004; Chang *et al.*, 2008; Garcia-Moyano *et al.*, 2015; Rastogi *et al.*, 2009; Wei *et al.*, 2009). Alternately Ascomycota and Basidiomycota function as decomposers of cellulose (the most abundant organic compound – 30-50 % of plant dry weight) with Basidiomycota also being able to degrade lignin (de Boer *et al.*, 2005; Lundell *et al.*, 2010; Lynd *et al.*, 2002). Some Basidiomycota also have the ability to sequester toxic metals such as Cd, Co, Pb, Zn and Hg in their fruiting bodies (Baldrian *et al.*, 2003).

3.8 Conclusion

It has been suggested that the most beneficial rehabilitation asset on mine sites is the top 10 cm of soil, where 50 % of microbial biomass resides. Hence not knowing or understanding the microbial status of the soil results in only having part of the picture of it as an asset (Wildman, 2017). Thus the purpose of this study was to evaluate the *V. karroo* rhizosphere microbial diversity of areas surrounding mine tailings through preliminary molecular analysis.

Once the phylogenetic diversity of the microorganisms in these environments has been described, the adaptation, physiological and metabolic potential of said microbial communities can then ultimately be ascertained/revealed (Xie *et al.*, 2011). Methods for ascertaining the physiological and metabolic characteristics of the microorganism community include metatranscriptomics, metaproteomics, functional gene arrays, metagenomics and novel isolation methods, among others (Cardenas and Tiedje, 2008).

The establishment of woody vegetation takes longer than grasses, however, once established, woody vegetation requires very little input/maintenance whereas grasses require continuous input and maintenance and often perish without anthropogenic assistance despite being established (Straker *et al*., 2007; Weiersbye *et al.*, 2006). This is important when considering this study as the host plant of the rhizosphere was a woody legume (*V. karroo*) and it is important to determine the rhizobiome of this plant as it may be utilised to aid in the establishment (with regards to success rates and time taken) of this woody plant amongst others in other environments with similar adverse conditions.

Additionally amending topsoil on mine spoils with hay/straw and processed sewage sludge is more effective in stimulating bacterial growth than simply inoculating topsoil with bacteria (Lindemann *et al.*, 1984), therefore it may be suggested that implementing the method of amending topsoil alongside the addition of microbes identified from similar sites would achieve the greatest efficiency of land reclamation. However, further research is required and should be performed to decisively determine if that is true. It has also been shown that the addition of chitin to soil promotes bacteria and fungi that posses the ability to degrade pathogenic fungal cell walls

and can therefore naturally improve soil suppressiveness against plant pathogens (Frąc *et al.*, 2018). Thus this can be used alternatively to fungicides that eliminate all fungi, including beneficial species.

Several microorganisms that were expected to be present in greater quantities were poorly observed from the genes utilised and may be better represented by alternate gene regions. However, it would appear that in this study there was a greater diversity of microorganisms observed associated with *V. karroo* rhizosphere than in previous studies analysing other plant species. This may demonstrate a positive step towards fully uncovering the rhizobiome in the future.

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Chapter 4

Correlation between soil chemistry and possible reasons for identified microbes

This chapter was written for the purpose of publication and thus several methods and materials and results are repeated briefly in order to present a comprehensive overview of the rhizobiome of *Vachellia karroo* located around mine storage facilities.

4.1 Introduction

In South Africa mining resulted in the construction of slime dams (also known as mine tailings, mine dumps and tailings storage facilities (TSF)) that consist of excess sludge (slime), created through the extraction of ore/mineral, for instance gold, from mined rock. The mine tailing purpose was to discard the slime since it is industrially nonfunctional. Additionally the mine tailings have been used to discard excess cyanide solution and water from underground, as well as manganese sludges from uranium plants (Adamson, 1972). Heavy metals (HMs), such as uranium, lead, arsenic and mercury, and trace elements (TEs) can be found in the mine tailings. Recently pollution by mine tailings has become a concern as, when mine tailings were first constructed none of the toxicity and the subsequent effect were known. Mine tailings may themselves be wind-dispersed or may leach toxic TEs and HMs into underground water supplies and thus leaves a large contaminated footprint (Straker *et al.*, 2007; Weiersbye and Witkowski, 2007). Toxic effects on plants include growth inhibition and protein alteration leading to the enzymatic reaction disruption (Clemens, 2006; Göhre and Paszkowski, 2006).

The main focus of research on mine tailing and affected area rehabilitation has been on the development of accessible, sustainable, affordable and environmentally-friendly techniques. One such method that incorporates these requirements is the use of plants (phytoremediation) (Krämer, 2005; Peuke and Rennenberg, 2005). This technique makes use of sustainable on-site methods which is in contrast to physical displacement or transport and storage that involves impractical, off-site methods. Research in this area is critical for the successful rehabilitation of existing and newly formed mine tailings.

Mine tailings of the Vaal River and West Wits operation sites (South Africa) are predominantly colonised by woody, semi-woody, slow-growing, perennial plant types (Weiersbye et al., 2006). Among the volunteer plants, *Vachellia karroo* is a good candidate plant for mine tailings revegetation because it can induce soil fertility through N fixation and decomposing foliage (Smit, 1999), as well as due to its ability to reducing soil erosion. Therefore, such a legume plant should facilitate a plant ecological succession. However, *V. karroo* grows slowly, and spoil inoculation with plant growth promoting microorganisms might be a strategy to speed up mine spoil reclamation (Glick et al., 2012), and therefore the first step for rhizosphere bioaugmentation interventions require the analysis of the rhizosphere microbial communities of *V. karroo*.

Any microorganisms in the soil associated with plants occur in the region called the rhizosphere. In fact, the rhizosphere rhizobiome is shaped by the plant rhizodepositions (Mendes *et al.*, 2014, Valverde *et al.*, 2016) and soil characteristics, and in turn the microbial activity can influence the plant growth by expressing specific plant beneficial traits (Mendes et al., 2013; Philippot et al., 2013). A specific ecological interaction that can help plant to colonize hostile environments is the mycorrhization that help the plant roots to exploit the soil nutrients (Berendensen et al., 2012; Frey-Klett et al., 2007; Raaijmakers et al., 2009) and mitigate the impact of potentially toxic elements (PTEs). Because the vast majority of soil microbes is unculturable (Kellenberger, 2001), soil and rhizosphere microbial communities are currently analyzed by bio-molecular methods based on the analysis of whole soil DNA, followed by sequencing and classification using genomic databases. Among the various bio-molecular approaches available to study the bacterial and fungal microbial communities, for the purpose of this study, the 16S rRNA V1-V3 hypervariable regions and the ITS2 hypervariable region will suffice in order to gain a broad view of microbial communities of the *V. karroo* rhizobiome. The ITS gene sequences were tested against other ribosomal genes. This approach was found to be the representative of multiple fungal taxa (Schoch et al., 2012) including arbuscular mycorrhizal (AM) fungi, and because the ITS gene has the universal barcode status it was used preferentially over other target gene sequences such as those amplified by the WANDA and AML primers. The rationale behind this work was to improve revegetation of mine tailings and the surrounding areas through the

rhizobiome augmentation, with the ultimate goal of recreating a self-sustaining ecosystem needing reduced stewardship over time.

4.2 Methods and materials

4.2.1 Sample sites and *Vachellia* trees sampled

The two mine tailing sites utilised in the study were Vaal River (S 26°54'58.89" E 26°45'7.00") and West Wits (S 26°26'4.61" E 27°21'37.92") operations, approximately 72 km apart. From each niche site 3 trees were sampled in the vicinity of the mine tailings at varying distances and for each control 2 trees, sampled at a distance from the mine tailings, were sampled. Briefly the revegetation strategies and construction of each niche site will be outlined, however, it is noteworthy to mention that all sites at VR and WW operations have received vast quantities of tailings dust over the last 80 years and are thus being progressively acidified and salinised by the acid mine drainage and the tailings/tailings dust (I. W. Weiersbye, *pers. comm.*).

4.2.1.1 Vaal River Operations

Madala site (VR1) (approximately 12044-16516 m from controls): comprised of acid mine drainage polluted soil which resulted from old tailings spills that were scraped off (from the adjacent West Complex TSF) and was developed through the planting of vegetation.
Black Reef site (VR2) (approximately 11759-16231 m from controls): comprised of acid mine drainage polluted soil (from the adjacent old mined reef) although no tailings spills occurred and was developed through the natural establishment and growth of vegetation.
East Pay Dam site (VR3) (approximately 9116-13588 m from controls): land/soil that was originally covered by the East Pay Dam TSF that was scraped off and is thus acid mine drainage polluted soil and was developed through the planting of vegetation.
R. G. Williams site (VR4) (approximately 13298-17770 m from controls): comprised of acid mine drainage polluted soil (from the up-gradient TSFs) although no tailings spills occurred and was developed through the natural establishment and growth of vegetation (I. W. Weiersbye, *pers. comm.*).

Vaal River Control (VRC): naturally occurring *V. karroo* at a distance from the VR operations mine tailings.

4.2.1.2 West Wits Operations

Tailing Storage Facilities lower slope, over soil, retaining wall (WW1) (approximately 2652-2860 m from controls): comprised of acid mine drainage polluted soil (from the adjacent TSF) mixed with old tailings and was developed through the planting of vegetation.
Toe-Paddock West Wits Old North Tailings Storage Facilities North Toe Paddock – Eastern Boundary (WW2) (approximately 4974-5182 m from controls): comprised of acid mine drainage polluted soil which resulted from old tailings spills that have not yet been scraped off (from the adjacent TSF) and was developed through the natural establishment and growth of vegetation.
Toe-Paddock West Wits Old North Tailings Storage Facilities North Toe Paddock North-Eastern Boundary (WW3) (approximately 5194-5402 m from controls): comprised of acid mine drainage polluted soil which resulted from old tailings spills that have not yet been scraped off (from the adjacent TSF) and was developed through the natural establishment and growth of vegetation.
Varkenslaagte Woodlands Blocks site (WW4) (approximately 3791-3999 m from controls): comprised of acid mine drainage polluted soil which resulted from old tailings spills that were scraped off (from the adjacent West Complex TSF) and was developed through the planting of vegetation (I. W. Weiersbye, *pers. Comm.*).
West Wits Control (WWC): naturally occurring *V. karroo* at a distance from the WW operations mine tailings.

4.2.2 Sampling strategy

Root and soil samples were collected from areas around TSFs found in the VR and WW Operations. Sampling was performed by digging up complete root systems as well as sections of root systems that provided a representative of the complete root system (fine and course roots) of *Vachellia karroo* with immediately surrounding soil. Three plants were sampled from each niche site from around MTFs and Figures 5 and 6 show the exact sites of sampling.

4.2.3 Soil physico-chemical analysis

4.2.3.1 pH and conductivity

pH (H_2O) and conductivity were measured by a modified version of that outlined by Anderson and Ingram (1989). pH (1M KCl) was measured by the procedure of van Reeuwjik (2002).

4.2.3.2 Nutrient and elemental analysis

Soil samples were placed in prepared sample cups up to the indicated mark on the inside of the cup. For every 10 samples placed in the XRF spectrometer a certified reference material (CRM) sample was used as regulatory control. Prior the analysis run the samples were gas flushed using helium. Specific CRMs used were NCS 87103, NCS 73315 and GBW 07312, and acquired from the China National Analysis Center for Iron and Steel. Specific elements included in the analysis were selected based on the CRM percentage agreement and all those included in Table 4.2 had a CRM percentage agreement above 75 % (with the exception of phosphorus and uranium, which have a percentage agreement of 65 % and 54 %, respectively).

Agricultural Research Council Institute for Soil, Climate and Water analysed the soil samples for available P content and the cation exchange capacity.

4.2.3.3 Statistical analysis

The soil characteristics pH (water and KCl) and conductivity values were subjected to a Two-Way ANOVA; followed by a Tukey-Kramer analysis (IBM SPSS Statistics 24). The mean of each chemical analysis of the soil samples from each niche site was determined and subjected to a Two-Way ANOVA; followed by a Tukey-Kramer analysis (IBM SPSS Statistics 24).

4.2.4 Extraction and analysis of soil DNA

Following the sample collection, samples then underwent preparation prior total soil DNA extraction. This preparation involved the following: 5 g of soil (equal proportions), without root

material, from each tree was weighed and bulked for each niche site and control; placed in a 50 mℓ centrifuge tube with lid (15 g total soil for niche sites and 20 g for control samples); and placed on a vortex mixer at max speed for 1 hr to ensure thorough dispersal of soil particulates.

From each bulk sample, 2 technical replicates were performed for the DNA extraction. DNA extraction from 500 mg of soil was carried out using using the DNeasy® PowerSoil® Kit (Qiagen), according to the manufacturer's protocol. DNA quality and concentration were measured by a ND-1000 spectrophotometer (NanoDrop, Wilmington, Delaware USA). In total 20 DNA samples were then sent to Molecular Research LP MR DNA (http://www.mrdnalab.com), Texas, in order to be sequenced. Sequencing targeted partial bacterial 16S gene amplicons of the V1-V3 hypervariable region using the primers 27F (5' –AGR GTT TGA TCM TGG CTC AG– 3') and 519R (5' –GTN TTA CNG CGG CKG CTG– 3') (Valverde *et al.*, 2014; Valverde *et al.*, 2016) and partial fungal ITS amplicons of the ITS2 hypervariable region using the primers ITS1F (5' –CTT GGT CAT TTA GAG GAA GTA A– 3') and ITS4 (5' –TCC TCC GCT TAT TGA TAT GC– 3') (Hartman *et al.*, 2012; Valverde *et al.*, 2016).

Total soil DNA was processed at MR DNA whereby the bacterial 16S rRNA V1-V3 hypervariable region (primers 27F/519R) and fungal ITS2 hypervariable region (primers ITS1F/ITS4) with barcode on the forward primer were used in a 28 cycle PCR (5 cycle used on PCR products) using the HotStarTaq Plus Master Mix Kit (Qiagen, USA). PCR products were checked using 2 % agarose gels in order to ascertain the relative intensity of the bands and the success of the amplification. Multiple samples were pooled together (for instance 100 samples) in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were then purified using calibrated Ampure XP beads. Following this the pooled and purified PCR products are used to prepare Illumina DNA libraries. The PCR products were sequenced at MR DNA (www.mrdnalab.com, Shallowater, Texas, USA) using Paired-End Illumina MiSeq according to the manufacturer's guidelines.

4.2.5 DNA sequencing

A gene library is comprised of the total number of reads post DNA sequencing and is frequently referred to as the depths of coverage (McMurdie and Holmes, 2014). Library size can vary greatly within sequencing runs and thus methods (generally normalisation methods such as rarefaction; Sanders, 1968) for processing the generated sequence libraries have been developed and are essential for quality purposes (McMurdie and Holmes, 2014). Thus processing of sequences is vital in order to produce reliable unbiased results. Additionally, technical replicates become important due to this issue.

Initial sequence processing (demultiplexing) was performed using a binning program available from MR DNA (http://www.mrdnafreesoftware.com/) on the fastq file provided containing all sequenced and combined contigs with barcodes and primers, thus unlike the study done by Valverde and colleagues (2016) it was unnecessary to process the raw data using *shhh.flows* due to contigs having been provided by MR DNA so that only the primer and barcoding regions need be removed. This follows that the first step in quality processing of the binned gene sequences without barcodes and primers, was performed using the online tool Galaxy Community Hub (https://usegalaxy.org/) whereby the quality of each technical replicate sequence library was determined and deemed satisfactory. Each technical replicate was then quality processed using the online tool Ribosomal Database Project (RDP; http://pyro.cme.msu.edu/) so that only the sequences within a specific size range were kept.

Following initial quality processing, chimeras were then removed from all technical replicate sequence libraries using the uchime tool on RDP. Following which the total number of sequences for each library was compared and technical replicate libraries were sub-sampled (rarefied) to be equal to the size of the smallest library in order to equalise sampling depth and allow for samples to be comparable to one another. This was performed using the *sub.sample* command in mothur (v.1.35.1) (Schloss *et al.*, 2009).

It was determined previously using mothur with both the *chimera.uchime* and *chimera.vsearch* commands that the ITS2 sequence libraries did not contain chimeras. The total number of

sequences for each library was thus compared and technical replicate libraries were sub-sampled (rarefied) to be equal to the size of the smallest library in order to equalise sampling depth and allow for samples to be comparable to one another. This was performed using the *sub.sample* command in mothur.

4.2.6 Data analysis

OTU tables for the 16S region were then constructed using the *Classifier* on RDP as well as rarefaction curves (*Rarefaction* under Analysis Tools) and the Chao1 and Shannon indices (*Shannon & Chao1 index* under Analysis Tools) were ascertained.

OTU tables for the ITS2 region were then constructed using the *Classifier* on RDP but rarefaction curves and Chao1 and Shannon indices were ascertained using mothur. This was done as follows, using mothur, OTUs were constructed from distances calculated following exact pairwise alignment (*pairwise.seqs*) by the Needleman-Wunsch algorithm (Huson *et al.*, 2007) and then clustered using the *cluster.seqs* command. The clustered sequences were then used to ascertain rarefaction curves, Chao1 and Shannon indices using the *rarefaction.single* and *summary.single* commands, respectively.

Modified OTU tables were then used to produce nMDS plots with weighted UniFrac distances and heat maps and using R v3.4.3 (R Foundation for Statistical Computing; http://www.R-project.org). Although attempted, differences in community structure could not be assessed by ANOSIM analysis using the *anosim* function in vegan (cran.r-project.org/package = vegan) as no R factor was produced. The compositions of major fungal and bacterial genera were compared using UPGMA clustering on Hellinger-transformed Bray-Curtis or Morisita-Horn distances together with a heatmap of abundance data created with *heatmap.2* in gplots.

4.3 Results

4.3.1 pH and conductivity

The pH and conductivity were analysed for each soil sample and the mean for each niche site was determined (Table 4.1). It was determined that there were only significant differences between niche sites for pH measured with water. The pH for all soil samples was acidic with the lowest pH being determined to be from the WWC (4.13 ± 0.032) from pH (KCl) and the highest being determined from VRC (6.35 ± 0.25) from pH (water). The overall trend showed that the pH measured with water was higher than that measured with KCl except in the case of VR3 which demonstrated the opposite. The highest conductivity value was determined to be from WW3 (826.33 ± 518.74) and the lowest value was from VRC (67.55 ± 24.69) (an overall a 12.23-fold difference). Both control soil samples had the lowest conductivity values.

Table 4.1: The pH and conductivity readings of areas surrounding Vaal Reef and West Wits mine residue deposits.

Analysis	Vaal River					West Wits				
	VR1	**VR2**	**VR3**	**VR4**	**VRC**	**WW1**	**WW2**	**WW3**	**WW4**	**WWC**
pH (Water)	5.13 ± 0.22^{ab}	5.13 ± 0.093^{ab}	5.28 ± 0.10^{ab}	4.67 ± 0.24^{a}	6.35 ± 0.25^{b}	5.27 ± 0.36^{ab}	5.88 ± 0.26^{ab}	4.75 ± 0.63^{a}	4.93 ± 0.23^{a}	4.92 ± 0.060^{a}
pH (1M KCl)	4.78 ± 0.13	4.94 ± 0.39	5.39 ± 0.14	4.45 ± 0.46	5.18 ± 0.34	4.63 ± 0.50	5.64 ± 0.33	4.58 ± 0.44	4.20 ± 0.25	4.13 ± 0.032
Conductivity (µSiemens/cm)	106 ± 26.16	90.67 ± 5.81	236.67 ± 25.89	121 ± 41.73	67.55 ± 24.69	369.67 ± 153.64	316.67 ± 78.15	826.33 ± 518.74	208.67 ± 51.06	101.93 ± 14.04

The means with different superscripts are significantly different ($p<0.050$) between sites according to the Tukey Kramer analysis.

4.3.2 Nutrient and elemental analyses

The total concentration of various elements was determined for each niche site and using XRF spectrophotometer (Table 4.2 and 4.3). Table 4.3 records the elements considered important in the study but didn't have a CRM agreement high enough to be analysed statistically, however, 3 elements, namely nickel (Ni), cobalt (Co) and thallium (Tl), were not recorded in this table as they were within normal soil ranges. Several elements measured and had satisfactory CRM percentage agreement were not recorded in Table 4.2 as their concentrations were determined to be within normal soil ranges. These elements include silicon (Si), potassium (K), titanium (Ti), vanadium (V), iron (Fe), copper (Cu), bromine (Br), gallium (Ga), rubidium (Rb), strontium (Sr), yttrium (Y), Zirconium (Zr), Niobium (Nb) and barium (Ba). From Table 4.2 it was determined that aluminium (Al) was present in the highest concentration and thorium (Th) was present in the lowest concentrations for all niche sites. All elements in Table 4.2 had at least one site that was significantly different to the other sites. From Table 4.3 it may be determined that antimony (Sb) was the highest concentration in this case and cadmium (Cd) was the lowest concentration in this case. Elements in Table 4.3 did not undergo statistical analysis as the results would not be reliable as indicated by a lower CRM percentage agreement.

During the XRF analysis a few chemicals were not reported, either due to not being present or they were present in concentrations that were too low to be detected and an alternative method of analysis may be better suited. The undetected elements include sodium (Na), magnesium (Mg) phosphorus (P), chlorine (Cl), germanium (Ge), selenium (Se), silver (Ag), tin (Sn), tellurium (Te), iodine (I), caesium (Cs), lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), ytterbium (Yb), hafnium (Hf), tantalum (Ta), tungsten (W) and mercury (Hg).

Table 4.2: Total concentrations (in $\mu g.g^{-1}$) of selected elements, using X-ray fluorescence, of areas surrounding Vaal Reef and West Wits mine residue deposits.

Element	Vaal River					West Wits				
	VR1	VR2	VR3	VR4	VRC	WW1	WW2	WW3	WW4	WWC
Aluminum	44793.33 ± 3778.20^{a}	44586.67 ± 1898.92^{a}	70230.00 ± 6011.50^{ab}	59986.67 ± 3039.79^{ab}	69782.50 ± 4271.54^{ab}	69050.00 ± 17647.81^{ab}	68253.33 ± 4030.95^{ab}	48066.67 ± 10465.63^{ab}	82920.00 ± 4391.97^{ab}	82332.50 ± 8798.54^{b}
Calcium	1769.33 ± 371.88^{ac}	461.20 ±35.76^{a}	4191.67 ± 292.34^{bd}	1186.00 ± 201.96^{ae}	2357.25 ± 408.20^{ad}	2436.67 ± 724.30^{ad}	3673.67 ± 358.61^{cd}	3107.33 ± 488.34^{cde}	728.84 ± 96.82^{a}	1810.50 ± 456.40^{ad}
Sulphur	1092.00 ± 22.19^{ab}	1005.40 ± 21.43^{ab}	1452.33 ± 65.02^{ab}	1170.67 ± 72.45^{ab}	445.83 ± 29.76^{b}	2251.00 ± 263.25^{ab}	1227.33 ± 18.84^{ab}	2601.00 ± 1356.78^{a}	1010.12 ± 278.91^{ab}	799.10 ± 236.83$_{ab}$
Manganese	2798.67 ± 354.62^{abc}	525.23 ± 56.99^{a}	5051.67 ± 1613.43^{b}	8497.67 ± 508.33^{c}	3581.75 ± 517.33^{bd}	937.70 ± 280.23^{ad}	568.50 ± 44.54^{a}	442.23 ± 139.64^{a}	268.88 ± 42.41^{a}	785.33 ± 113.68^{a}
Chromium	111.67 ± 15.30^{ad}	81.93 ± 5.10^{a}	132.30 ± 1.61^{ae}	118.80 ± 5.31^{af}	111.98 ± 6.46^{a}	196.00 ± 8.47^{be}	284.83 ± 11.04^{g}	201.30 ± 36.04^{ce}	170.86 ± 19.50^{def}	184.18 ± 9.12^{bef}
Arsenic	2.50 ± 0.17^{a}	5.50 ± 0.71^{ab}	19.57 ± 4.39^{bde}	4.07 ± 0.24^{ad}	4.50 ± 0.60^{ab}	32.17 ± 5.59^{ce}	41.33 ± 0.58^{c}	42.10 ± 9.26^{c}	12.67 ± 1.75^{ab}	13.98 ± 1.12^{ab}
Zinc	30.00 ± 8.81^{a}	26.10 ± 1.33^{a}	121.73 ± 21.08^{ab}	48.33 ± 11.81^{ab}	112.40 ± 47.41^{ab}	117.70 ± 56.04^{ab}	182.37 ± 16.68^{b}	65.10 ± 8.94^{ab}	38.07 ± 2.86^{a}	67.23 ± 11.86^{ab}
Thorium	4.37 ± 0.23^{ac}	2.83 ± 0.22^{a}	9.23 ± 1.42^{bcd}	4.27 ± 0.62^{ac}	4.98 ± 0.38^{ace}	9.60 ± 1.55^{bd}	9.77 ± 0.09^{bde}	8.50 ± 1.97^{cdf}	10.54 ± 0.67^{bf}	11.20 ± 1.14^{bf}
Lead	8.40 ± 0.59^{a}	12.10 ± 1.15^{a}	36.33 ± 5.93^{bc}	13.00 ± 1.11^{a}	15.00 ± 0.89^{a}	45.93 ± 1.79^{be}	50.43 ± 2.88^{b}	49.23 ± 12.75^{b}	18.76 ± 1.12^{ac}	27.13 ± 2.39^{ace}
Uranium	0.30 ± 0.00^{a}	0.30 ± 0.00^{a}	19.37 ± 7.65^{bd}	0.30 ± 0.00^{a}	0.33 ± 0.00^{a}	23.10 ± 5.40^{b}	49.90 ± 3.39^{c}	29.60 ± 3.77^{b}	4.68 ± 3.07^{ad}	0.33 ± 0.00^{a}

The means with different superscripts are significantly different ($p<0.050$) between sites according to the Tukey Kramer analysis.

Table 4.3: Total concentrations (in $\mu g.g^{-1}$) of selected elements, using X-ray fluorescence, of areas surrounding Vaal Reef and West Wits mine residue deposits.

Element	Vaal River					West Wits				
	VR1	VR2	VR3	VR4	VRC	WW1	WW2	WW3	WW4	WWC
Molybdenum	0.79 ± 0.46	3.93 ± 0.34	4.63 ± 0.91	3.43 ± 0.65	4.10 ± 0.31	5.83 ± 0.73	4.80 ± 0.26	0.94 ± 0.78	5.66 ± 0.45	5.63 ± 0.34
Cadmium	0.67 ± 0.00	0.67 ± 0.00	0.67 ± 0.00	0.57 ± 0.10	0.67 ± 0.00	0.91 ± 0.24	0.54 ± 0.12	0.50 ± 0.17	0.25 ± 0.05	0.67 ± 0.00
Antimony	1.33 ± 0.83	5.70 ± 2.36	6.60 ± 2.81	6.07 ± 3.55	9.90 ± 1.14	2.07 ± 1.07	2.67 ± 0.84	8.60 ± 2.90	6.34 ± 1.16	8.03 ± 1.94

Two analyses were performed by the Agricultural Research Council Institute for Soil, Climate and Water, namely, Bray no. 1 for P and cation exchange capacity for K, Ca, Mg and Na. The results are reported in Table 4.4.

Significant differences between niche sites were only observed for P and K with the highest P and K concentrations occurring in soil from VR3 (71.43 ± 37.49 and 268 ± 41.33, respectively), which were significantly different to every niche site as well as controls with the exception of the VR1 and the lowest concentrations of both elements were from WW3 and WW4. From the table it can be seen that VR3 had the highest concentration for all cations with the exception of Na which had the greatest concentration at WW1. Both control soil samples had the lowest Na concentrations.

Table 4.4: The exchangeable cations (in µg.g^{-1}), phosphorus, potassium, calcium, magnesium and sodium of areas surrounding Vaal Reef and West Wits mine residue deposits.

Analysis	Vaal River					West Wits				
	VR1	**VR2**	**VR3**	**VR4**	**VRC**	**WW1**	**WW2**	**WW3**	**WW4**	**WWC**
Phosphorus	19.07 ± 3.38^{ab}	3.5 ± 0.74^{a}	71.43 ± 37.49^{b}	2.63 ± 1.46^{a}	6.6 ± 0.16^{a}	1.03 ± 0.24^{a}	1.66 ± 0.81^{a}	0.55 ± 0.19^{a}	0.55 ± 0.09^{a}	10.85 ± 3.57^{a}
Potassium	162.67 ± 26.30^{ab}	122.67 ± 13.98^{a}	268.00 ± 41.33^{b}	153.67 ± 8.41^{a}	94.00 ± 5.60^{a}	72.33 ± 4.33^{a}	107.67 ± 32.46^{a}	55.33 ± 10.48^{a}	65.00 ± 10.41^{a}	141.25 ± 11.43^{a}
Calcium	525.67 ± 166.30	385.33 ± 31.80	1163.67 ± 123.01	267.00 ± 28.00	542.00 ± 92.06	569.67 ± 171.81	840.00 ± 263.12	1018.33 ± 587.77	249.67 ± 19.06	518.00 ± 117.44
Magnesium	130.67 ± 32.20	106.00 ± 17.21	158.00 ± 22.03	85.33 ± 2.33	73.25 ± 7.87	102.00 ± 31.79	95.00 ± 29.55	64.00 ± 24.21	63.33 ± 13.33	129.75 ± 10.91
Sodium	8.20 ± 1.00	2.97 ± 0.46	5.40 ± 0.20	2.27 ± 0.30	1.05 ± 0.39	52.83 ± 35.06	6.63 ± 1.58	5.80 ± 2.57	27.43 ± 13.68	1.03 ± 0.04

The means with different superscripts are significantly different ($p<0.050$) between sites according to the Tukey Kramer analysis.

4.3.3 Analysis of rhizobiome diversity

After preliminary analysis (removal of ambiguous bases and elimination of unusually short or long sequences) and before subsampling and subsequent classification, the sequence totals for each niche site and control were plotted to show any trends that may have occurred in sequencing of amplicons.

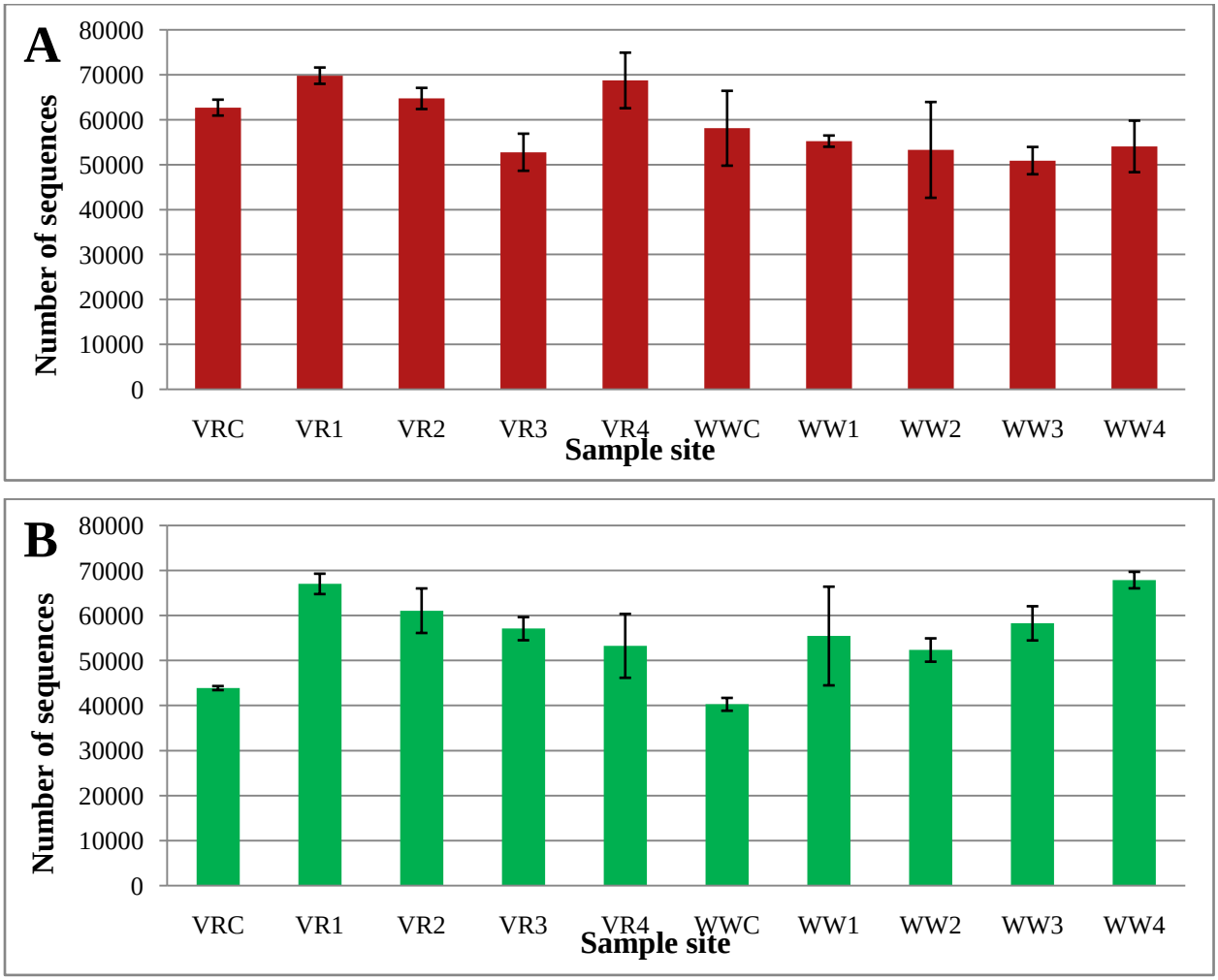
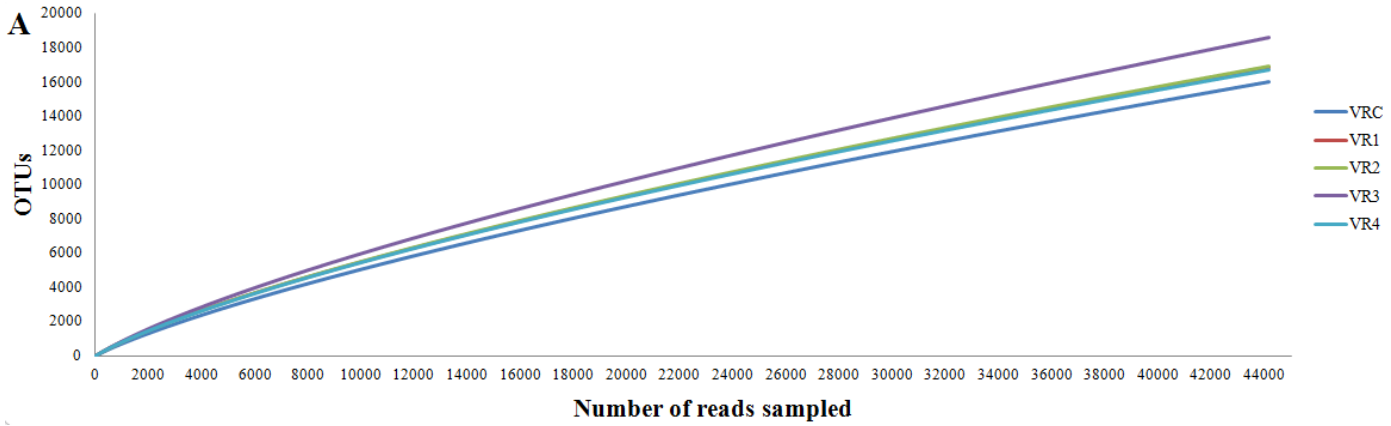


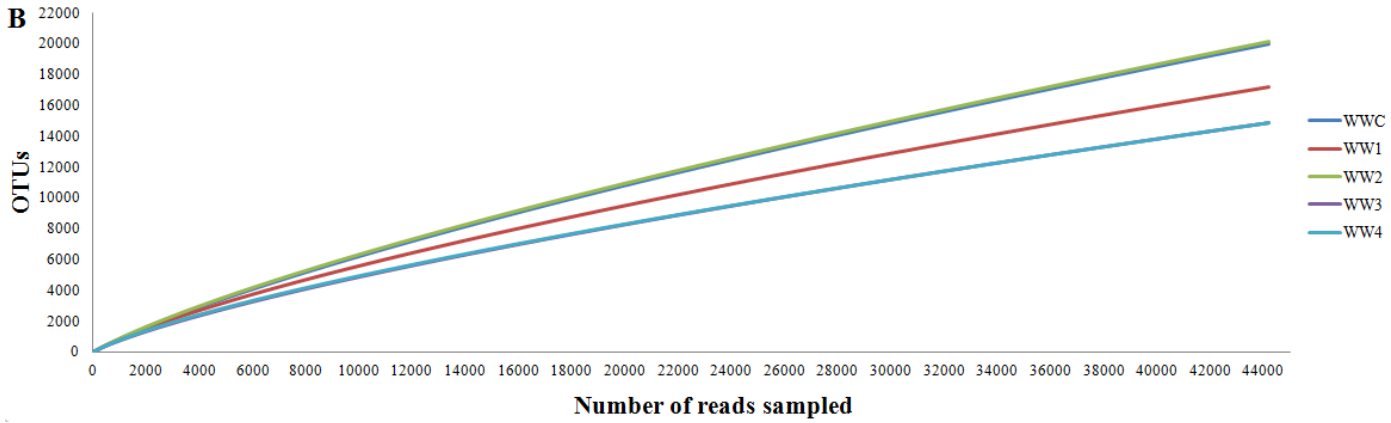
Figure 4.1: Bar graphs illustrating total number of sequences remaining post initial processing for A) 16S V1-V3 and B) ITS2 for DNA isolated from soil from different niche sites

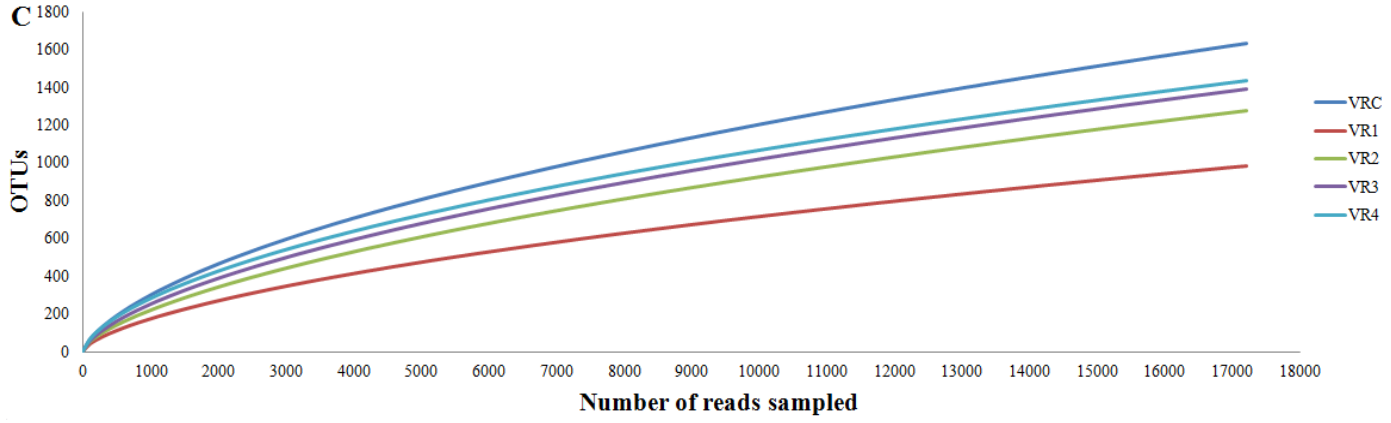
It would appear that a trend develops in the case of ITS2 sequences whereas for the 16S V1-V3 sequences no trend is apparent. The controls for the ITS2 have the lowest number of remaining sequences when compared to the sample sites for the ITS2 region. Once sub-sampled all sequence libraries for ITS2 region contained 39270 sequences and all the sequence libraries for 16S V1-V3 region contained 44231 sequences.

4.3.4 Alpha diversity

Rarefaction curves were constructed at a 0.03 cutoff in order to compare and ascertain the depth of sampling and are shown in Figure 4.2.







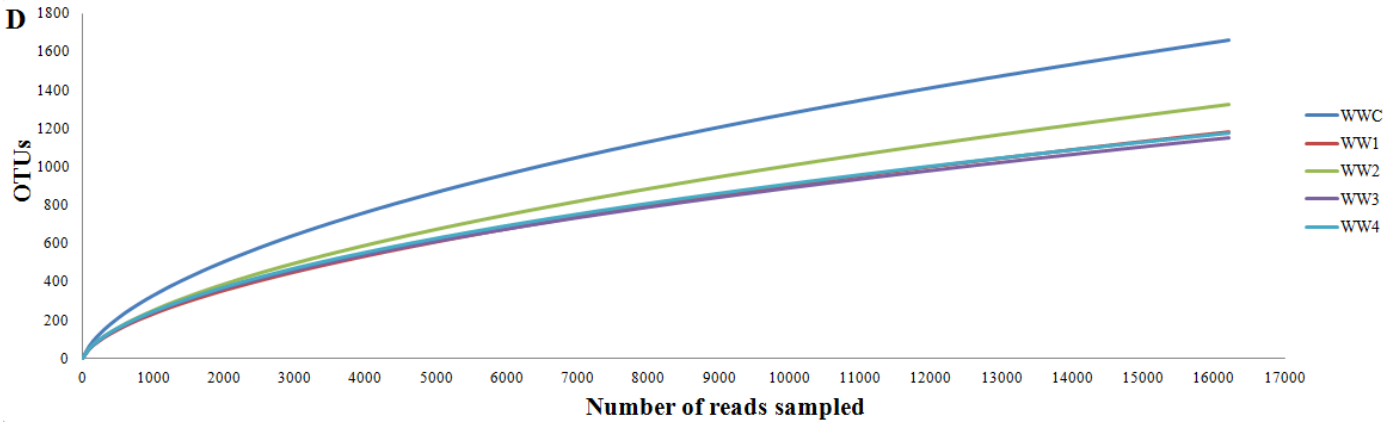


Figure 4.2: Rarefaction curves describing OTUs as a function of sampling effort for OTUs defined at a 0.03 distance cutoff of genes sequenced from soil DNA from different niche sites with A and B) 16S V1-V3 and C and D) ITS2

As can be seen from the generally shallow curves that although the niche sites were well sampled they did not appear to be completely/fully sampled as the curves did not completely level out and continued to climb with increasing sequencing effort. It would also appear that the 16S sequences had steeper rarefaction curves and were thus not sampled as well as the ITS2 sequences.

Out of all the rarefaction curves, the graph illustrating the 16S sequences from VR operations is the only one that has the curve of the control clustering with sample sequences and the sample from VR3 is the curve that does not cluster with the other curves.

The Chao1 and Shannon indices were determined for both the 16S and ITS2 regions at a 0.03 cutoff and are represented in the tables below. The indices determined using mothur (the ITS2 sequences) followed a method that performed clustering through several iterations and the cluster values (OTU number) as well as the indices were those of the final iteration and were thus lower than those performed by RDP on the 16S V1-V3 region.

Table 4.5: Measures of alpha diversity for the 16 V1-V3 gene region sequenced from soil from different niche sites at 0.03 cutoff for OTUs and/or clustering.

Sample site	Number of sequences	Cluster/OTUs	Chao1	Shannon (H')
VRC	44231	16013	58590.3792	8.084215
VR1	44231	16833.5	58579.15045	8.6882
VR2	44231	16912	58649.41172	8.684595
VR3	44231	18610	70576.34826	8.926365
VR4	44231	16723	57134.97468	8.63155
WWC	44231	20017.5	76768.60345	9.017725
WW1	44231	17229.5	62533.95709	8.76787
WW2	44231	20162	76815.79227	9.122495
WW3	44231	14912	50095.42369	8.27733
WW4	44231	14857	52756.5619	8.397785

Table 4.6: Measures of alpha diversity for the ITS2 gene region sequenced from soil from different niche sites at 0.03 cutoff for OTUs and/or clustering.

Sample site	Number of sequences	Cluster/OTUs	Chao1	Shannon (H')
VRC	39270	1787	3880.796	5.1751235
VR1	39270	986.5	2494.3765	3.6592165
VR2	39270	1356	3295.6792	4.4842245
VR3	39270	1512.5	3511.653	4.727112
VR4	39270	1556	3482.927	5.131101
WWC	39270	1859	3957.1119	5.414581
WW1	39270	1250	2743.2029	4.585419
WW2	39270	1416	3283.4855	4.7484285
WW3	39270	1201.5	2713.833	4.5256455
WW4	39270	1210	2549.1873	4.5461125

According to the Chao1 index for both the 16S and ITS analyses, the number of OTUs is less than that estimated by the Chao1, by 3.36-3.85 and 2.11-2.53 times respectively. The Shannon index for the ITS analysis is above average whereas the index for the 16S analysis is at least double the value expected. It is also noteworthy to mention in the case of ITS2 analysis the Chao1 indices have higher estimates for both controls when compared to the indices from the experiment niche sites.

4.3.5 Beta diversity

Upon review of the OTU tables generated by RDP several taxa were removed in order to create a view of the core biome (taxa that occur over all niches/biomes analysed), thus any taxa that didn't occur in 1 or more niche sites from the Operation sites (had a value of 0 sequences) were completely removed from the OTU tables. nMDS plots from all niche sites and controls from both Operation sites were constructed and are shown in Figures 4.3 and 4.4.

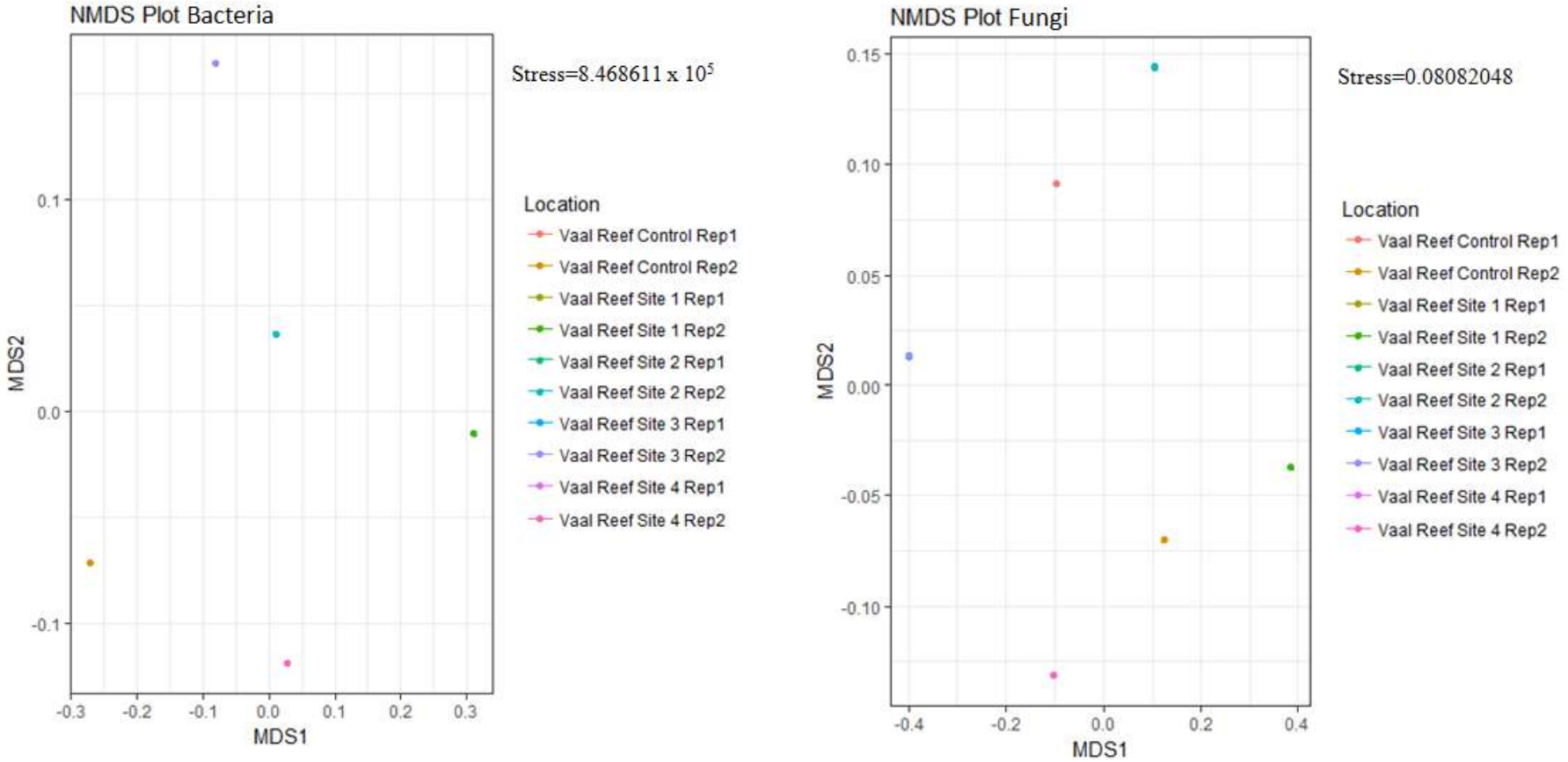


Figure 4.3: nMDS ordination plot (UniFrac dissimilarity matrix). Each point represents either the bacterial or fungal community of an individual sample replicate from Vaal River Operation site.

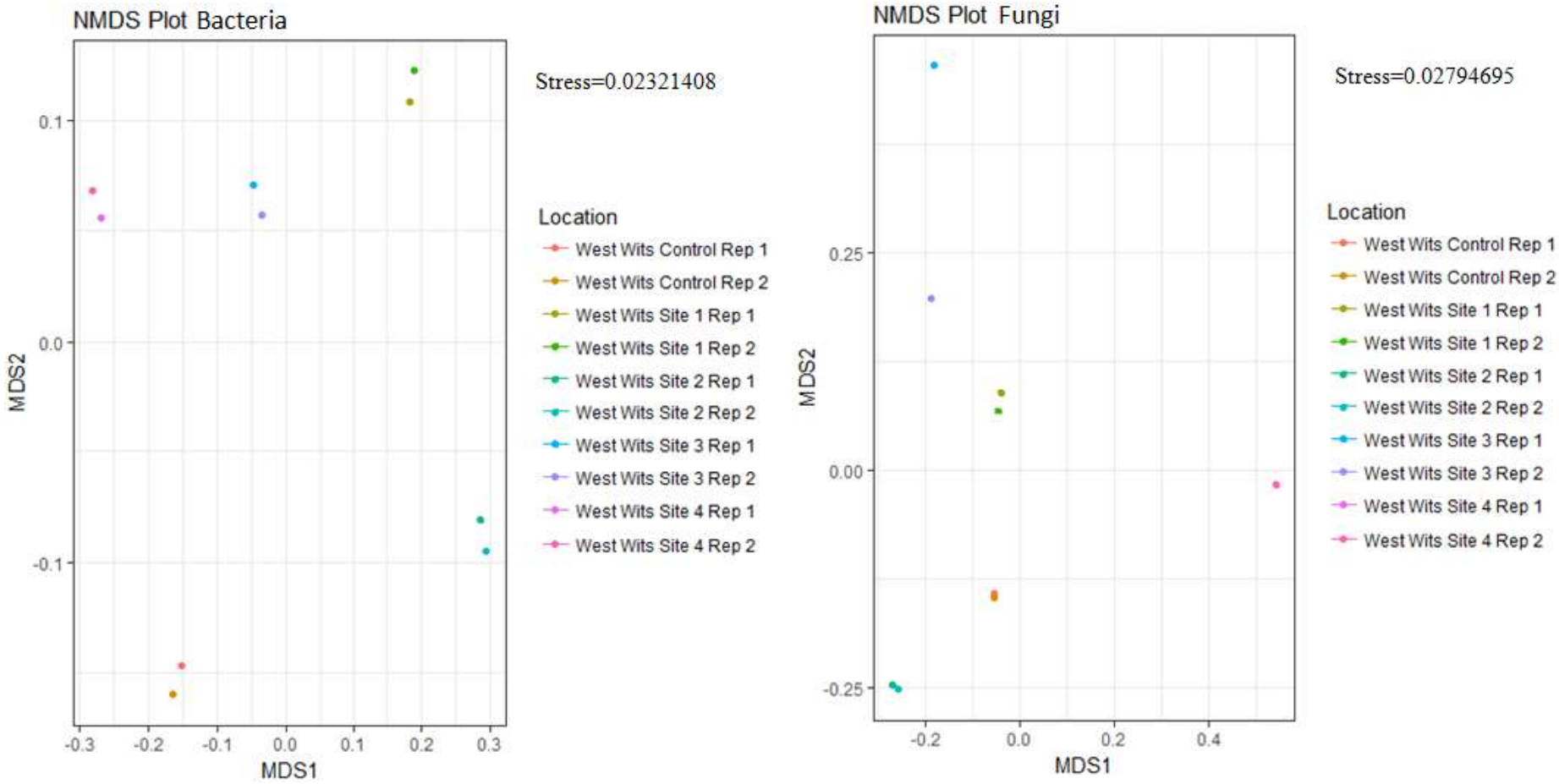
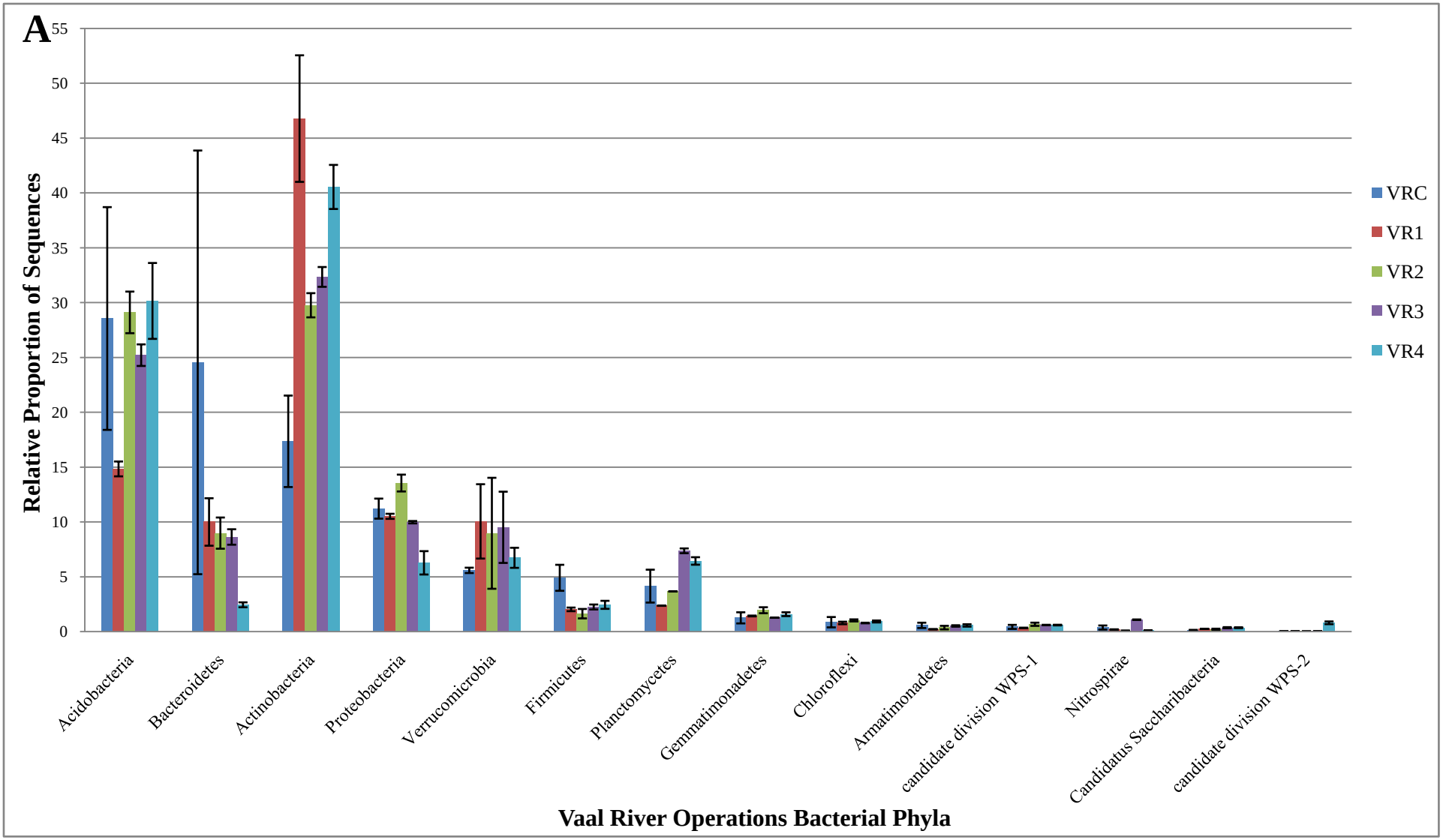


Figure 4.4: nMDS ordination plot (UniFrac dissimilarity matrix). Each point represents either the bacterial or fungal community of an individual sample replicate from West Wits Operation site.

From the nMDS plots no apparent patterns of clustering occurred and due to this ANOSIM could not be determined. From the alpha diversity indices it would have been expected that the control samples would have been separate from the experiment samples and the experiment samples would have clustered together, however, this was not observed. Several technical replicates also did not align with each other in the case of 16S from WW Operations and ITS2 from both VR and WW Operations. The technical replicates for 16S were still closely related unlike some of the replicates from the ITS2 where VRC replicates and WW3 replicates were located in different locations on the nMDS plot in Figure 2.5 and 2.6, respectively. OTU tables containing the core microbiome data and had discrepant taxa removed did not produce reliable nMDS plots due to the fact that data provided by the modified table was insufficient.

The overall bacterial communities at the phyla level were analysed, while the overall fungal communities at the class level were analysed and are illustrated in Figures 4.5 and 4.6. Phylum level taxa were used in the case of bacteria and class level taxa were used in the case of fungi as they portrayed the most information in a summarised form. From the graphs the bacteria in the core microbiome are represented by 14 and 15 phyla from VR and WW Operations, respectively. In the case of fungi, 5 phyla were identified in the core microbiome and the phylum that occurred in the lowest relative proportion was Chytridiomycota (below 0.5 % in all niche and control sites) and the most abundant phylum was predominantly Ascomycota (62-91 %) followed by either Basidiomycota or Zygomycota with the exception of VR1 which showed Zygomycota (66 %) as the most abundant phylum and WW4 which showed Fungi-unidentified (45 %) (only 1 % above Ascomycota at 44 %) as the most abundant phylum (data not shown).


A
Relative Proportion of Sequences
55
50
45
40
35
30
25
20
15
10
5
0
VRC
VR1
VR2
VR3
VR4
Acidobacteria
Bacteroidetes
Actinobacteria
Proteobacteria
Verrucomicrobia
Firmicutes
Planctomycetes
Gemmatimonadetes
Chloroflexi
Armatimonadetes
candidate division WPS-1
Nitrospirae
Candidatus Saccharibacteria
candidate division WPS-2
Vaal River Operations Bacterial Phyla

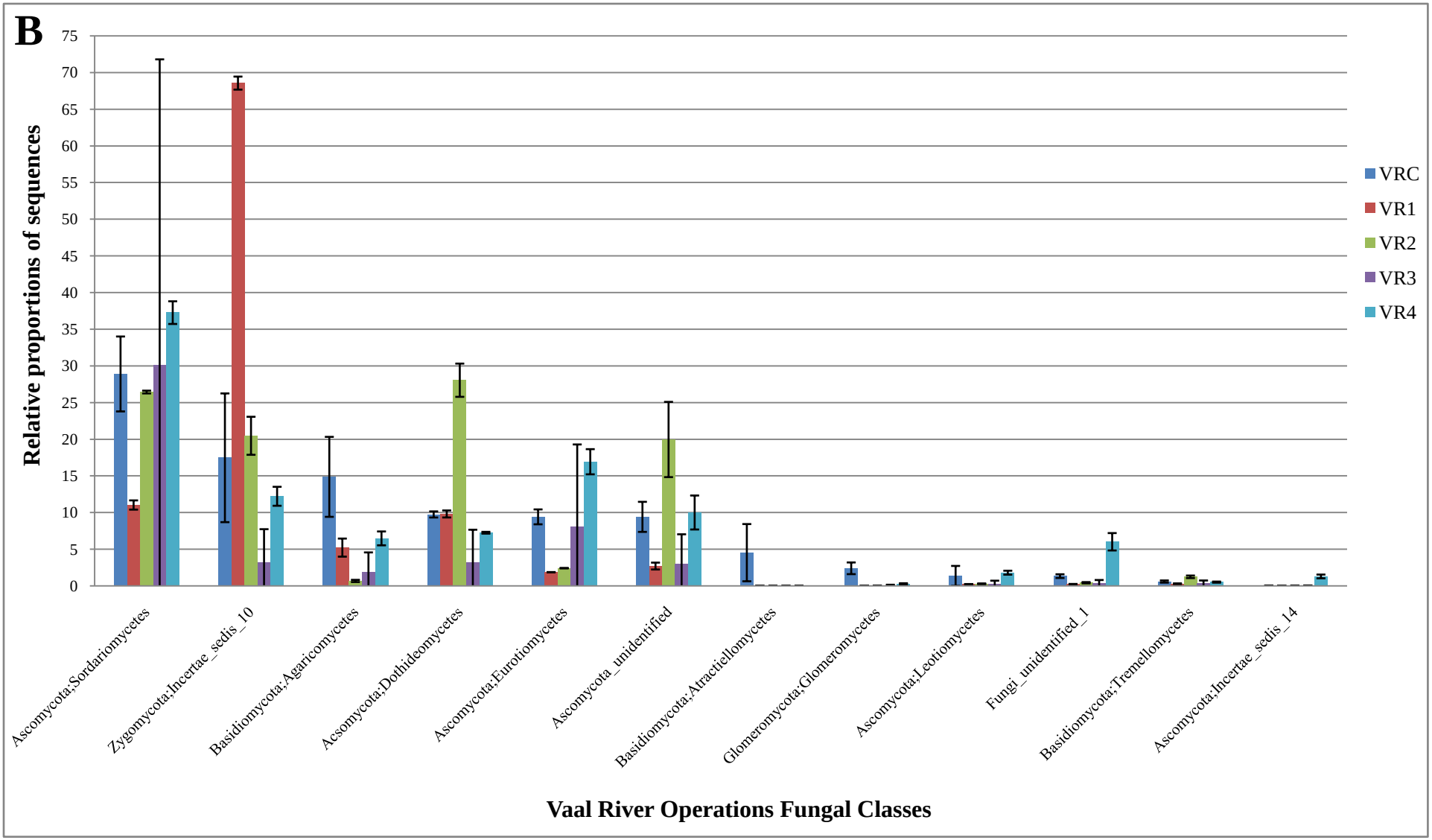
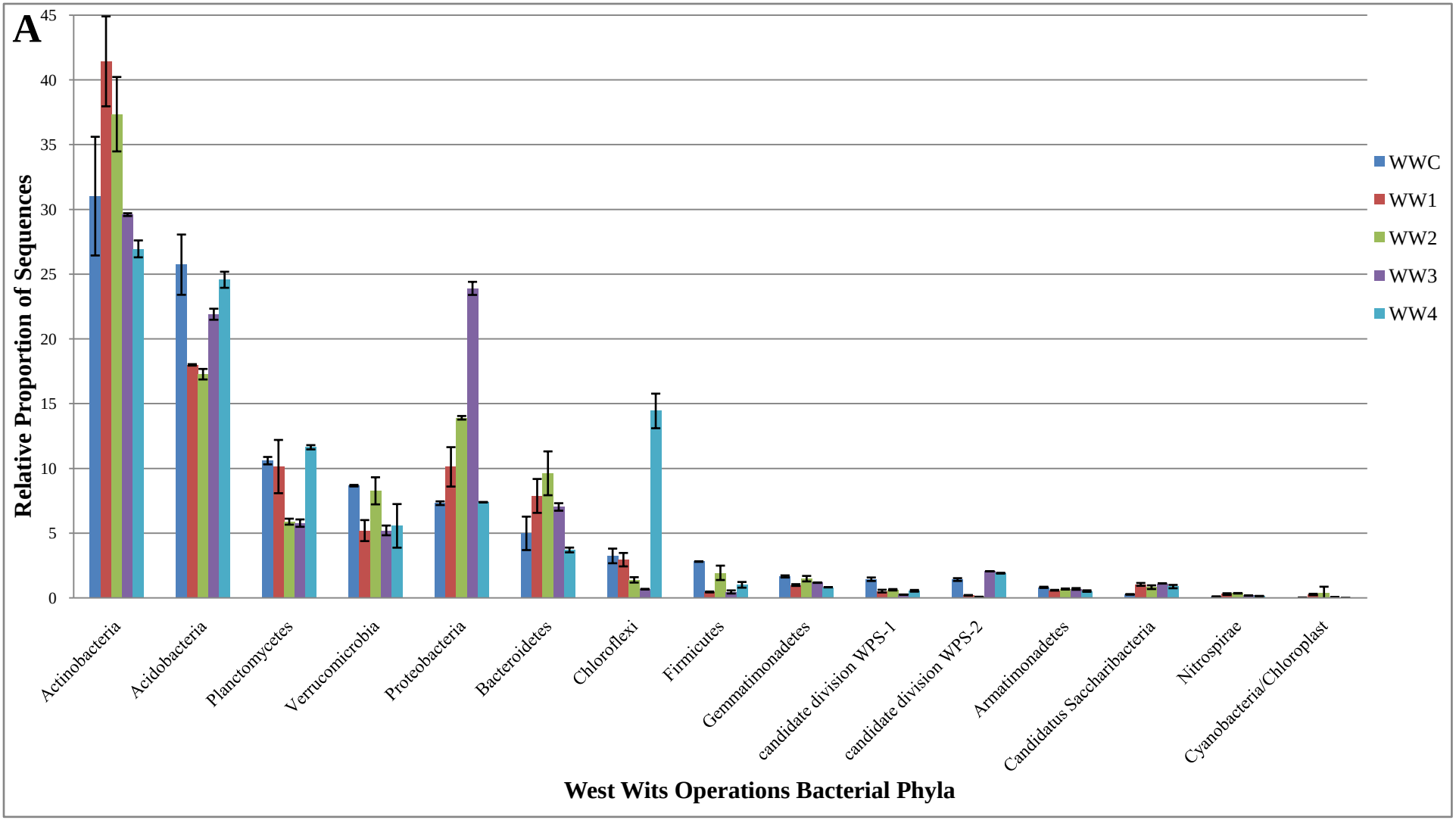


Figure 4.5: Bar graphs illustrating relative proportions of the A) bacteria and B) fungi associated with the *Vachellia karroo* rhizosphere from the Vaal River Operations site.


A
Relative Proportion of Sequences
45
40
35
30
25
20
15
10
5
0
WWC
WW1
WW2
WW3
WW4
Actinobacteria
Acidobacteria
Planctomycetes
Verrucomicrobia
Proteobacteria
Bacteroidetes
Chloroflexi
Firmicutes
Gemmatimonadetes
candidate division WPS-1
candidate division WPS-2
Armatimonadetes
Candidatus Saccharibacteria
Nitrospirae
Cyanobacteria/Chloroplast
West Wits Operations Bacterial Phyla

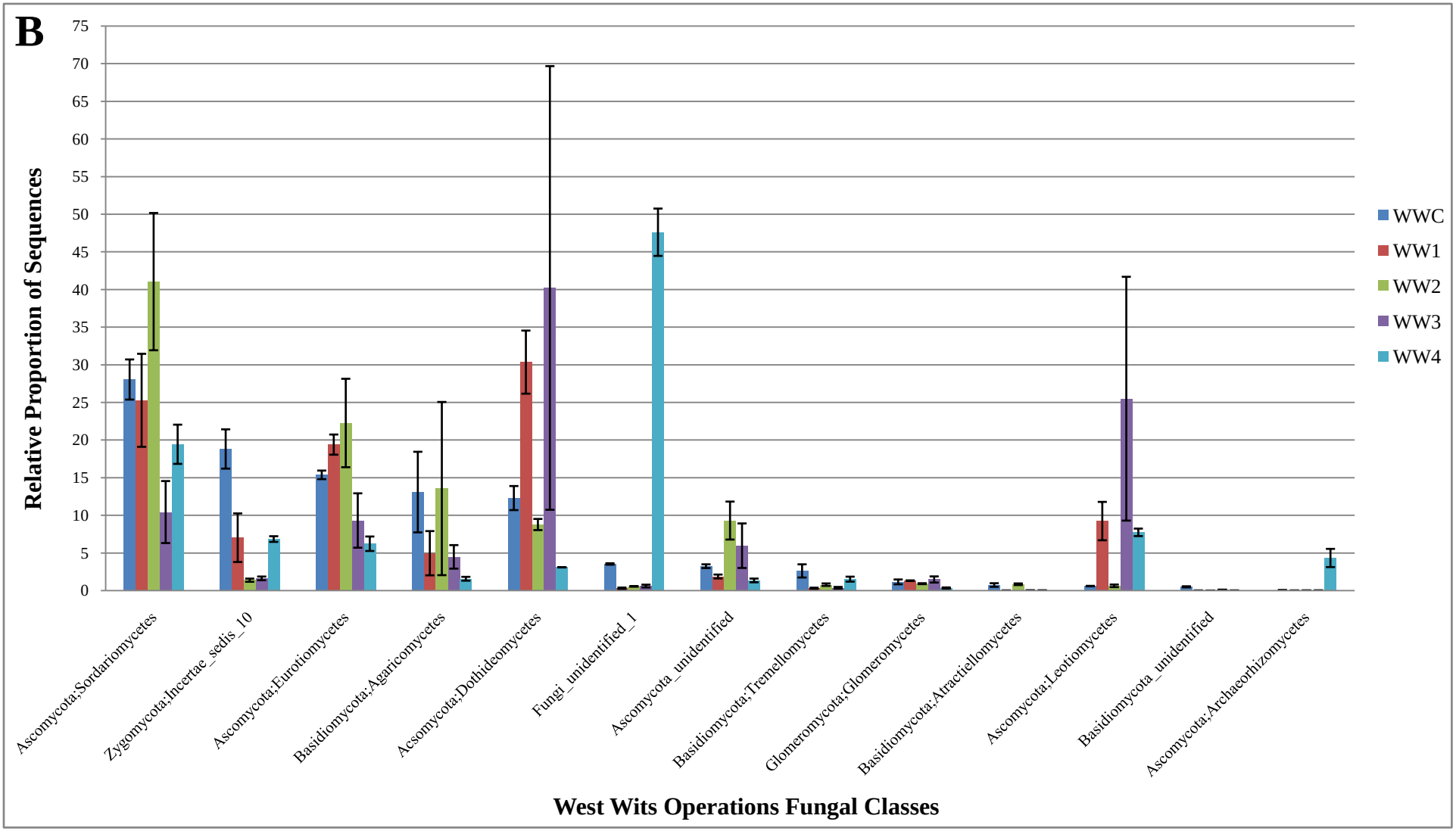


Figure 4.6: Bar graphs illustrating relative proportions of the A) bacteria and B) fungi associated with the *Vachellia karroo* rhizosphere from the West Wits Operations site.

Heat maps were constructed using OTU tables from the core biome of both major bacterial and fungal genera after taxa that had large discrepancies between sequence numbers (by at least 2 times difference) between replicates were removed (Figures 4.7 and 4.8). The technical replicates were maintained in the construction of the heat maps so that it may be observed whether the technical replicates paired together and were thus reliable.

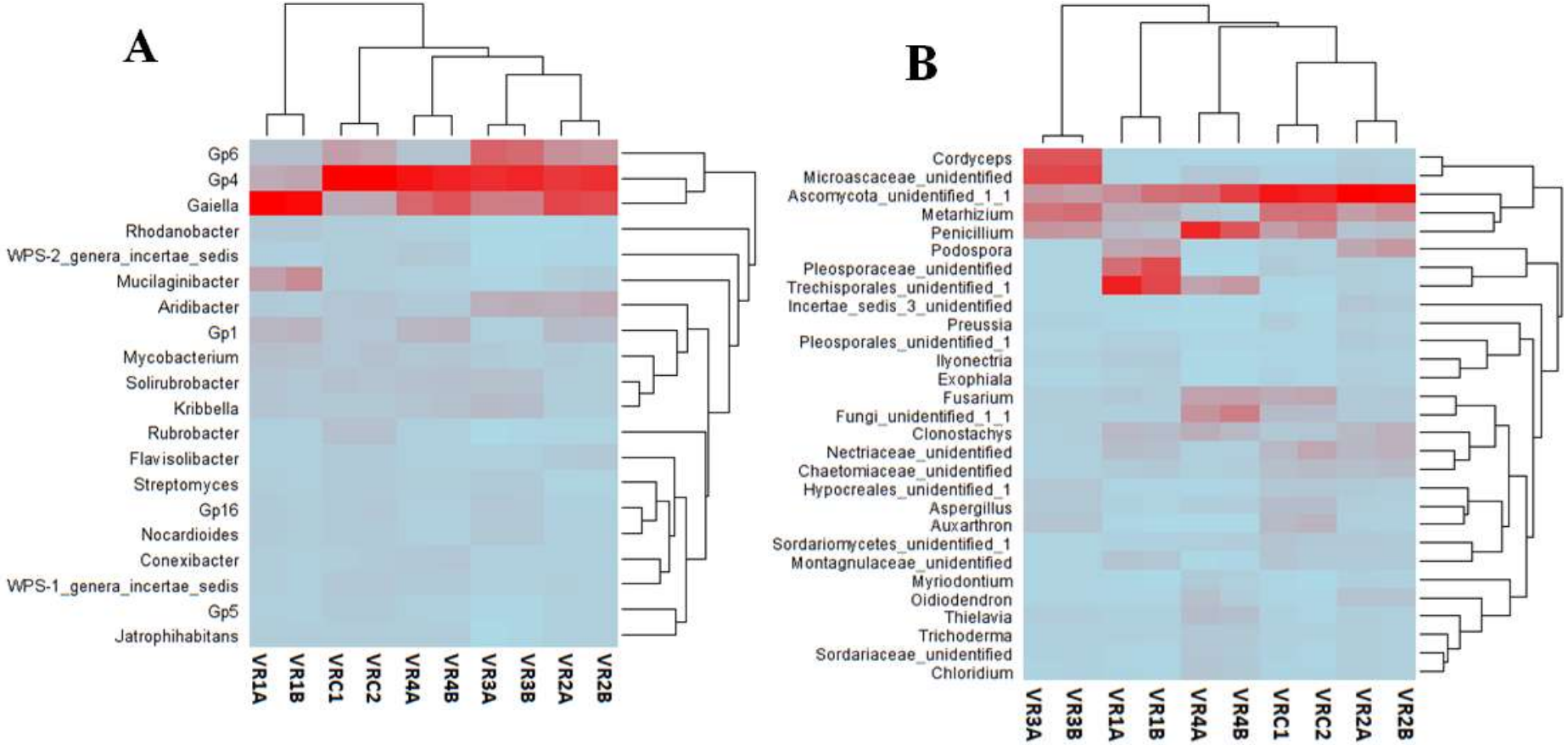


Figure 4.7: Heatmaps displaying the most abundant taxa of A) bacteria and B) fungi of the core microbiome after removal of discrepant taxa of soil of controls and niche sites from Vaal River Operations. Samples were clustered based on the percent relative abundance of the dominant taxa shown in rows in the figure. Key for colour scale: Increase in red in the increase in abundance and increase in light blue is decrease in abundance.

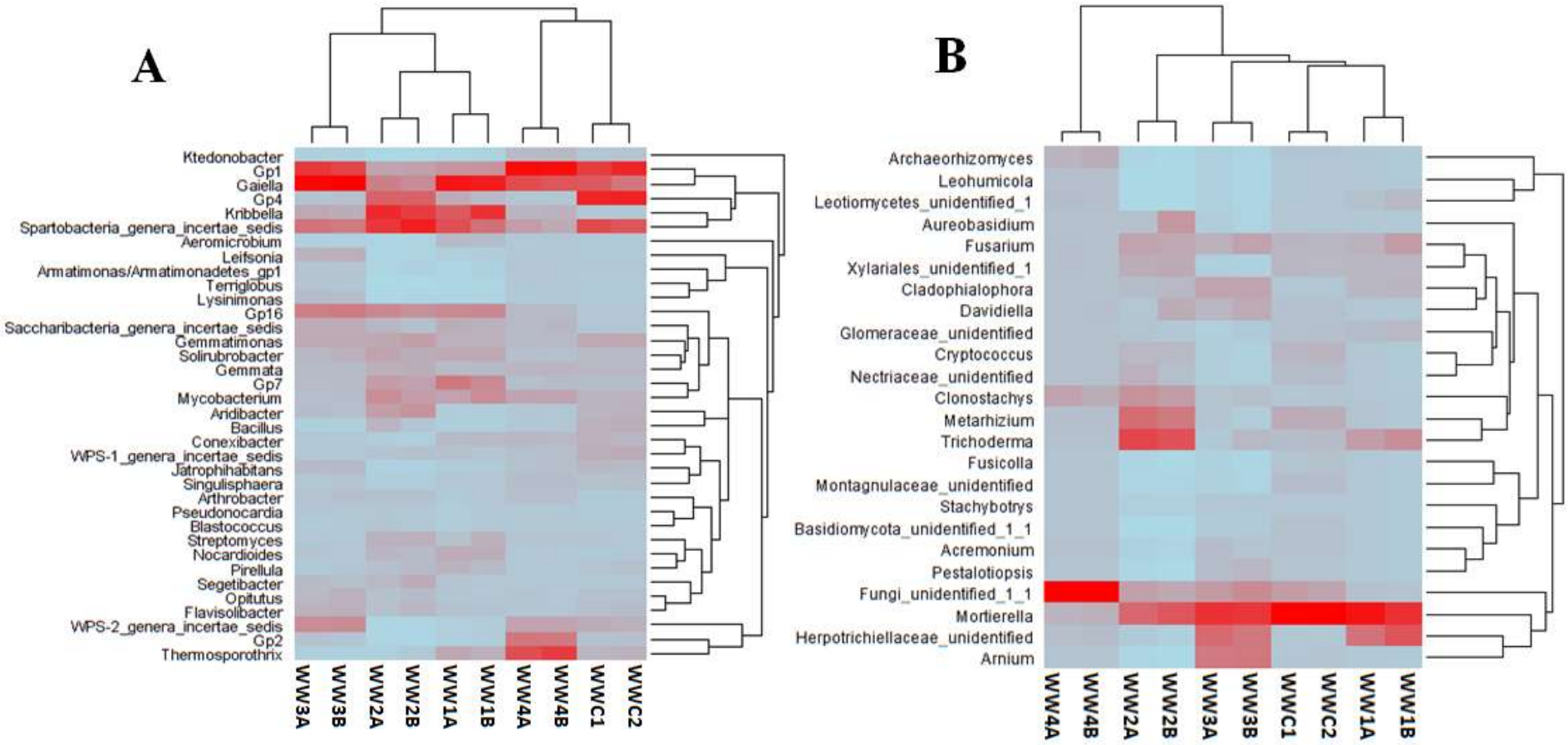


Figure 4.8: Heat maps displaying the most abundant taxa of A) bacteria and B) fungi of the core microbiome after removal of discrepant taxa of soil of controls and niche sites from West Wits Operations. Samples were clustered based on the percent relative abundance of the dominant taxa shown in rows in the figure. Key for colour scale: Increase in red in the increase in abundance and increase in light blue is decrease in abundance.

From the heat maps only 6 of the 14 bacterial phyla were represented in the dominant genera from VR Operations and 12 of the 15 bacterial phyla were represented in the dominant genera from WW Operations. Regarding the VR Operations, the Actinobacteria were represented by 9 genera; the Acidobacteria were represented by 6 genera; the Bacteroidetes were represented by 2 genera; and the Proteobacteria, Candidate division WPS-1 and Candidate division WPS-2 were each represented by a single genus. With regards to the WW Operations, the Actinobacteria were represented by 14 genera; the Acidobacteria were represented by 7 genera; the Planctomycetes were represented by 3 genera; the Chloroflexi, Bacteroidetes and Verrucomicrobia were represented by 2 genera; and the Armatimonadetes, Candidatus Saccharibacteria, Gemmatimonadetes, Firmicutes, Candidate division WPS-1 and Candidate division WPS-2 were all represented by a single genus.

In the case of the fungal heat maps all 5 phyla were represented by genera before discrepant taxa were removed; however, after removal, 5 phyla were represented in WW Operations and only 3 phyla were represented in VR Operations with both Glomeromycota and Zygomycota were no longer represented in the VR Operations. For the phyla that remained at VR Operations, the Ascomycota were represented by 27 genera and both Basidiomycota and Fungi_unidentified were represented by a single genus each. For the WW Operations, the Ascomycota were represented by 19 genera; the Basidiomycota were represented by 2 genera; and Glomeromycota, Zygomycota and Fungi_unidentified were each represented by a single genus.

The heat maps also determined the phylogenetic relatedness of each sample site and controls. Interestingly, the control replicates did not cluster separately from the experiment sample replicates and in each heat map an experiment sample clustered separately from the rest of the replicates.

4.4 Discussion

4.4.1 Soil physico-chemical analysis

4.4.1.1 pH and conductivity

Chemical processes and TEs within the soil are indirectly and directly influenced by pH (Hooda, 2010). Previous soil analyses have determined that the pH varies between 4 and 8 and generally soils that have a pH above this range contain a percentage of sodium ions and soils that have a pH below this range contain sulphuric acid (Thompson and Troeh, 1978). All sites tested here contained pH values within the typical range. The most desirable soil pH range is approximately between 6.25 and 7.75, however, typically agricultural soils range between 5.5 and 8.25 (Eash *et al.*, 2008). Soils containing a pH near either end of the pH range have been contaminated by anthropogenic activities (Eash *et al.*, 2008).

Mine tailing solution extracts typically have high conductivity (Weiersbye *et al.*, 2006), which was corroborated by a study performed on mine tailings in Welkom (Buck, 2014). Conductivity of these sites, however, whilst comparing the highest values and the lowest values, was up to 5.17 and 7.55-fold less than the conductivity from mine tailings in Welkom (Buck, 2014).

4.4.1.2 Chemical analysis

Chemicals analysed in the study due to being toxic to people and animals, are known soil contaminants, and/or because they are necessary for metabolism but were not found during analysis, include mercury (Hg), silver (Ag) and platinum (Pt).

Although XRF analysis ascertains elemental concentration it does not, however, determine chemical speciation and it is important to note that elemental speciation and mobility is determined by both the physical and chemical characteristics of soil (Kabata-Pendias and Pendias, 1992). Thus the soil elemental toxicity and availability to microorganisms and plants fluctuates with pH, organic matter content, soil texture, cation exchange capacity, redox potential of soil and the concentration and oxidation state of elements (Adriano, 1986).

4.4.2 Sequence processing

4.4.2.1 Initial processing

After the initial sequence processing a pattern was observed from the ITS2 sequences and although a pattern was observed here it was not observed in further analyses. The confidence of the measures changes with sample size (Hughes *et al.,* 2001). It would appear that in this study a large quantity of sequences were produced even after the initial processing and subsampling. Thus possible restrictions in the confidence of measures may be as a result of measures employed themselves. An example of a previous study, by Valverde *et al.* (2016), only utilised 3712 16S rRNA sequences and 27810 ITS2 sequences, which is far lower than each individual replicate sample in this case, especially since the smallest sample size for 16S rRNA (V1-V3 region) was 44231 sequences and smallest sample size for the ITS2 region was 39270 sequences. It is possible that the abundances of genes amplified by PCR are not true reflections of the original template DNA since primers have different binding and elongation efficiency (Raeymaekers, 1995; Reysenbach *et al.*, 1992).

4.4.2.2 Alpha diversity

Interestingly, despite the ITS2 control samples having the lowest number of sequences after initial processing they had the highest number of clusters/OTUs after subsampling. According to the tables the number of the OTUs clustered for the 16S rRNA and ITS2 genes is less than is possible (according to the Choa1 index), however, the value of the Shannon index implies that both the richness and the evenness of community is better than previous studies. Unfortunately, the Choa1 and Shannon index in this case were ascertained using the sequence data and not the classified data. The benefit of this is that the indices are not limited by sequence databases, however, the disadvantage is that until classifying sequence databases are comprised of all organism data, beta analyses are restricted to what is currently available.

4.4.2.3 Beta diversity

The fact that initial clustered sequences/OTUs (alpha diversity indices) did not necessarily match the clustering from the classified OTU table (i.e. classification reference database), ultimately suggests that the reference databases are lacking in information and in order for the beta diversity to be comparable to the alpha diversity cluster parameters serious improvements need to be made in the databases. Frequently taxonomy counts are utilised to estimate the relative abundance of organisms (Cox *et al.*, 2017). Metataxonomy addresses richness estimation and does not address how the diversity relates to functional diversity (Hughes *et al.*, 2001).

The ordination of the taxonomic profiles did not reveal a clear separation between control samples and experiment niche samples, which may suggest that the controls may not serve as appropriate taxonomic controls. The lack of differences between controls and experiments may be due to the fact that the plant species was consistent between all samples, and the lack of an ordination pattern may be due to the experiment samples having no consistent parameters.

4.4.3 Soil conditions and rhizobiome

4.4.3.1 Vaal River Control

This is the least acidic (most neutral) soil of all sites sampled. The Ca concentration is a concern as it is half the lowest value of the typical concentration range in soils. This may lead to Ca deficiency in *V. karroo*. S is unlikely to be a concern when regarding either deficiency or toxicity as the concentration falls within the typical the concentration range in soils. Mn becomes a concern when soils become acidic (equal to or less than pH 5.5), however, with the elemental concentration being 6-fold greater than the highest value in the mean average concentration it may be a cause of toxicity. Cr, Cd and Sb are all a major concerns as their concentrations are, just under double, 3-fold greater than and almost 10-fold greater than the maximum concentration of the typical concentration range, respectively. Zinc may also be a concern as it is above the average concentration of soils. It would appear that Al, As, Th, Pb, U and Mo were not a concern as they were either within normal range or below the average soil concentration. Looking at the microbial diversity, it would appear that the fungal diversity is greater than the

bacterial diversity according to the heat maps. From the overall bacterial phyla the most prominent taxa were Acidobacteria (28.6 %) followed closely by Bacteriodetes (24.6 %), then Actinobacteria (17.4 %) and Proteobacteria (11.2 %) and from the overall fungal classes the most prominent taxa were Sordariomycetes (Ascomycota; 28.9 %), Incertae_sedis_10 (Zygomycota; 17.5 %), Agaricomycetes (Basidiomycota; 14.9 %), Dothideomycetes (Ascomycota; 9.7 %) and Eurotiomycetes (Ascomycota) and Ascomycota_unidentified (both 9.4 %). Looking closely at the dominant taxa (from the heat maps) the top 9 bacterial genera (Gp4, Gp6, *Gaiella, Aridibacter,* Gp1, *Mycobacterium, Solirubrobacter, Kribbella* and *Rubrobacter*) are from Acidobacteria (4 genera) and Actinobacteria (5 genera) and none from Bacteriodetes. The top 12 fungal genera (*Ascomycota_unidentified_1_1, Metarhizium, Penicillium, Fusarium, Nectriaceae_unidentified, Auxarthron, Aspergillus, Fungi_unidentified_1_1, Chaetomiaceae_unidentified, Sordariomycetes_unidentified_1, Montagnulaceae_unidentified* and *Hypocreales_unidentified_1*) are from Sordariomycetes (7 genera), Eurotiomycetes (3 genera), Dothideomycetes (1 genus) and Fungi_unidentified (1 genus).

4.4.3.2 VR1

The soil from this site was acidic and with this Mn becomes a concern, and compacted with the concentration being 4-fold greater than the highest value in the mean average concentration, it is likely to cause toxicity. The Ca concentration is also a concern as it is less than half the lowest value of the typical concentration range in soils. This may lead to Ca deficiency in *V. karroo*. S, Cr, Cd and Sb are all a major concerns as their concentrations are, double (for both S and Cr), 3-fold greater than and above the maximum concentration of the typical concentration range, respectively. It would appear that Al, As, Zn, Th, Pb, U and Mo were not a concern as they were either within normal range or below the average soil concentration. Looking at the microbial diversity, it would appear that the fungal diversity is greater than the bacterial diversity according to the heat maps. From the overall bacterial phyla the most prominent taxa were Actinobacteria (46.8 %) followed by Acidobacteria (14.8 %), then Proteobacteria (10.5 %), Verrucomicrobia (10.1 %) and Bacteroidetes (10 %) and from the overall fungal classes the most prominent taxa were Incertae_sedis_10 (Zygomycota; 68.6 %), Sordariomycetes (Ascomycota; 11 %), Dothideomycetes (Ascomycota; 9.8 %) and Agaricomycetes (Basidiomycota; 5.2 %).

Looking closely at the dominant taxa (from the heat maps) the top 8 bacterial genera (Gaiella, Mucilaginibacter, Gp4, G1, Mycobacterium, Kribbella, Solirubrobacter and Gp6) are from Acidobacteria (3 genera) and Actinobacteria (4 genera) and one from Bacteriodetes. The top 9 fungal genera (*Trechisporales_unidentified_1*, *Pleosporaceae_unidentified*, *Ascomycota_unidentified_1_1*, *Podospora*, *Metarhizium*, *Penicillium*, *Clonostachys*, *Nectriaceae_unidentified* and *Montagnulaceae_unidentified*) are from Sordariomycetes (4 genera), Dothideomycetes (2 genera), Eurotiomycetes (1 genus) and Ascomycota_unidentified (1 genus). In comparison to VRC there appears to be less bacterial and fungal community evenness with the focus being on Actinobacteria and Incertae_sedis_10 being the predominant taxa.

4.4.3.3 VR2

The soil from this site was acidic and with this Mn may be a concern, however, the concentration is within mean average soil range. The Ca concentration is a major concern as it is 10-fold less than half the lowest value of the typical concentration range in soils. This will likely lead to Ca deficiency in *V. karroo*. S, Cr, Cd and Sb are all a major concerns as their concentrations are, double, above, 3-fold greater than and almost 6-fold greater than the maximum concentration of the typical concentration range, respectively. It would appear that Al, As, Zn, Th, Pb, U and Mo were not a concern as they were either within normal range or below the average soil concentration. From the overall bacterial phyla the most prominent taxa were Actinobacteria (29.8 %) and Acidobacteria (29.1 %), followed by Proteobacteria (13.5 %), Verrucomicrobia (9 %) and Bacteroidetes (9 %) and from the overall fungal classes the most prominent taxa were Dothideomycetes (Ascomycota; 28 %) followed closely by Sordariomycetes (Ascomycota; 26.4 %) and Incertae_sedis_10 (Zygomycota; 20.5 %). Looking closely at the dominant taxa (from the heat maps) the top 10 bacterial genera (Gp4, *Gaiella*, Gp6, *Aridibacter*, Gp1, *Flavisolibacter*, *Mucilaginibacter*, *Kribbella*, *Solirubrobacter* and *Mycobacterium*) are from Acidobacteria (4 genera), Actinobacteria (4 genera) and Bacteriodetes (2 genera). The top 8 fungal genera (*Ascomycota_unidentified_1_1*, *Metarhizium*, *Podospora*, *Clonostachys*, *Nectriaceae_unidentified*, *Chaetomiaceae_unidentified*, *Oidiodendron* and *Sordariomycetes_unidentified_1*) are from Sordariomycetes (6 genera), Dothideomycetes (1

genus) and Ascomycota_unidentified (1 genus). Looking at the microbial diversity, it would appear that the bacterial and fungal diversity are closely matched.

4.4.3.4 VR3

The soil from this site was acidic and with this, Mn becomes a concern, and compacted with the concentration being 10-fold greater than the highest value in the mean average concentration, it is likely to cause toxicity. The Ca concentration is a minor concern as it is just below the lowest value of the typical concentration range in soils and cause Ca deficiency in *V. karroo*. S, Cr, Zn, U, Cd and Sb are all a major concerns as their concentrations are, 3-fold greater than, just over double, above, 9-fold greater than, 3-fold greater than and 6-fold greater than the maximum concentration of the typical concentration range, respectively. It would appear that Al, Pb and Mo were not a concern as they were either or below the average or within the normal soil concentration range. There may be some concern from As and Th since they were both on the high end of the typical concentration range, although they were both within range. From the overall bacterial phyla the most prominent taxa were Actinobacteria (32.3 %) and Acidobacteria (25.2 %), followed by Proteobacteria (10 %), Verrucomicrobia (9.5 %) and Bacteroidetes (8.6 %) and from the overall fungal classes the most prominent taxa were Sordariomycetes (Ascomycota; 30 %), Eurotiomycetes (Ascomycota; 8.1 %) and Incertae_sedis_10 (Zygomycota) and Dothideomycetes (Ascomycota) both 3.2 %. Looking closely at the dominant taxa (from the heat maps) the top 9 bacterial genera (Gp4, Gp6, *Gaiella*, *Aridibacter*, *Kribbella*, *Solirubrubacter*, *Nocardioides*, Gp16, *Streptomyces*) are from Acidobacteria (4 genera) and Actinobacteria (5 genera). The top 8 fungal genera (*Microascaceae_unidentified*, *Cordyceps*, *Metarhizium*, *Penicillium*, *Ascomycota_unidentified_1_1*, *Hypocreales_unidentified_1*, *Aspergillus* and *Auxarthon*) are from Sordariomycetes (4 genera), Eurotiomycetes (3 genera) and Ascomycota_unidentified (1 genus). Looking at the microbial diversity, it would appear that the bacterial and fungal diversity are closely matched.

4.4.3.5 VR4

The soil from this site was acidic and with this Mn becomes a concern, and compacted with the concentration being 16-fold greater than the highest value in the mean average concentration, it is likely to cause toxicity. The Ca concentration is a major concern as it is 4-fold less than the lowest value of the typical concentration range in soils and may result in Ca deficiency in *V. karroo*. S, Cr, Cd and Sb are all a major concerns as their concentrations are, double the (for both S, Cr and Cd) and 6-fold greater than the maximum concentration of the typical concentration range, respectively. It would appear that Al, As, Th, Pb, U and Mo were not a concern as they were either below the average or within the normal soil concentration range. From the overall bacterial phyla the most prominent taxa were Actinobacteria (40.6 %) and Acidobacteria (30.2 %), followed by Verrucomicrobia (6.7 %), Proteobacteria (6.3 %), and Bacteroidetes (2.4 %) and from the overall fungal classes the most prominent taxa were Sordariomycetes (Ascomycota; 37.3 %), Eurotiomycetes (Ascomycota; 16.9 %) and Incertae_sedis_10 (Zygomycota; 12.2 %), Ascomycota_unidentified (10 %), Dothideomycetes (Ascomycota; 7.2 %), Agaricomycetes (Basidiomycota; 6.5 %) and Fungi_unidentified_1 (6 %). Looking closely at the dominant taxa (from the heat maps) the top 8 bacterial genera (Gp4, *Gaiella*, Gp1, *Solirubrobacter, Kribbella, Mycobacterium, WPS-2_genus_incertae_sedis* and *Conexibacter*) are from Actinobacteria (5 genera), Acidobacteria (2 genera) and candidate division WPS-2 (1 genus). The top 12 fungal genera (*Penicillium, Ascomycota_unidentified_1_1, Trechisporales_unidentified_1, Fungi_unidentified_1_1, Fusarium, Clonostachys, Thielavia, Microscaceae_unidentified, Trichoderma, Sordariaceae_unidentified, Chloridium* and *Oidiodendron*) are from Sordariomycetes (7 genera), Eurotiomycetes (1 genus), Dothideomycetes (1 genus), Ascomycota_unidentified (1 genus), Agaricomycetes (1 genus) and Fungi_unidentified_1. Looking at the microbial diversity, it would appear that the fungal diversity is more diverse than the bacterial diversity.

4.4.3.6 West Wits Control

This was the most acidic soil of all sites sampled and with this Mn becomes a minor concern as its concentration is greater than the highest value in the mean average concentration although it

is within the overall soil concentration range. The Ca concentration is a minor concern as it is just less than the lowest value of the typical concentration range in soils and Ca deficiency may occur in *V. karroo*. Al, S, Cr, Th, Cd and Sb are all a major concerns as their concentrations are, above (for both Al and S), 3-fold greater than, above, 3-fold and 8-fold greater than the maximum concentration of the typical concentration range, respectively. Even though As is within the typical soil concentration its concentration is on the high end of the range and may be a cause for concern. It would appear that Zn, Pb and Mo were not a concern as they were either below the average or within the normal soil concentration range. From the overall bacterial phyla the most prominent taxa were Actinobacteria (31 %) and Acidobacteria (25.7 %), then Plantomycetes (10.6 %), Verrucomicrobia (8.7 %), Proteobacteria (7.3 %), Bacteroidetes (5 %), Chloroflexi (3.2 %) and Firmicutes (2.8 %) and from the overall fungal classes the most prominent taxa were Sordariomycetes (Ascomycota; 28 %), Incertae_sedis_10 (Zygomycota; 18.8 %), Eurotiomycetes (Ascomycota; 15.4 %), Agaricomycetes (Basidiomycota; 13.1 %) and Dothideomycetes (Ascomycota; 12.3 %). Looking closely at the dominant taxa (from the heat maps) the top 13 bacterial genera (Gp4, Gp1, *Spartobacteria_genera_incertae_sedis, Gaiella, Gemmatimonas, Bacillus, WPS-1_genera_incertae_sedis, Conexibacter, Aridibacter, Mycobacterium, Solirubrobacter, Gemmata* and *Jatrophihabitans*) are from Actinobacteria (5 genera), Acidobacteria (3 genera), Verrucomicrobia (1 genus), Gemmatimonadetes (1 genus), Firmicutes (1 genus), Planctomycetes (1 genus) and candidate division WPS-1 (1 genus). The top 9 fungal genera (*Mortierella, Fungi_unidentified_1_1, Metarhizium, Cryptococcus, Fusarium, Xylariales_unidentified_1, Nectriaceae_unidentified, Trichoderma* and *Montagnulaceae_unidentified*) are from Sordariomycetes (5 genera), Dothideomycetes (1 genus), Tremellomycetes (1 genus), Incertae_sedis_10 (1 genus) and Fungi_unidentified_1 (1 genus).

4.4.3.7 WW1

The soil from this site was acidic and with this Mn becomes becomes a minor concern as its concentration is greater than the highest value in the mean average concentration although it is within the overall soil concentration range. The Ca concentration is a concern as it is half the lowest value of the typical concentration range in soils and Ca deficiency may occur in *V.*

karroo. S, Cr, As, Zn, U, Cd and Sb are all a major concerns for toxicity. S concentration was 4-fold greater than the maximum concentration of the typical concentration range. Cr concentration is over 3-fold greater than the maximum concentration of the typical concentration range. As concentration is above the maximum concentration of the typical concentration range and 5.5-fold greater than the average soil concentration. Zn concentration is above the maximum concentration of the typical concentration range. U concentration is 10-fold greater than the average soil concentration. Cd concentration is 4.2-fold greater than the maximum concentration of the typical concentration range. Sb concentration is double the maximum concentration of the typical concentration range. Even though the Th concentration is within the typical soil concentration range, its concentration is above the average and may be a cause for concern. It would appear that Al, Pb and Mo were not a concern as they were either below the average or within the normal soil concentration range. From the overall bacterial phyla the most prominent taxa were Actinobacteria (41.4 %) and Acidobacteria (18 %), then Plantomycetes and Proteobacteria (10.1 %), Bacteroidetes (7.9 %), Verrucomicrobia (5.2 %), Chloroflexi (2.9 %) and Candidatus Saccharibacteria (1 %) and from the overall fungal classes the most prominent taxa were Dothideomycetes (Ascomycota; 30.3 %), Sordariomycetes (Ascomycota; 25.3 %), Eurotiomycetes (Ascomycota; 19.3 %), Leotiomycetes (Ascomycota; 9.2 %), Incertae_sedis_10 (Zygomycota; 7 %), Agaricomycetes (Basidiomycota; 5 %), Ascomycota_unidentified (1.9 %) and Glomeromycetes (Glomeromycota, 1.3%). Looking closely at the dominant taxa (from the heat maps) the top 10 bacterial genera (*Gaiella, Kribbella, Spartobacteria_genera_incertae_sedis*, Gp1, Gp16, Gp7, *Mycobacterium, Solirubrobacter, Saccharibacteria_genera_incertae_sedis* and *Gemmatimonas*) are from Actinobacteria (4 genera), Acidobacteria (3 genera), Verrucomicrobia (1 genus), Gemmatimonadetes (1 genus) and Candidatus Saccharibacteria (1 genus). The top 8 fungal genera (*Mortierella, Herpotrichiellaceae_unidentified, Trichoderma, Fusarium, Xylariales_unidentified_1, Cladophialophora, Glomeraceae_unidentified* and *Leotiomycetes_unidentified_1*) are from Sordariomycetes (3 genera), Eurotiomycetes (2 genera), Incertae_sedis_10 (1 genus), Leotiomycetes (1 genus) and Glomeromycetes (1 genus). Interestingly even if a phylum or class occurs as a low percentage (for instance 1 %) a genus within the taxonomic classification can be shown as a dominant taxon as with the bacteria Candidatus Saccharibacteria

(*Spartobacteria_genera_incertae_sedis*) and the fungus Glomermycota (*Glomeraceae_unidentified*).

4.4.3.8 WW2

The soil from this site was acidic and with this Mn becomes a minor concern as its concentration is just above the maximum value in the mean average concentration although it is within the overall soil concentration range. The Ca concentration is a concern as it is less than the lowest value of the typical concentration range in soils and Ca deficiency may occur in *V. karroo*. S, Cr, As, Zn, U, Cd and Sb are all a major concerns for toxicity. S concentration was 2.5-fold greater than the maximum concentration of the typical concentration range. Cr concentration is almost 5-fold greater than the maximum concentration of the typical concentration range. As concentration is 6-fold greater than the maximum concentration of the typical concentration range. Zn concentration is almost double the maximum concentration of the typical concentration range. U concentration is 21.7-fold greater than the average soil concentration. Cd concentration is 2.5-fold greater than the maximum concentration of the typical concentration range. Sb concentration is 2.5-fold greater than the maximum concentration of the typical concentration range. Even though the Th concentration is within the typical soil concentration range, its concentration is above the average and may be a cause for concern. It would appear that Al, Pb and Mo were not a concern as they were either below the average or within the normal soil concentration range. From the overall bacterial phyla the most prominent taxa were Actinobacteria (37.4 %) and Acidobacteria (17.3 %), then Proteobacteria (13.9 %), Bacteroidetes (9.6 %), Verrucomicrobia (8.3 %), Plantomycetes (5.9 %), Firmicutes (1.9 %), Gemmatimonadetes (1.5 %) and Chloroflexi (1.4 %) and from the overall fungal classes the most prominent taxa were Sordariomycetes (Ascomycota; 41 %), Eurotiomycetes (Ascomycota; 22.3 %), Agaricomycetes (Basidiomycota; 13.5 %), Ascomycota_unidentified (9.3 %), Dothideomycetes (Ascomycota; 8.8 %) and Incertae_sedis_10 (Zygomycota; 1.4 %). Looking closely at the dominant taxa (from the heat maps) the top 13 bacterial genera (*Spartobacteria_genera_incertae_sedis, Kribbella*, Gp4, *Gaiella*, Gp16, Gp1, *Mycobacterium, Aridacter*, Gp7, *Gemmatimonas, Solirubrobacter, Gemmata* and *Streptomyces*) are from Actinobacteria (5 genera), Acidobacteria (5 genera), Verrucomicrobia (1 genus),

Gemmatimonadetes (1 genus) and Planctomycetes (1 genus). The top 9 fungal genera (*Trichoderma, Metarhizium, Mortierella, Clonostachys, Fusarium, Xylariales_unidentified_1, Fungi_unidentified_1_1, Cryptococcus* and *Cladophialophora*) are from Sordariomycetes (5 genera), Eurotiomycetes (1 genus), Incertae_sedis_10 (1 genus), Tremellomycetes (1 genus) and Fungi_unidentified_1 (1 genus). Again it was observed that even if a phylum or class occurs as a low percentage (for instance 1 % or less) a genus within the taxonomic classification can be shown as a dominant taxon as with the bacteria Gemmatimonadetes (*Gemmatimonas*) and the fungus Tremellomycetes (0.8 %) (*Cryptococcus*).

4.4.3.9 WW3

The soil from this site was acidic. The Ca concentration is a concern as it is less than the lowest value of the typical concentration range in soils and Ca deficiency may occur in *V. karroo*. S, Cr, As, U and Sb are all a major concerns for toxicity. S concentration was 5-fold greater than the maximum concentration of the typical concentration range. Cr concentration was 3.3-fold greater than the maximum concentration of the typical concentration range. As concentration is 6-fold greater than the maximum concentration of the typical concentration range. U concentration is 13-fold greater than the average soil concentration. Sb concentration is 8-fold greater than the maximum concentration of the typical concentration range. Even though the Th concentration is within the typical soil concentration range, its concentration is above the average and may be a cause for concern. Cd may also be a concern as its concentration is slightly above the maximum of the typical soil concentration range. It would appear that Al, Mn, Zn, Pb and Mo were not a concern as they were either below the average or within the normal soil concentration range. From the overall bacterial phyla the most prominent taxa were Actinobacteria (29.6 %), Proteobacteria (23.9 %), Acidobacteria (21.9 %), then Bacteroidetes (7 %), Plantomycetes (5.8 %), Verrucomicrobia (5.2 %), candidate division WPS-2 (2 %) and Gemmatimonadetes (1.2 %) and from the overall fungal classes the most prominent taxa were Dothideomycetes (Ascomycota; 40.2 %), Sordariomycetes (Ascomycota; 10 %), Eurotiomycetes (Ascomycota; 9.3 %), Ascomycota_unidentified (6 %), Agaricomycetes (Basidiomycota; 4.5 %), and Glomeromycetes (Glomeromycota; 1.5 %). Looking closely at the dominant taxa (from the heat maps) the top 20 bacterial genera (*Gaiella*, Gp1, *Spartobacteria_genera_incertae_sedis*, Gp16,

WPS-2_genera_incertae_sedis, Leifsonia, Kribbella, Saccharibacteria_genera_incertae_sedis, Solirubrobacter, Gemmata, Gp7, *Mycobacterium, Aridibacter, Lysinimonas, Armatimonas/Armatimonadetes_gp1, Terriglobus, Jatrophihabitans, Opitutus, Flavisoibacter* and Gp2) are from Actinobacteria (7 genera), Acidobacteria (6 genera), Verrucomicrobia (2 genera), candidate division WPS-2 (1 genus), Candidatus Saccharibacteria (1 genus), Planctomycetes (1 genus), Armatimonadetes (1 genus) and Bacteroidetes (1 genus). The top 7 fungal genera (*Mortierella, Herpotrichiellaceae_unidentified, Arnium, Fungi_unidentified, Cladophialophora, Fusarium* and *Davidiella*) are from Sordariomycetes (2 genera), Eurotiomycetes (2 genera), Incertae_sedis_10 (1 genus), Dothideomycetes (1 genus) and Fungi_uniditified_ 1 (1 genus). The bacterial diversity is over double the fungal diversity from this site.

4.4.3.10 WW4

The soil from this site was acidic. The Ca concentration is a major concern as it is 5-fold less than the lowest value of the typical concentration range in soils and Ca deficiency may likely occur in *V. karroo*. Al, S, Cr, Th, U and Sb are all a major concerns for toxicity. The Al and Th concentrations were just above the average soil concentrations. S concentration was double the maximum concentration of the typical concentration range. Cr concentration was 3-fold greater than the maximum concentration of the typical concentration range. U concentration was double the average soil concentration. Sb concentration is 6-fold greater than the maximum concentration of the typical concentration range. It would appear that Mn, As, Zn, Pb, Cd and Mo were not a concern as they were either below the average or within the normal soil concentration range. From the overall bacterial phyla the most prominent taxa were Actinobacteria (26.9 %) and Acidobacteria (24.6 %), then Chloroflexi (14.4 %), Plantomycetes (11.6 %), Proteobacteria (7.4 %), Verrucomicrobia (5.6 %), Bacteroidetes (3.7 %), candidate division WPS-2 (1.9 %) and Firmicutes (1 %) and from the overall fungal classes the most prominent taxa were Fungi_unidentified_1 (47.6 %), Sordariomycetes (Ascomycota; 19.4 %), Leotiomycetes (7.7 %), Incertae_sedis_10 (Zygomycota; 6.8 %), Eurotiomycetes (Ascomycota; 6.2 %), Archaeorhizomycetes (Ascomycota; 4.3%), Dothideomycetes (Ascomycota; 3.1 %), Agaricomycetes (Basidiomycota; 1.6 %), Tremellomycetes (Basidiomycota; 1.5 %) and

Ascomycota_unidentified (1.3 %). Looking closely at the dominant taxa (from the heat maps) the top 11 bacterial genera (Gp1, *Gaiella, Thermosporotrhix*, Gp2, *WPS-2_genera_incertae_sedis, Spartobacteria_genera_incertae_sedis, Mycobacterium, Ktedonobacter, Gemmata, Kribbella* and *Conexibacter*) are from Actinobacteria (4 genera), Acidobacteria (2 genera), Chloroflexi (2 genera), Verrucomicrobia (1 genus), candidate division WPS-2 (1 genus), and Planctomycetes (1 genus). The top 6 fungal genera (*Fungi_unidentified_1_1, Clonostachys, Archaeorhizomyces, Mortierella, Leotiomycetes_unidentified_1* and *Herpotrichiellaceae_unidentified*) are from Fungi_uniditified_1 (1 genus), Sordariomycetes (1 genus), Eurotiomycetes (1 genus), Archaeorhizomycetes (1 genus), Incertae_sedis_10 (1 genus) and Leotiomycetes (1 genus). The bacterial diversity evenness and number is greater than the fungal diversity from this site especially as one fungal taxa, namely Fungi_unidentified_1 was the predominant taxon, which unfortunately doesn't lead to any possible functional analysis unless the fungi within this taxon are classified.

4.5 Conclusion

Bulk soil analysis was not performed, which means that one factor contributing to/influencing the rhizobiome was not considered (Mendes *et al.*, 2014). The study by Mendes *et al.* (2014) reported that when comparing the functional and taxonomic microbial community of the rhizosphere and bulk soil, the rhizosphere communities resembled each other more than the bulk soil communities, which stands to reason that the microbial community structure differed between the bulk soil and the rhizosphere.

A review by Peay *et al.* (2016) suggests the predominant fungi within soil are Basidiomycota and mycorrhiza whereas Ascomycota are associated with aerial parts of the plants. This is contradictory to the findings in this study and it was shown that in fact Ascomycota was the predominant fungal taxa in the soil sampled and Basidiomycota and mycorrhiza were seen as rare taxa, however, this may be due to the choice of gene used for the identification. On the other hand, the study by Valverde *et al.* (2016) showed similar overall diversity results. Looking at the predominance/brightness of the red band in heat maps the predominant genera of the dominant

genera were looked at in more detail. It is important to remember that the rare taxa also participate in the overall rhizosphere community and its functions. Gradually as technology develops and improves, analysis of the rare taxa will become more accessible.

Surprisingly the bacterial genera that causes nodulation in legumes, namely *Rhizobium* and *Bradyrhizobium* were not reported as dominant taxa in the heat maps but this may be due to the fact that the soil used in the DNA extraction did not contain root material. It is known that once a nodule senesces several rhizobia are released (Bottomley, 1992; Moawad *et al.*, 1984), however, the details of this process are not yet known (Denison and Kiers, 2011). Oddly the Acidobacteria subgroups Gp4, Gp6, Gp7 and Gp16 were isolated and identified from the soil samples. This is unusual as these subgroups are associated with alkali conditions (pH around 8.5) and not with acidic conditions. The expected subgroups were both Gp1 and Gp2 as they are generally associated with acidic soils with pH ranging from 3.5 to 5.5 (Jones *et al.*, 2008), however, Gp2 was not identified as frequently as Gp1. Unfortunately the functions of each subgroup is not known (Engel, 2015).

With time the disturbed soil has the potential to be habitable/safely utilized particularly if revegetation of disturbed sites is successful. The success of revegetation may be influenced by the selection of plants used and the associated microbial communities, hence this study has provided some insight into possible options and alternatives for future revegetation of disturbed sites. Several microorganisms that were expected to be present in greater quantities were poorly observed from the genes utilised and may be better represented with the use of alternate gene regions. However, it would appear that in this study there was a greater diversity of microorganisms observed associated with *V. karroo* rhizosphere than in previous studies analysing other plant species. This may demonstrate a positive step towards fully uncovering the rhizobiome in the future.

With the initial identification of rhizosphere microorganisms along with soil chemical conditions having been performed, possible functionality in pathogen resistance and stress tolerance in plants may be fully determined in future studies. With this, one may efficiently and reliably utilise microbes for industrial and revegetation applications in the future.

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Appendices

Appendix 1

Appendix 1 – Tree numbers and corresponding GPS co-ordinates

A table illustrating the trees and their respective GPS co-ordinates selected from Vaal River Operations and West Wits Operations.

Site	Niche site	Tree number	GPS co-ordinates	
			Longitude	Latitude
Vaal River Operations	Madala	1	26.69433333	-26.93516667
		2	26.69458333	-26.93466667
		3	26.69473333	-26.93458333
	Black Reef	4	26.69188333	-26.92073333
		5	26.69190000	-26.92075000
		6	26.69201667	-26.92085000
	East Pay Dam	7	26.74088333	-26.94808333
		8	26.74066667	-26.94805000
		9	26.74071667	-26.94800000
	R. G. Williams	10	26.70270000	-26.96691667
		11	26.70276667	-26.96690000
		12	26.70303333	-26.96708333
	Control 1	13	26.80491667	-26.88920000
	Control 2	14	26.84152778	-26.86572222
West Wits Operations	Tailing Storage Facilities lower slope, over soil, retaining wall	1	27.35912547	-26.43001179
		2	27.35912547	-26.43001179
		3	27.35912547	-26.43001179
	Toe-Paddock West Wits Old North Tailings Storage Facilities North Toe Paddock – Eastern Boundary	4	27.38526863	-26.42729430
		5	27.38526863	-26.42729430
		6	27.38526863	-26.42729430
	Toe-Paddock West Wits Old North Tailings Storage Facilities North Toe Paddock North-Eastern Boundary	7	27.38662440	-26.42531617
		8	27.38662440	-26.42531617
		9	27.38662440	-26.42531617
	Varkenslaagte Woodlands Blocks	10	27.37157914	-26.42738943
		11	27.37157914	-26.42738943
		12	27.37157914	-26.42738943
	Control 1	13	27.340500000	-26.44711111
	Control 2	14	27.339111111	-26.44855556

Appendix 2

Appendix 2 – Trial PCR and the results

Nested PCR

Nested PCR protocol was selected as it provides increased specificity for the amplification of AM fungal DNA (Winn and Koneman, 2006). This is attributed to the fact that two primer sets were used, with the target of the second primer set residing within that of the first (i.e. it is within the product of the first primer set) (Winn and Koneman, 2006). If the first set of primers were to amplify the wrong locus, it was unlikely that the second set of primers would be able to amplify the product thus decreasing the possibility that the incorrect sequences were amplified. Hence, the specificity of the process was increased as a greater proportion of product from the desired site was produced (Winn and Koneman, 2006).

The primary PCR reaction (Bio-Rad MyCycler™ Thermal Cycler) utilized the universal eukaryotic nuclear primers NS1 and NS4 (White *et al.*, 1990).

NS1: 5' – GTAGTCATATCGTTGTCTC – 3' (White *et al.*, 1990)
NS4: 5' – CTTCCGTCAATTCCTTTAAG – 3' (White *et al.*, 1990)

The components of the PCR reaction (20μℓ) were:
10 μℓ of 2x Phire® Plant PCR buffer
6 μℓ double distilled (nuclease free) water
0.8 μℓ NS1 stock solution 10 μM
0.8 μℓ NS4 stock solution 10 μM
0.4 μℓ Phire® Hot Start II DNA polymerase
2 μℓ DNA sample

The primary PCR was carried out as follows: the initial denaturation at 98 °C for 5 min; followed by 40 cycles with the denaturation steps at 98 °C for 5 s each; the primer annealing steps at 54 °C (Lee *et al.*, 2008) for 5 s each; the elongation step at 72°C for 20 s each; and after the 40

cycles a final extension step at 72 °C for 1 min. The reduced times for the PCR protocol are as a result of the Phire® Hot Start II DNA polymerase. The product produced by the first PCR was used as the template for the nested PCR reaction.

The nested PCR reaction (Applied Biosystems 2720 Thermal Cycler) utilized the AM fungal primers AML1 and AML2 (Lee *et al.*, 2008). These primers are AM fungi-specific and target the less variable SSU rRNA gene to allow for discrimination against non-AM fungi sequences and sufficient resolution down to species level in AM fungi (Lee *et al.*, 2008).

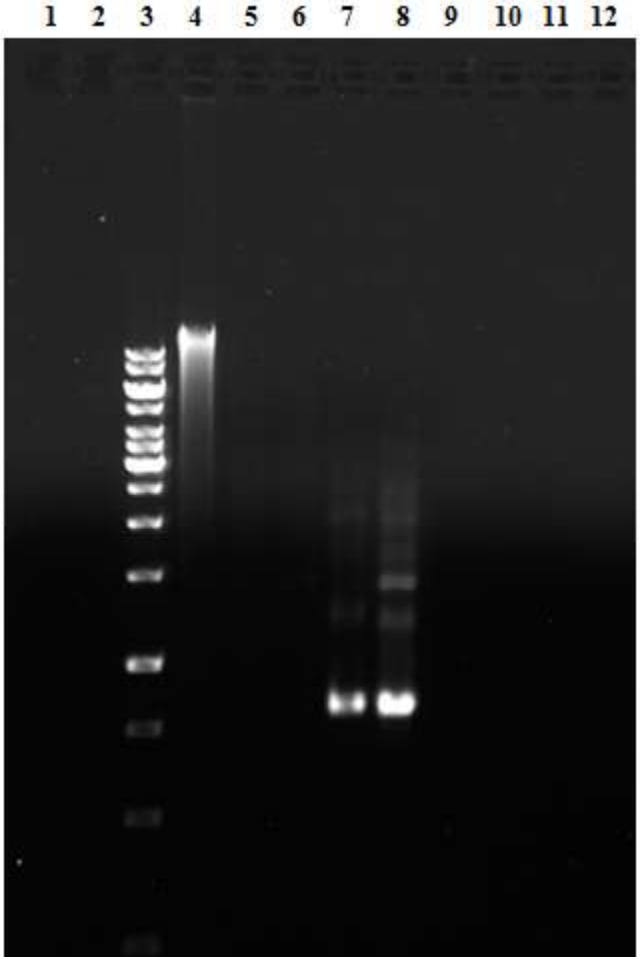
AML1: 5' – ATC AAC TTT CGA TGG TAG GAT AGA – 3' (Lee *et al.*, 2008)
AML2: 5' – GAA CCC AAA CAC TTT GGT TTC – 3' (Lee *et al.*, 2008)

The components of the PCR reaction (20 µℓ) were:
10 µℓ of 2x Phire® Plant PCR buffer
6 µℓ double distilled (nuclease free) water
0.8 µℓ AML1 stock solution 10 µM
0.8 µℓ AML2 stock solution 10 µM
0.4 µℓ Phire® Hot Start II DNA polymerase
2 µℓ primary PCR product as DNA template

The nested PCR was carried out as follows: the initial denaturation at 98 °C for 5 min; followed by 40 cycles with the denaturation steps at 98 °C for 5 s each; the primer annealing steps at 63 °C (Lee *et al.*, 2008) for 5 s each; the elongation step at 72 °C for 20 s each; and after the 40 cycles a final extension step at 72 °C for 10 min. The DNA product produced by the nested PCR reaction was analysed on an agarose gel and a few samples were selected for cloning, according to the clarity and strength of the DNA band on the agarose gels and representative samples of the different hosts in the study.

<u>NanoDrop results</u>
Concentration = ± 64 ng/µℓ
260/280 = 1.68

Agarose gel results



A figure illustrating a 1 % agarose gel showing original extracted DNA and nested PCR DNA results.

Lanes: (3) O' GeneRuler™ 1kb DNA ladder; (4) Original total soil DNA; (5-6) primary PCR DNA results using NS primers; (7-8) nested PCR DNA results using AML primers.

Appendix 3

Appendix 3 –DNA yield and preliminary quality ascertained with the use of a ND-1000 spectrophotometer (NanoDrop, Wilmington, Delaware USA) using 1-2 µℓ of the sample.

A table illustrating the DNA replicates from Vaal River Operations and West Wits Operations and the results analysed using the NanoDrop.

Site	Sample	DNA concentration (ng/µℓ)	260/280 ratio
Vaal River Operations	VRC Replicate 1	54.94	1.93
	VRC Replicate 2	65.67	1.89
	VR1 Replicate 1	30.6	1.98
	VR1 Replicate 2	23.5	2.06
	VR2 Replicate 1	51.22	1.89
	VR2 Replicate 2	34.78	1.9
	VR3 Replicate 1	41.98	1.96
	VR3 Replicate 2	34.84	1.92
	VR4 Replicate 1	25.9	1.81
	VR4 Replicate 2	28.83	1.86
West Wits Operations	WWC Replicate 1	145.04	1.9
	WWC Replicate 2	123.9	1.92
	WW1 Replicate 1	21.6	1.85
	WW1 Replicate 2	32.3	1.7
	WW2 Replicate 1	53.1	1.8
	WW2 Replicate 2	49.5	1.89
	WW3 Replicate 1	17	1.6
	WW3 Replicate 2	8.98	1.78
	WW4 Replicate 1	17.95	1.72
	WW4 Replicate 2	12.85	1.79