

**Bacterial diversity of soil irrigated with Gypsiferous mine water as determined by culture-
dependent and -independent techniques.**

By

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

Signed: _____

Date: _____



DEDICATION

This thesis is dedicated to my late

Mother, Miriam Maleoa Lekhanya (1945-2007)

And

Sister, Pinky Maleshoeane Mokgere (1970-2007)

You are forever with me.

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“James 1:5”

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LIST OF ABBREVIATIONS

Al:	Aluminium
ALD:	Anoxic Limestone Drain
AMD:	Acid Mine Drainage
ARDRA:	Amplified Ribosomal DNA Restriction Analysis
ARISA:	Automated Ribosomal Intergenic Spacer Analysis
ARUM:	Acid Reduction Using Microbiology
AWCD:	Average Well Colour Development
bp:	Base Pair
Ca ²⁺ :	Calcium
CaCO ₃ :	Calcium Carbonate (limestone)
CaO:	Calcium Oxide
Ca(OH) ₂ :	Calcium Hydroxide
CaSO ₄ .H ₂ O:	Calcium Sulphate (Gypsiferous mine Water)
cfu:	Colony Forming Unit
Cl ⁻ :	Chloride
CLPP:	Community Level Physiological Profiles
DGGE:	Denaturing Gradient Gel Electrophoresis
DNA:	Deoxy Ribonucleic Acid
EC:	Electrical Conductivity
FAME:	Fatty Acid Methyl Ester
FISH:	Fluorescent In Situ Hybridisation
Fe ³⁺ :	Ferric iron
Fe ²⁺ :	Ferrous iron
FeS ₂ :	Pyrite
G+C:	Guanine plus Cytosine
GN:	Gram Negative
GP:	Gram Positive
H ⁺ :	Hydrogen
H ₂ O:	Water

K ⁺ :	Potassium
Mg ²⁺ :	Magnesium
mRNA:	Messenger Ribonucleic Acid
Na ⁺ :	Sodium
NADH:	reduced Nicotinamide Adenine Dinucleotide
NaOH	Sodium Hydroxide
O ₂ :	Oxygen
OD:	Optical Density
OTU:	Operational Taxonomic Unit
PBR:	Permeable Reactive Barriers
PCA:	Principal Component Analysis
PCR:	Polymerase Chain Reaction
PLFA:	Phospholipid Fatty Acid
RAPD:	Randomly Amplified Polymorphic DNA
RFLP:	Restriction Fragment Length Polymorphism
RISA:	Ribosomal Intergenic Spacer Analysis
RNA:	Ribonucleic Acid
rDNA:	Ribosomal Deoxy Ribonucleic Acid
rRNA:	Ribosomal Ribonucleic Acid
rpm:	Revolutions Per Minute
SCSU:	Sole Carbon Source Utilisation
SO ₄ ²⁻ :	Sulphate
TDS:	Total Dissolved Salts
TGGE:	Temperature Gradient Gel Electrophoresis
T-RFLP:	Terminal Restriction Fragment Length Polymorphism
WCFA:	Whole Cell Fatty Acid

CHAPTER 1

1. INTRODUCTION

Soil contains more microbial species and genera than most habitats. Soil microbes are an essential part of soil microflora and are present in abundance. Torsvik *et al.* (1990) estimated a minimum of 4 000 different bacterial genomes per gram of soil. These microbes play a vital role in soil functions and quality since most of them are involved in the biochemical cycling of elements (carbon, nitrogen, sulphur etc.) and trace elements (iron, nickel, mercury, etc.) (Ranjard *et al.*, 2000). These microorganisms are also involved in degradation of complex organic matter and are involved in plant productivity (Nannipieri *et al.*, 2003).

Anthropogenic, agricultural, and other man-made practices, often have a negative effect on soil microbial communities and ecological balance, resulting in a decrease in biodiversity, an extinction of sensitive species or selection of those better adapted to the environment (Kozdrój and van Elsas, 2001). An example of the above-mentioned practices is the use of lime-treated acid mine water, better known as gypsiferous water, for irrigation purposes. This practice is most common in areas where there is a shortage of rain fall and the treated mine water is used as an alternative (Jovanoic *et al.*, 1998). The mining industry produces an acidic and sulphur-rich wastewater, commonly referred to as acid mine drainage (AMD). AMD is produced through the oxidation of iron pyrite and sulphur minerals by oxygen and water in the presence of *Acidothiobacillus* species (Johnson, 2003). This water is regarded as a major environmental threat because of its high levels of acidity and salinity and needs to be treated before it is discharged into the environment (Annandale *et al.*, 2002). For treatment, the acidic water is often allowed to react with alkaline reagents, such as limestone, and this results in neutral-pH water rich in both calcium and sulphate (Jovanoic *et al.*, 1998; Annandale *et al.*, 2002). This lime-treated water is then used for the irrigation of crops (Jovanoic *et al.*, 1998)

An understanding of the structural and functional diversities of soil microbial communities is imperative in order to understand how microbes respond to various

anthropogenic disturbances (Ranjard *et al.*, 2000). However this has been hampered by two challenges in the field of soil microbiology:

- (1) The development of efficient methods to determine the actual microbial composition and;
- (2) Methods to study microbial functions *in situ* (Hill *et al.*, 2000).

Viable cell count has been a traditional culture-dependent method used for years to study soil bacterial communities (Kirk *et al.*, 2004). The method determines the total culturable heterotrophic bacteria present in a given environment. However, over the years, the approach to studying soil microbial communities changed drastically with the development of culture-independent techniques (also referred to as molecular techniques). These techniques presented soil microbiologists with a platform to profusely study and analyse soil microorganisms (Hill *et al.*, 2000). Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) is one of the most widely used molecular method to study microbial diversity from environmental samples (Ferris *et al.*, 1996).

Numerous studies have been conducted on the response of soil microbial communities to various disturbances and stress. In their study, Juck *et al.* (2000) observed and compared microbial communities of two Canadian soil ecosystems contaminated with petroleum. They found that samples from the one soil community were negatively affected by the contamination whereas those from the second community showed an increase in diversity, following the introduction of the contaminant. Rasmussen *et al.* (2001) studied the microbial community of soil contaminated with mercury. They observed an initial decrease in microbial diversity followed by an immediate increase after exposure to the contaminant. On the other hand, Evans *et al.* (2004) found that the bacterial community structure, of an acidic cambisol under Atlantic forest, remained consistent after the introduction of oil. The above-mentioned studies reveal the unique characteristic of soil microorganisms to easily adapt and take advantage of the new conditions as previously studied by Atlas (1991).

The use of lime-treated acid mine water for irrigation purposes has gained much popularity in South Africa, especially in semi-arid areas such as Mpumalanga Province (Jovanoic *et al.*, 1998). A number of studies have focused on the response of microbial activity and/or biomass to various sulphide minerals (Bajpai *et al.*, 1976, Carter, 1986, Ehrlich, 1996, Lindsay *et al.*, 2009). This study investigates the effect of lime-treated acid mine water, also referred to as gypsiferous mine water, on soil bacterial communities using both culture-dependent and culture-independent techniques. The specific objectives were to:

- Determine the microbial community structures of gypsum-irrigated and non-gypsum irrigated (control) soil samples using DGGE;
- Examine the carbon utilisation patterns of gypsum irrigated and non-gypsum irrigated (control) soil samples using community-level physiological profiles and;
- Compare the genetic, functional and culturable heterotrophic diversities among soil samples collected from different pivots; all irrigated with gypsiferous mine water.

CHAPTER 2

2. LITERATURE REVIEW

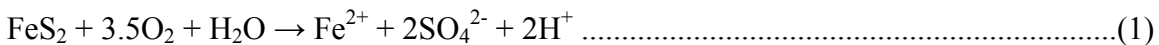
2.1) Acid mine water

The mining industry is one of the most important industries in South Africa, both from the point of gross national product as well as job creation. However, during the mining of mineral resources, pollution problems are created and these pose a threat to the already scarce water resources in South Africa (Jovanoic *et al.*, 1998). The disposal of mine wastewater is a critical problem wherever operating and closed mines are found. Besides the extremely low levels of pH, other constituents present in the water, such as salts and metals, make it impossible for a direct discharge of the water into river systems (Pulles *et al.*, 1996; Jovanoic *et al.*, 1998).

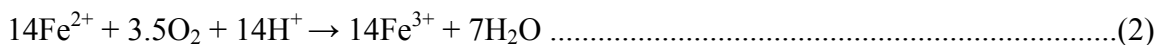
Acid mine wastewater is the by-product of a variety of industrial operations; with the mining industry being the main producer. Water draining from both active and abandoned mines is often extremely acidic and contains elevated concentrations of metals (iron, aluminium, manganese and possibly other heavy metals). Such water is generally referred to as Acid Mine Drainage (AMD) (Johnson and Hallberg, 2005).

The production of these acidic metal-rich mine drainage waters is described in great detail by Johnson (2003). In Brief, acid mine water is produced through the accelerated oxidation of iron pyrite (FeS₂) and other sulphidic minerals by oxygen and water. In most cases, the process is often accelerated by the presence of oxidising bacteria such as *Thiobacillus ferrooxidans*. Although sulphidic minerals are stable in dry and anoxic environments, exposure to both oxygen and water causes them to oxidize spontaneously (Johnson, 2003).

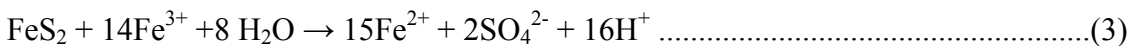
The following chemical reactions explain the process of pyrite oxidation (Barker and Banfield, 2003):



In most conditions, pyrite is oxidised by ferric iron (Fe³⁺) rather than molecular oxygen.



The ferric iron is then reduced to ferrous iron by pyrite:



The sum of reactions in equation 2 and 3 produce the chemical reaction in equation 1.

The oxidation of pyrite is dependent upon the regeneration of ferric iron (Fe^{3+}), which is reduced to ferrous iron (Fe^{2+}) on reaction with pyrite (Johnson, 2003).

The high concentrations of SO_4^{2-} , accompanied by the presence of potentially toxic metals (e.g., aluminium, copper, zinc and cadmium) necessitate the treatment of AMD prior to discharge into rivers and streams (Rose and Elliot, 2000). Often, the sulphuric acid may be neutralised naturally by reacting with base minerals. However, in cases where inadequate natural neutralisation potential is present, AMD needs to be treated by other means (such as active and passive treatment systems) (Johnson, and Hallberg, 2005).

2.2) Treatment of Acid Mine Drainage

Acid mine drainage treatment processes are divided into “active” and “passive” techniques. The former process refers to the continuous application of alkaline agents to neutralise the acidity and precipitate the metals. The latter process involves the use of natural and constructed wetland ecosystems (Jage *et al.*, 2001, Johnson and Hallberg, 2005). However, a more useful classification is the division of treatment processes into abiotic and biological groups with each major group further divided into “active” and “passive” processes (Figure 2.1) (Johnson and Hallberg, 2005).

2.2.1) Abiotic treatment processes

- *Active system*

This method involves physical addition of chemical-neutralising agents such as sodium hydroxide (NaOH), calcium hydroxide ($\text{Ca}(\text{OH})_2$), calcium oxide (CaO) and calcium carbonate (CaCO_3). These chemicals raise the pH, accelerate the oxidation of

ferrous iron and cause acid-soluble metals to precipitate as hydroxides and carbonates (Jage *et al.*, 2001, Johnson and Hallberg, 2005). The end product is an iron-rich sludge which may contain other metals, depending on the chemistry of the treated mine (Johnson and Hallberg, 2005).

- *Passive system*

Another method for adding alkalinity to acidic mine waters is the pre-treatment within an anoxic limestone drain (ALD) (Gazea *et al.*, 1996). During the process, alkali is added to AMD while maintaining the iron in its reduced state to avoid oxidation of ferrous iron and ferrous hydroxide precipitation (Johnson and Hallberg, 2005). In an ALD, mine water flows through limestone gravel within a drain impermeable to both water and air (Johnson and Hallberg, 2005).

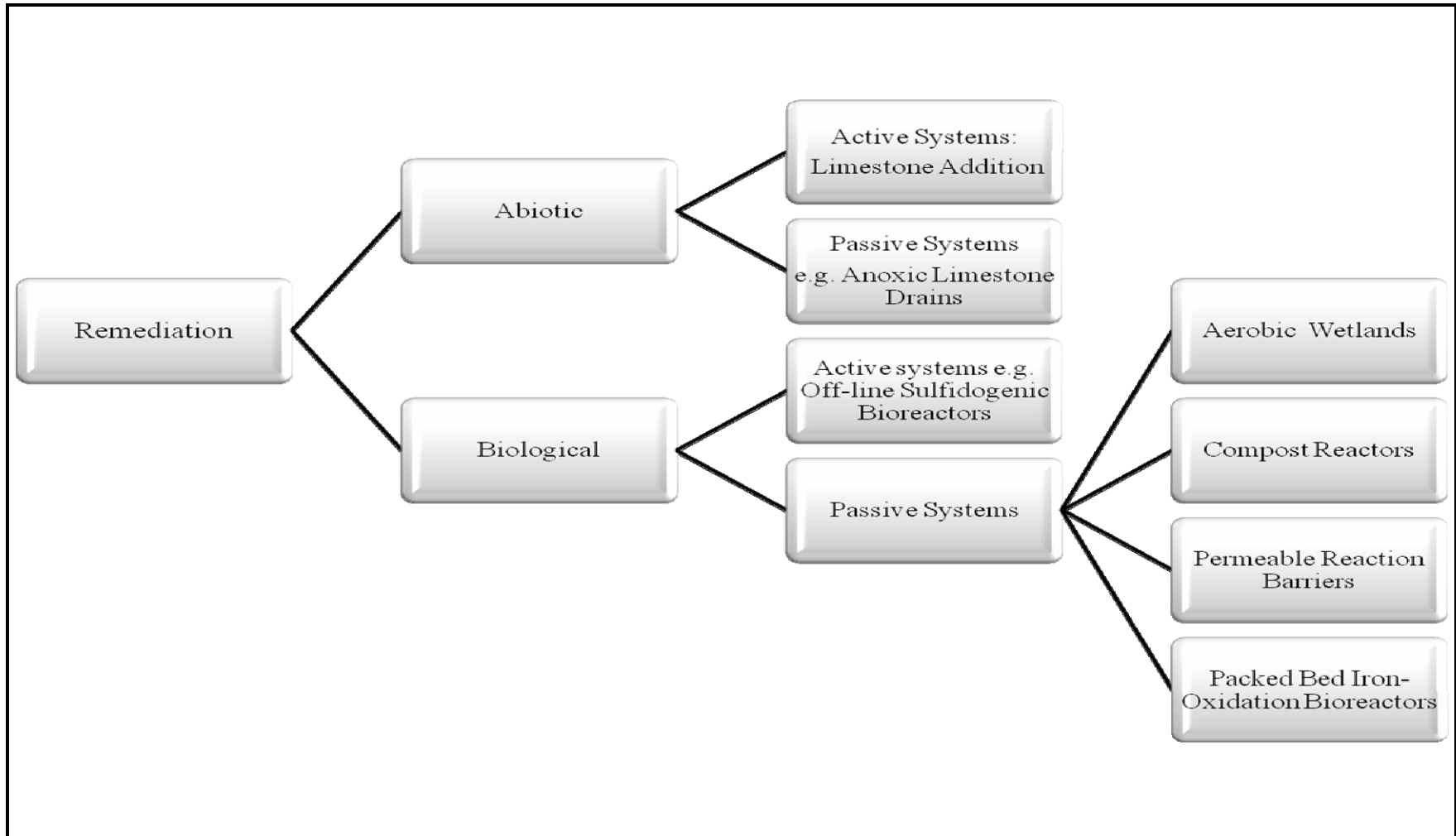


Figure 2.1 Different treatment technologies for acid mine drainage (Johnson and Hallberg, 2005).

2.2.2) Biological treatment processes:

- **Passive biological systems:**

Microbial processes

Several microorganisms are capable of producing alkalinity and immobilising metals, thereby reversing the process of AMD generation (Johnson and Hallberg, 2005). This is achieved through a consortium of different microorganisms which work together to generate alkalinity (Cloete and Coetser, 2006) through microbiological processes such as denitrification, methanogenesis as well as sulphate and iron reduction (Johnson and Hallberg, 2005). Photosynthetic microorganisms (e.g., cyanobacteria) produce net alkalinity by consuming bicarbonate and producing hydroxyl ions. During dissimilatory reduction of sulphate to sulphide, alkalinity is generated through microbial transformation of sulphuric acid to hydrogen sulphide (Johnson and Hallberg, 2005).

Aerobic Wetlands

Aerobic wetland systems are employed for the treatment of alkali water. In these systems, the pH of the water is raised as it comes into contact with carbonate rocks and the ferrous iron is oxidised in the aerated water and subsequently hydrolysed and precipitated as ferrous hydroxide (Gazea *et al.*, 1996; Johnson and Hallberg, 2005). Aerobic wetland treatment was one of the first passive designs to be used on a large scale (Jage *et al.*, 2001). Macrophytes are planted in aerobic wetlands to regulate water flow as well as to filter and stabilise the ferric precipitates. They also provide additional surface area for the precipitation of ferric ion compounds and minerals (Johnson and Hallberg, 2005).

Anaerobic wetlands/compost bioreactors

Contrary to aerobic wetlands, compost bioreactors/anaerobic wetlands rely on the mitigation of acidic water under anaerobic conditions. These systems generate alkalinity through a combination of sulphate-reducing bacteria and the addition of limestone. In order to create the anoxic conditions, sulphate-reducing bacteria require

a rich organic substrate. The most common materials used as substrates for the sulphate-reducing bacteria include natural products as well as wastes such as horse/cow manure, peat, wood chips and saw dust (Gazea *et al.*, 1996).

Composite aerobic and anaerobic wetlands

Other passive systems include the combination aerobic and anaerobic wetlands. These wetlands utilise a combination of aerobic and anaerobic conditions to treat AMD; an example is a system called Acid Reduction Using Microbiology (ARUM). The system is made up of two oxidising cells, in which the ferrous iron is oxidised and precipitated. AMD passes through a holding cell and through two ARUM cells where the alkaline and sulphide are generated (Johnson and Hallberg, 2005).

Permeable reactive barriers (PBR)

These are used for the treatment of a wide range of polluted groundwater. The system operates the same as compost bioreactors. They are constructed by digging a pit in the flow path of the contaminated groundwater, filling the hole with a mixture of organic material and limestone and landscaping the surface (Johnson and Hallberg, 2005). The processes within PBR generate alkalinity and remove metals as sulphides, hydroxides and carbonates.

Iron-oxidising bioreactors

In these systems, a number of iron oxidising bacteria are used. These are known to accelerate the oxidation of ferrous iron to ferric in acidic mine waters. The most common and well studied of iron oxidising bacteria is *Acidithiobacillus ferrooxidans*. In iron oxidising bioreactors, *At. Ferrooxidans* is immobilized onto a solid matrix, forming the basis of packed bed reactors (Johnson and Hallberg, 2005).

- **Active biological systems**

- Sulphidogenic bioreactors*

Sulphidogenic bioreactors offer a different and unique approach for the treatment of AMD. These bioreactors use hydrogen sulphide (H₂S) to produce alkalinity and remove metals such as insoluble sulphides (Johnson and Hallberg, 2005). These systems have the following advantages:

- (i) easier and more controlled performance;
- (ii) metals such as copper and zinc present in AMD can be recovered and controlled and;
- (iii) sulphate concentrations in processed water are significantly low.

2.3) Microbial populations inhabiting metal-rich environments

It has been established that acidic, metal-rich environments, such as mine spoils, tailings and AMD, can harbour a diverse range of microbial life. Despite the adverse conditions, the microbial community structures and interactions found in these environments are diverse and complex (Johnson, 2003). A large number of these microorganisms are obligate acidophilic microorganisms which thrive under low pH levels.

2.3.1) Acidophilic microorganisms

Most of known acidophiles are mesophilic (growth temperature range 20-40 °C), some are thermotolerant (growth temperature range 40-60 °C) and others are thermophilic (growth temperature > 60°C) (Johnson, 2003).

- *Mineral-oxidizing acidophiles*

The most important and studied of acidophiles are those which are able to oxidise ferrous iron and/or reduced forms of sulphur and accelerate the oxidative dissolution of sulphidic minerals (Table 2.1). *Acidithiobacillus ferrooxidans* is the best known of all mineral-degrading bacterium and the first pyrite-oxidising bacterium to be discovered (Johnson, 2003). Because of its widespread distribution in natural and man-made environments, and relative ease of culturing in the laboratory, it was

assumed the most significant sulphide mineral-oxidising bacterium. However a second iron-oxidising bacterium, *Leptospirillum ferrooxidans* is now known to be more abundant and active than *At. ferrooxidans* in numerous situations. *L. ferrooxidans* has a higher substrate affinity, greater tolerance to ferric iron and low pH (<2) than *At. Ferrooxidans* (Johnson 2003).

○ *Iron-and Sulphate-reducing Acidophiles*

Under anoxic environments, both ferric iron and sulphate can act as alternative electron acceptors to oxygen and this applies to both acidic as well as neutral pH environments. In these environments, anaerobic respiration using ferric ion appears to be an attractive alternative to aerobic metabolism due to elevated concentrations of iron, the solubility of ferric iron at low pH as well as the high redox potential of the ferrous/ferric couple (+770 mV at pH 2). Although a number of researchers have proved the biological reduction of sulphate to sulphide and the presence of neutrophilic sulphate reducing bacteria, a few have reported on the isolation and characterisation of acidic sulphate reducing bacteria (Johnson, 2003).

○ *Other acidophiles*

There are other acidophilic microorganisms present in mine tailings which have no impact on the cycling of iron and sulphur but are still of great ecological significance. These are heterotrophic prokaryotes, acidophilic eukaryotes and acidophilic protozoa (Johnson, 2003).

Table 2.1 Sulphide mineral-oxidising bacteria (Johnson, 2003).

	Iron-oxidisers	Ion/sulphur-oxidisers	Sulphur-oxidisers
Mesophiles	<i>Leptospirillum ferrooxidans</i>	<i>Acidithiobacillus ferrooxidans</i>	<i>Acidithiobacillus thiooxidans</i>
	<i>Ferroplasma spp</i>	<i>Thiobacillus prosperus</i>	<i>Thiomonas cuprina</i>
	<i>Ferrimicrobium acidophilum</i>	<i>Sulfobacillus montserratensis</i>	
Moderate	<i>Acidimicrobium ferrooxidans</i>	<i>Sulfobacillus thermosulfidooxidans</i>	<i>Acidithiobacillus caldus</i>
Thermophiles	<i>Leptospirillum thermoferrooxidans</i>	<i>Sulfobacillus acidophilus</i>	
Thermophiles		<i>Acidianus spp.</i>	<i>Metallophaera spp</i>
		<i>Sulfolobus metallicus</i>	
		<i>Sulfurococcus yellowstonensis</i>	

2.4) Soil as a habitat for microorganisms

Soil is fundamental and irreplaceable; it governs plant productivity and maintains biogeochemical cycles. The living biota present in soil is very diverse and includes the microflora, the mesofauna as well as macrofauna; the presence of which is dependent upon the soils physical and chemical properties (Nannipieri *et al.*, 2003). Bacteria are the most abundant group of microorganisms present in soil. They are highly versatile as they are capable of carrying out all known biological reactions, including the degradation of virtually all organic compounds (Nannipieri *et al.*, 2003). The soil system has the following distinctive properties:

1. It consists of a diverse microbial population. In the early 1990's, Torsvik *et al.* (1990) estimated the presence of approximately 4,000 different bacterial genomes in a forest soil in Norway. Later in the decade, Torsvik *et al.* (1996) calculated the presence of almost 6000 different bacterial genomes per gram, using the genome size of *Escherichia Coli* as a unit.
2. It is structured and heterogeneous system, with microorganisms living in discrete microhabitats most of are generally poor in nutrients and energy (Nannipieri *et al.*, 2003).
3. The system has distinguished zones referred to as 'hot spots'. These are zones of increased biological activity due to presence of high levels of organic matter (Nannipieri *et al.*, 2003).
4. The solid phase has a unique ability to adsorb important molecules such as proteins, nucleic acids and extracellular enzymes, essential for sustaining microbial activity.
5. The minerals present (e.g., manganese, iron, etc) are themselves responsible for a number of chemical reactions. Iron oxides in are involved in electron transfer reactions such as the oxidation of phenols and polyphenols (Nannipieri *et al.*, 2003).

2.4.1) The role of microorganisms in soil

Bacteria are an essential part of the soil community due to their immense presence (up to 10^9 cells per gram soil) (Ranjard *et al.*, 2000); their high species diversity (a minimum of 4000 completely different genomes per gram soil) (Torsvik *et al.*, 1990) as well as the magnitude of their metabolic activities (Ranjard *et al.*, 2000). Microbial characteristics have been regarded as sensitive indicators of soil health because of the clear correlation between microbial diversity and soil health and/or quality. The relationship between microbial diversity and soil functionality is important; considering the fact that 80-90% of processes in soil are mediated by microbes (Coleman and Crossley, 1996). These microbes play important roles in the biogeochemical cycles of the main elements (carbon, nitrogen, sulphur, etc.) and trace elements (iron, nickel, mercury, etc.) and are therefore critically involved in energy and nutrient exchanges within soil. Microorganisms can also be used to determine the past history of a given environment (Ranjard *et al.*, 2000). They are nature's original recyclers; many of which are able to convert toxic organic compounds to harmless products, often carbon dioxide and water. Since the discovery that microbes are able to transform and/or degrade xenobiotics, scientists have been exploring the microbial diversity, especially of contaminated areas in search of organisms that can degrade a wide range of pollutants. Due to the importance of microorganisms in soil, it is therefore vital to understand the interrelationships between microbes and their environment by studying the structural and functional diversity of microbial communities and how they respond to natural and man-made disturbances (Ranjard *et al.*, 2000).

2.5) Microbial diversity

In microbiology, the term diversity is used to describe the qualitative variation among microbial populations. Microbial diversity often includes the amount and distribution of genetic information within microbial species; the diversity of bacterial and fungal species in microbial communities and ecological diversity (Nannipieri *et al.*, 2003). Torsvik and Øvreås (2002) defined microbial diversity as the complexity and variability of microbes at different levels of biological organisations. It encompasses genetic variability within taxons (species); the number (richness) as well as the relative abundance (evenness) of

taxons in communities. Important aspects of diversity at ecosystem level are the range of processes, the complexity of interactions and the number of trophic levels. Diversity can be defined as the range of significantly different kinds of organisms and their relative abundance in a natural assemblage or habitat. Theoretically, it can be regarded as the amount and distribution of genetic information in a community (Øvreås and Torsvik, 1998).

A representative estimate of microbial diversity is a prerequisite in understanding the functional activities of microorganisms in ecosystems (Garland & Mills, 1994).

Microbial diversity can be divided into different levels, including genetic, taxonomic and functional diversity:

- Genetic diversity is defined as the amount and distribution of genetic information in a community (Johnsen *et al.*, 2001);
- Taxonomic diversity is defined as the number of different bacterial types and their relative abundance present in a community (Johnsen *et al.*, 2001) and;
- Functional diversity is defined by the range of activities and carbon utilisation activities in a community (Torsvik and Øvreås, 2002). It is determined in terms of the presence, absence or rate of substrate utilisation.

2.5.1) The significance of studying microbial diversity

Microorganisms comprise a huge and unexplored reservoir of resources, which offers innovative applications useful to man. Microorganisms have been evolving for almost 4 billion years and are capable of exploiting a vast range of energy sources and able to survive and thrive in almost every habitat (Kapur and Jain, 2004).

Microorganisms represent the richest repertoire of molecular and chemical diversity in nature. They have an enormous impact and role in our daily lives, from maintaining the biosphere to improving our lifestyles (Hunter-Cervera, 1998). Decomposition processes are dominated by microbial activities and are as fundamental as primary production for

the long-term functioning of an ecosystem. Microbial diversity analyses are therefore essential in order to (Øvreås, 2000):

- increase the knowledge of the diversity of genetic resources in a community;
- understand the relative distribution of organisms;
- increase the knowledge of the functional role of diversity;
- understand the regulation of biodiversity and;
- understand the consequences and importance of biodiversity (to what extent does ecosystem functioning and sustainability depend on maintaining a specific level of diversity).

2.5.2) Response of microorganisms to anthropogenic factors

A number of factors, such as stress and disturbances, are known to affect the soil microbial community structure. Atlas (1984) postulated that various interactions among populations in a specific habitat bring about a more stable and organised community. Microorganisms are known to respond to perturbations the same way as plants and animals, only much faster. This is partly attributed to their faster growth rate, as compared to higher organisms. Another reason is that the microbial community often consist of members which are ready to take advantage of the new situation (Øvreås, 2000). A number of studies have been centred on the response of microorganisms to various stresses/disturbances, such as hydrocarbons (Juck *et al.*, 2000; Maila *et al.*, 2005), herbicides (Fantroussi *et al.*, 1999) as well as heavy metals and antibiotics (Müller *et al.*, 2001; Müller *et al.*, 2002). Often after the disturbance, the microbial communities are able to recover and take advantage of the new conditions; thus illustrating the adaptability of microorganisms (Øvreås, 2000).

2.6) Common precincts in studying microbial diversity

Throughout the study of soil microbiology, a number of problems associated with studying bacterial and fungal diversity in soil have been identified. These are not only due to methodological limitations, but also stem from lack of sufficient taxonomic knowledge. It has been difficult to study the diversity of a group of microorganisms when

it is not understood how to categorise or identify the species present (Kirk *et al.*, 2004). The following are some of the hindrances in studying bacterial diversity:

Spatial heterogeneity

In most instances, 1 to 5 g of soil is used as sample to measure diversity, and from which conclusions about the community are made. This approach has a number of limitations such as the spatial distribution of the microorganisms in soil as well as the soil-particle-cell-particle interactions (Trevors, 1998). Microbial communities exist on such a small scale that 1 to 5 g of soil could be bias and favour the detection of only the dominant populations in the sample (Grundmann and Gourbière, 1999). Grundmann and Gourbière (1999) suggested a micro-sampling procedure which reduces the errors associated with soil heterogeneity and spatial distribution of bacteria in soil matrix. Another problem of using 1 to 5 g soil to study microbial diversity is that soil is heterogeneous, containing many microhabitats suitable for microbial growth and bacteria are often found as aggregates in soil. Furthermore, the spatial distribution of soil bacteria has been found to be impacted by plants (Kirk *et al.*, 2004), as shown by an increase in bacterial numbers in and around the rhizosphere.

Inability to culture soil microorganisms

The vast phenotypic and genetic diversity found in soil bacterial communities makes it one of the most difficult communities to study (Kirk *et al.*, 2004). Torsvik *et al.* (1990) approximated that at least 99% of bacteria observed under the microscope can not be cultured by common laboratory techniques. Rondon *et al.* (1999) suggested that the unculturable community are microorganisms which are phylogenetically similar to the culturable minority (1%) but whose physiological state eludes our ability to culture them. However, it might also be possible that the 99% are phylogenetically different from the 1% and can not be cultivated in standard media. According to this view, there is wealth of microbial communities that have not yet been discovered and that only the minority of the population is represented (Rondon *et al.*, 1999).

Taxonomic ambiguity of microbes

Another problem associated with measuring biodiversity in soil is the inability to access taxonomic diversity, as there is no official definition of a bacterial species (Trevors, 1998). The traditional species definition was based on higher plants and animals and does not apply to prokaryotes (Kirk *et al.*, 2004). The genetic plasticity of bacteria, allowing DNA transfer through plasmids, bacteriophages and transposons, further complicates the concept of bacterial species.

2.7) Species diversity indices

Diversity indices are used to measure the diversity of a community in which members belong to different and unique species. A diversity index is a mathematical measure of species diversity in a community. Diversity indices not only provide information about community composition (i.e., the number of species present); but also consider the relative abundances of different species present in a community (Beals *et al.*, 2000).

Diversity indices provide information regarding the scarcity and/or commonness of species in a particular community. An index of diversity measures the degree of uncertainty that an individual, picked randomly from a multi-species assemblage, will belong to a particular group within the community. The greater the heterogeneity of the assemblage, the greater the diversity of the community (Atlas, 1984). Species diversity represents a count of the number of different species in a community. *Viz* communities with many different species have high diversity and those with few different species have low diversity (Atlas, 1984). The capacity to quantify diversity in such a manner is an important tool for microbiologists trying to understand and study microbial community structure.

Over the years, numerous indices have been developed and are used in ecological literature, however the Simpson's index of diversity (D) and Shannon-weaver index (H) are by far the most common and frequently used (Zahl, 1977).

2.7.1) Simpson's index

The Simpson index was first proposed by the British statistician Edward Simpson in a paper in Nature (Simpson, 1949). Simpson's index takes into account both species richness and species evenness. Richness is a measure of the number of different kinds of organisms present in a particular area. The more species present in an area, the richer the sample. Evenness is a measure of the relative abundance of the different species making up the richness of an area (Offwell woodland & wildlife trust, 2004).

In ecology, the Simpson's index is used to quantify the biodiversity of a particular habitat. The term Simpson's Diversity Index may refer to any of the following closely related indices:

1. The Simpson's index (D) measures the probability that two individuals picked randomly and independently from a population will belong to the same species or group (Atlas, 1984). Simpson's index can be calculated using two different equations of which either is acceptable.

$$D = \sum (n / N)^2 \quad \dots\dots\dots (1)$$

Or

$$D = \frac{\sum n (n - 1)}{N (N-1)} \quad \dots\dots\dots (2)$$

Where n is the number of organisms of a particular species and N is the total number of organisms of all the species.

With the Simpson's index, 0 represents infinite diversity and 1 represents no diversity, i.e. the higher the D value, the lower the diversity.

2. Simpson's Index of Diversity 1 - D. The value of this index also ranges between 0 and 1. However, in this case the higher the value, the greater the sample diversity.
3. Simpson's Reciprocal Index 1 / D, or the inverse of the Simpson's index:

$$D = \frac{N (N-1)}{\sum n (n - 1)}$$

The value of this index starts with 1 as the lowest figure. This figure would represent a community consisting of only one species. The higher the value, the greater the level of diversity. The maximum value represents the number of species present in a sample (Offwell woodland & wildlife trust, 2004).

2.7.2) Shannon-Weaver index

The Shannon-Weaver formula was originally designed in a study of communications and information theory (Shannon and Weaver, 1948) and stems from a common question in communication: ‘How to predict the next letter in a message or communication?’ The uncertainty is measured by the Shannon function ‘H’. The index has been modified as an index of community diversity and is currently widely accepted (Dickman, 1968).

$$H' = - (\sum p_i \ln p_i)$$

where p_i is the proportion of the total number of specimen i expressed as a proportion of the total number of species in the ecosystem. The product of $(p_i \ln p_i)$ for each species in the community is summed and multiplied by -1 to give Shannon index (H'). The H' value is at a maximum when there is a complete distribution of species within a community and is at the minimum when a community is composed of one species. Thus, the higher the H' value, the higher the diversity.

2.8) Microbial diversity analyses

According to Kirk *et al.* (2004) methods used to measure microbial diversity in soil can be categorised into two groups, i.e., culture-dependent methods (biochemical techniques) and culture-independent methods (molecular based techniques). The former is based on culturing and growing microorganisms on a nutrient agar plate and measuring the rate of substrate use. The latter involves the application of molecular methods, including the direct extraction of genomic DNA from the soil.

2.9) Culture-dependent and Biochemical techniques

For years, the enumeration of bacteria from environmental samples has depended upon conventional laboratory techniques which relied on growing the bacteria on a suite of different media. These culturing techniques were often combined with a simultaneous or

subsequent differentiation of the colonies based on physiological and biochemical tests (Theron and Cloete, 2000). Culturing techniques have been used to measure microbial communities in different environments and soil parameters (De Leij *et al.*, 1993, Maloney *et al.*, 1997).

2.9.1) Dilution plating and culturing methods

The study and analysis of soil microbial communities has relied on culturing techniques using a vast range of culture media designed to maximise the growth of different microbial species (Hill *et al.*, 2000). Culturing methods are easy, fast and inexpensive and provide information on the viable, heterotrophic component of the population (Kirk *et al.*, 2004). However the methods do not accurately reflect the actual bacterial community structure, but rely on the selectivity of growth media for certain bacteria. Moreover, after plating the organism several times to obtain a pure culture, the organism may diverge from its original physiology. Only a minor fraction (0.1 to 10%) of bacteria can be cultivated using these standard techniques (Hill *et al.*, 2000).

2.9.2) Community-level physiological profile (CLPP)

The sole carbon source utilisation system (e.g. API and Biolog) was initially developed as a tool for identifying bacteria up to specie level, based upon a broad survey of their metabolic properties. During the analysis of the community level substrate utilisation fingerprint, total soil microbial communities are inoculated onto wells containing different carbon substrates (Garland & Mills, 1991). Growth of aerobic, heterotrophic microorganisms in the wells is indicated by a colour development, which can be quantified colometrically. The end result of the analysis is a substrate utilisation fingerprint of 95 substrates, which can be interpreted in relation to metabolic activities of specific populations or communities in a sample (Garland & Mills, 1991). The application of the community-level approach to provide microbial function offers a more sensitive and meaningful measure of heterotrophic microbial community structure (Garland and Mills, 1991).

In most cases, multivariate statistical techniques are needed to analyse and compare samples taking into account the large number of variables (95) per sample. Principal component analysis (PCA) is the most common multivariate statistical technique. The purpose of which is to arrange samples of multidimensional space into a low-dimensional space in such a way that similar samples are close by and different samples are far apart (Garland, 1996). Garland and Mills (1991; 1994) used PCA to reduce complex multidimensional data into a smaller number of variables, or principal components, which represent a subset of the original data.

Different BIOLOG™ microplates for the assessment of bacterial and fungal diversity

Gram negative (GN) microplates are used for the identification of gram-negative bacteria and contain carbon sources appropriate to the group. Gram positive (GP) microplates are available for the identification of gram-positive bacteria. Both GP and GN microplates consist of a set of 95 substrates and tetrazolium dye (Preston-Mafham, 2002). As the bacteria utilise the substrates, the dye is subsequently reduced and produces color, which is measured colometrically (Garland and Mills, 1991).

For the assessment of fungal activity, SF-N and SF-P microplates have been developed. These contain the same carbon sources as GN and GP but lack the dye, since many fungal species are unable to reduce the dye. These plates are observed for changes in turbidity instead of colour change (Dobranic and Zak, 1999). BIOLOG™ FF plates are now also available for the assessment of fungi and contain several carbon sources different from those present on GN and GP plates and a different tetrazolium dye that can be utilised by fungi. To prevent interference of turbidity or colour development by fungi, an appropriate combination of antibiotics (e.g. gentamycin, streptomycin, rifampicin), are included (Preston-Mafham, 2002).

Ecoplates are now produced and are specifically tailored for ecological applications. These comprise of three replicate sets of 31 environmentally applicable substrates, nine of which are considered plant root exudate constituents (Preston-Mafham, 2002).

MT microplates contain redox chemicals but no substrates providing researchers with the opportunity to customise the plates. This is taking into consideration the fact that the selection of carbon sources in GN/GP plates may be biased towards simple carbohydrates. Only a few substrates present in GN/GP actually contribute towards community separation of environmental samples and many of them are redundant to the analysis (Preston-Mafham, 2002).

Almost 75% of papers on community analysis by BIOLOG™ plates over the last 10 years have used GN plates. This was mainly because they were the only appropriate type available. However, even with the availability of Ecoplates or fungal plates, GN plates are still favoured although evidence suggests greater relevance and analytical power could be achieved when using other alternatives (Preston-Mafham, 2002).

2.9.3) Fatty acid analysis

The use of fatty acid analysis for the estimation of microbial biomass has been extensively used in the study of soil microbial communities. Fatty acids are defined as carboxylic acid derivatives of long chain aliphatic molecules (Werker *et al.*, 2003). Fatty acid analysis is based on the assumption that phospholipids make up a constant proportion of cell biomass and that any variation in fatty acids may result in markers which can be used to interpret community-level profiles (Ibekwe and Kennedy, 1998). There are mainly two methods for the extraction and analysis of fatty acids from samples, namely phospholipid fatty acid analysis (PLFA) and whole cell fatty acid analysis (WCFA). WCFA analysis is commonly known as fatty acid methyl ester (FAME) analysis. PLFA takes into account those fatty acids that are linked to membrane phospholipids. WCFA on the other hand, considers all cellular fatty acids from all membrane sources (Werker *et al.*, 2003).

Phospholipid fatty acid analysis

Phospholipid fatty acid (PLFA) analysis has been used extensively as a method to assess the structure of soil microbial communities and to determine changes relating to soil disturbances (Hill *et al.*, 2000). PLFA are considered useful signature makers because of

their presence in all living cells and because they make up a constant proportion of the biomass of organisms (Kozdrój and van Elsas , 2001). An important consideration in the use of PLFA in describing microbial communities is that unique fatty acids are indicative of specific groups of organism (Table 2.2). Different groups of bacteria are characterised by specific phospholipids fatty acid profiles and a change in phospholipid profile would indicate a change in microbial composition (Ibekwe and Kennedy, 1998).

Table 2.2 Marker fatty acids of microorganisms inhabiting soil ecosystems (Kozdrój *et al.*, 2001).

Microbial group	Fatty acids
Gram negative bacteria	16:1 ω 7t, 16:1 ω Sc, 18:1 ω 7,cy 17:0, cy 19:0,
Gram positive bacteria	<i>i</i> 15:0, A15:0, <i>i</i> 16:0, <i>i</i> 17:0, a17:0
<i>Actinomycetales</i>	10 Me16:0, 10 Me17:0, 10 Me 18:0
<i>Cytophagaflavobacterium</i>	16:1 ω 5c
<i>Pseudomonas</i>	16:0, 16:1 ω 7c, 18:1 ω 7c,18:1 ω 9t, 18:1 ω 12t
<i>Anthrobacter</i>	a15:0 and a17:0
Fungi	16:1 ω Sc (arbuscular fungi), 18:1 ω 9c, 18:2 ω 6
Eukaryotic algae and <i>protozoa</i>	16:1 ω 4, 16:3, 18:4 ω 3, 20:4, 20:5,22:6

Fatty acid methyl ester analysis

Fatty acid methyl ester (FAME) analysis is a biochemical method which involves the extraction and use of fatty acids from all membrane sources of an organism (Werker *et al.*, 2003). Since fatty acids make up a relatively constant proportion of cell biomass and are different for specific groups, they are useful in distinguishing major taxonomic groups within a community (Kirk *et al.*, 2004). This method is able to detect changes in the composition of the bacterial and/or fungal community, as well as enables one to follow signature fatty acids of different groups of microorganisms. For FAME analysis, fatty acids are extracted directly from soil or any environmental sample, methylated and analysed by gas chromatography (Kirk *et al.*, 2004). FAME profiles of different soils are compared using multivariate analysis such as principal component analysis.

Table 2.3 provides a summary of both the advantages and disadvantages of each of the above-mentioned culture-dependent/biochemical methods.

Table 2.3 Advantages and disadvantages of culture-dependent and biochemical methods in studying soil microbial diversity. (Kirk *et al.*, 2004).

Method	Advantages	Disadvantages
Plate counts	<ul style="list-style-type: none"> - Fast - Inexpensive 	<ul style="list-style-type: none"> - Unculturable microorganisms not detected, - Bias towards fast growing individuals
Community Level Physiological Profiling (CLPP)	<ul style="list-style-type: none"> - Fast - Highly reproducible - Relatively inexpensive - Differentiates between microbial communities - Generates large amount of data 	<ul style="list-style-type: none"> - Only represents culturable fraction of community - Favors fast growing organisms - Only represents those organisms capable of utilizing available carbon sources - Sensitive to inoculum density
Fatty Acid Methyl Ester analysis	<ul style="list-style-type: none"> - No culturing of required, direct extraction - Follow specific organisms or communities 	<ul style="list-style-type: none"> - If using fungal spores, a lot of soil is needed - Can be influenced by external factors - Results can be confounded by other microorganisms

2.10) Culture- independent/Molecular techniques

Some of the most enduring challenges in soil microbiology have been the development of effective methods to determine the microbial which microorganisms are present in soil and to determine microbial functions *in situ*. These challenges are worsened by difficulties in separating microorganisms from the soil matrix and plant tissues; common morphological traits shared among soil microorganisms and the constantly changing microbial taxonomies (Hill *et al.*, 2000). However, the past 10 years have witnessed dramatic developments in the study of soil microbial communities. The development of molecular approaches has provided soil microbiologists with an opportunity to gain access and study microorganisms present in soil and to better understand the soil microbial communities (Hill *et al.*, 2000). The application of these molecular techniques is becoming increasingly common in microbial ecology as they provide a wide range of resolution, from broad-scale measures to discrimination of microbes at strain level. These techniques provide tools for the analysis of the entire microbial community including those which could not be cultured in the laboratory (Amann *et al.*, 1995).

There are mainly two types of molecular analyses for studying microbial communities using DNA extracted from the soil (Figure 2.2). Partial community DNA analyses only investigate parts of the information by focusing on genome sequences targeted and amplified by PCR. Whole community DNA analyses focuses on all the genetic information contained in the extracted DNA (Ranjard *et al.*, 2000).

2.10.1) Partial community DNA analyses

These approaches consist of the analysis of PCR-amplified sequences. The most commonly used target sequences are the genes of the ribosomal operon. These methods include:

- ❖ PCR fragment cloning followed by restriction and/or sequencing analysis, which enables the assessment of the community in terms of the number of different species and to a lesser extent, the relative abundance of these species;
- ❖ Genetic fingerprinting which provides a global picture of the genetic structure microbial community (Ranjard *et al.*, 2000).

PCR fragment cloning and characterization

This approach is used to investigate the diversity of bacterial communities by producing a library of clones obtained by PCR from DNA. Cloning separates sequences so that they can be characterised individually using PCR/RFLP (polymerase chain reaction/restriction fragment length polymorphism) and/or by sequencing. Sequencing allows identification of uncultured bacteria as well as an estimation of their relatedness to known culturable species (Ranjard *et al.*, 2000).

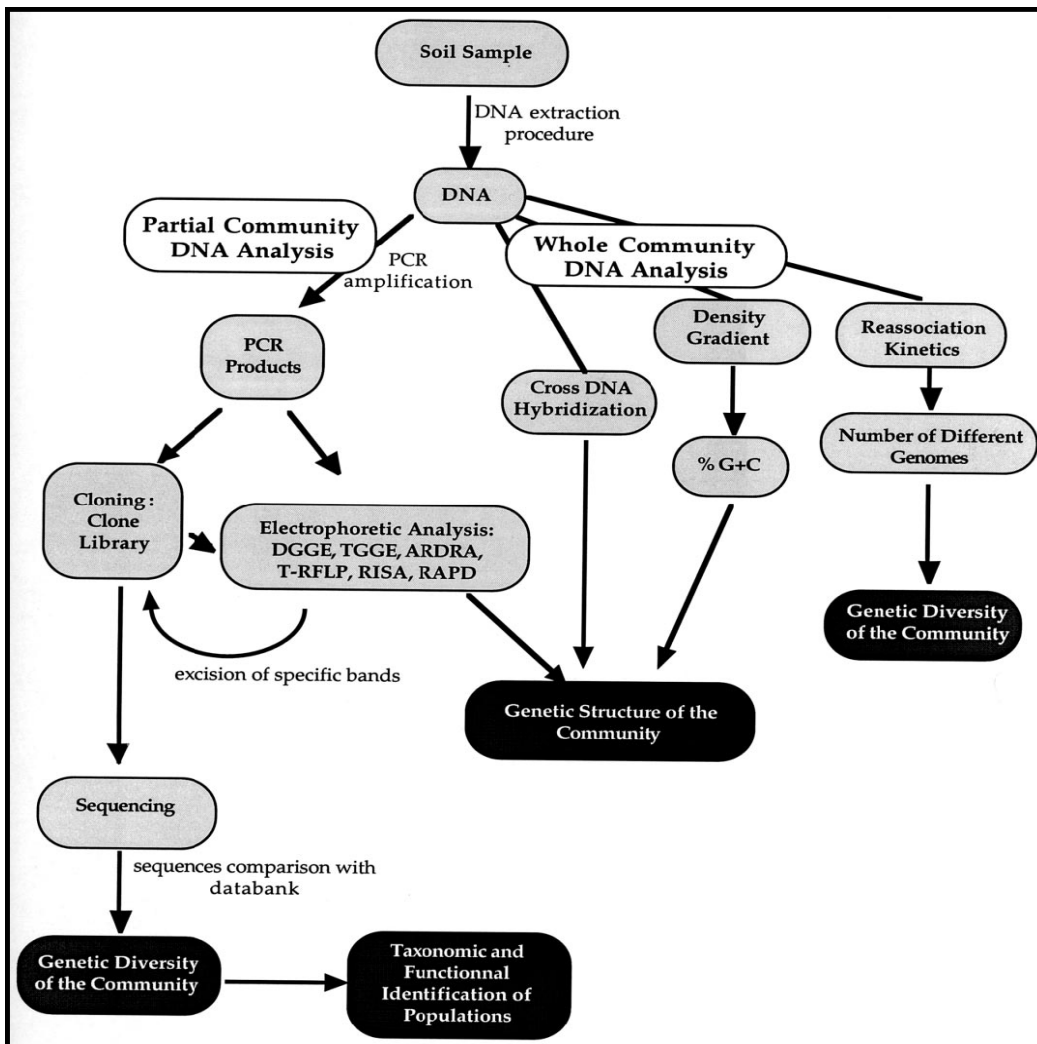


Figure 2.2 Schematic representation of commonly used molecular approaches in microbial ecology (Ranjard *et al.*, 2000).

Genetic fingerprint techniques

These techniques are also based on PCR amplification, but do not require a clone library. Instead, they are based on the principle of resolving the diversity of the amplified sequences by differential electrophoretic migration on agarose or polyacrylamide gels, depending on either size (ARDA, RISA) or sequence (DGGE, TGGE). The genetic fingerprints provide complex band profiles, which yield a representative of the genetic structure of the community (Ranjard *et al.*, 2000).

- *Amplified Ribosomal DNA Restriction Analysis (ARDRA)/Restriction Fragment Length Polymorphism (RFLP)*

Amplified ribosomal DNA restriction analysis (ARDRA), also known as restriction fragment length polymorphism (RFLP) is used to study microbial diversity by producing DNA polymorphisms (Kirk *et al.*, 2004). The method is based on the restriction digestion of amplified 16S rRNA or 18S rRNA regions by restriction endonucleases. The separation of DNA fragments from different populations requires that they differ in sequence at the restriction endonuclease sites, or differ in length of DNA flanked by common restriction sites (Theron and Cloete, 2000).

- *Ribosomal Intergenic Spacer Analysis (RISA)*

Ribosomal intergenic spacer analysis (RISA) provides ribosomal-based fingerprinting of microbial community. The method involves PCR amplification of the intergenic region between the small (16S) and the large (23S) subunit rRNA operon using oligonucleotide primers targeting the conserved regions in the 16S and 23S genes (Øvreås, 2000). The PCR product is electrophoresed on a polyacrylamide gel (under denaturing conditions) and the DNA visualised by silver staining (Kirk *et al.*, 2004). The result is a complex banding pattern that provides a community-specific profile, with at least one band corresponding to one individual organism present in the sample.

Although RISA provided relatively rapid estimates of microbial community compositions, it had its limitations and these led to the development of an improved

version of RISA referred to as automated RISA (ARISA). In the automated approach, the initial steps of DNA extraction and PCR are the same as in RISA. However in this case, PCR is conducted with a fluorescence-tagged oligonucleotide primer and the electrophoretic step is performed with an automated system (Kirk *et al.*, 2004).

○ *Terminal Restriction Fragment Length Polymorphism (T-RFLP)*

Terminal restriction fragment length polymorphism (T-RFLP) is an improvement of the ARDRA method and only measures the terminal restriction fragment of the 16S rRNA (Kozdrój and van Elsas, 2001). PCR is performed using universal primers, one of which is fluorescently labelled (Øvreås, 2000). The fluorescently end-labelled PCR product is then digested by restriction enzymes. The resulting fragments are separated by gel electrophoresis using an automated sequence analyser with laser detection to detect the labelled fragments. Upon analysis, only the terminal end-labelled restriction fragments are detected and each unique fragment is counted as an Operational Taxonomic Unit (OTU) (Øvreås, 2000).

○ *Randomly Amplified Polymorphic DNA (RAPD)*

Randomly amplified polymorphic DNA (RAPD) employs short primers (about 10bp) of random sequences to amplify portions of the sample DNA by PCR. Since each primer is short, it will anneal to a lot of sites throughout the target DNA, producing a spectrum of PCR fragments of various lengths. PCR fragments are further resolved on agarose gel (Ranjard *et al.*, 2000).

○ *Denaturing Gradient Gel Electrophoresis (DGGE)*

Muyzer *et al.* (1993) introduced denaturing gradient gel electrophoresis (DGGE) as a new genetic fingerprinting technique in 1993. Sequence variation in rRNA is used for understanding phylogenetic relationships between organisms (Muyzer *et al.*, 1993). DGGE is used to determine the bacterial genetic diversity and specifically the predominant populations in a given sample (Muyzer *et al.*, 1993).

The technique is based on the extraction of total genomic DNA from an environmental sample; the extracted DNA is used as a template for PCR amplification. During PCR, the ribosomal DNA is targeted as it is the most conserved region of the genome (Ercolini, 2004). Amplified double stranded DNA fragments (200-700bp), with the same length but different base pair sequences are separated (Ferris *et al.*, 1996) via gel electrophoresis through a polyacrylamide gel containing a linearly increasing gradient of denaturants (Muyzer *et al.*, 1993). The most commonly used denaturants are heat (constant at 60 °C), formamide (0 - 40%) and urea (0 - 7M) (Helms, 1990). Initially, fragments move through the gel according to their molecular weight. However, as the denaturing gradient increases, they begin to partially melt in discrete regions called “melting domains” (Helms, 1990). The melting temperatures of the domains are sequence specific (Ercolini, 2004). This partial melting reduces the mobility of the DNA molecule through the gel; thus DNA fragments of the same size but different base pair composition will have different responses to the denaturing gradient (Muyzer *et al.*, 1993).

The rate of mobility within the polyacrylamide gel is dependent on the shape of the fragment, which in turn depends on the denaturant gradient and fragment sequence. Partially melted fragments move more slowly than double stranded (Helms, 1990). A ladder of bands forms, each one corresponding to an individual PCR product of a specific sequence. The resulting bands can be probed with diagnostic oligonucleotides to identify specific sequences or bands may be excised, re-amplified and sequenced (Ferris *et al.*, 1996). Resolution of the process can be enhanced by the addition of a 30 - 40 base pair GC-rich sequence to the 5'-end of one of the primers in order to better enhance the melting behaviour of the fragment by ensuring that the DNA fragment remains partially double-stranded and to allow for the majority of sequence variation to be detected in the denaturing gel (Muyzer *et al.*, 1993; Ercolini, 2004).

DGGE can be performed in either perpendicular or parallel denaturing gradient gels. In perpendicular gels, the denaturing gradient is perpendicular to the direction of the electrophoresis and the gradient range is broad, such as 0-100% or 20-100%.

Perpendicular gels are commonly used to detect the melting behaviour of DNA fragments and to experimentally determine the optimal denaturing range to use in parallel electrophoresis experiments (Ercolini, 2004). In perpendicular gradient gels, only one sample can be loaded or a mixture of amplicons for which the melting behaviour is to be studied. In parallel DGGE, the denaturing gradient is parallel to the electric field and the range of denaturants is narrowed, allowing better separation of sequences. Parallel gels are most preferred and are commonly employed as they allow multiple samples to be loaded on the same gel (Ercolini, 2004).

2.10.2) Whole genomic community DNA analyses

Nucleic acid hybridisation techniques

Nucleic acid hybridisation using probes is both a qualitative and quantitative tool in molecular ecology (Kirk *et al.*, 2004). Whole cell DNA or RNA are extracted from the environmental sample, fixed to a nylon membrane and allowed to hybridise with oligonucleotide or polynucleotide probes of known sequences and tagged with fluorescent markers at the 5'-end (Kirk *et al.*, 2004). Hybridisation relies on the specific binding of nucleic acid probes to complementary DNA or RNA (target nucleic acid). These probes may either be used to detect genes in the bacterial genome (southern blots) or used to detect rRNA or mRNA (northern blots) (Theron and Cloete, 2000). The probes are single strands of nucleic acids with detectable marker molecules highly specific to complementary target sequences (Theron and Cloete, 2000).

- ***Fluorescent In Situ Hybridisation (FISH)***

Fluorescent in situ hybridisation (FISH) allows for rapid identification of taxonomic microbial groups using oligonucleotide probes (Hill *et al.*, 2000). The technique involves *in-situ* hybridisation of target nucleic acids (RNA and DNA) extracted from environmental samples with labelled probes (Kirk *et al.*, 2004). Traditionally, radioactive isotopes were used to label the oligonucleotide probes however most recently, fluorescent probes are used (Kirk *et al.*, 2004). These fluorescently-labelled probes are designed to be complementary to a specific part

- of the 16S rRNA of the target microorganisms. If an organism contains the specific sequence that is complementary to the sequence of the probe, the probe will hybridise to the rRNA in the specific target cells and the bound probes will fluoresce and become visible (Theron and Cloete, 2000).
- *Cross-DNA hybridisation/Reciprocal hybridisation*

Cross-hybridisation of total community DNA is an approach used to determine whether two samples have the same kinds of organisms (Theron and Cloete, 2000). The technique involves cross-hybridisation of DNA between one sample with that from another sample. The DNA from one sample is radioactively labelled and used as template and the extent to which the labelled DNA anneals to the filter-bound target DNA reflects the similarity of probe and target, and the extent to which the population structure of bacterial communities is similar (Ranjard *et al.*, 2000). Significant hybridisation only occurs between identical or closely related organisms (Theron and Cloete, 2000).
 - *Thermal reassociation of DNA*

DNA reassociation has been used to investigate genomic sequence complexity and to assess the diversity of natural microbial communities (Torsvik *et al.*, 1990). In this analysis, total community DNA is extracted, purified, denatured and allowed to re-anneal. The renaturation of DNA is dependent upon random collisions of complementary single-stranded DNA strands and follows second order kinetics (Øvreås, 2000). The principle of DNA renaturation kinetics is that the rate at which DNA reassociates is proportional to the concentration of complementary DNA sequences and inversely proportional to the total amount of different sequences in the sample (Theron and Cloete, 2000). Thus, as the microbial community diversity/complexity (heterogeneity) increases, the rate of reassociation of DNA decreases (Kirk *et al.*, 2004)

Using this method, Torsvik and collaborators estimated about 4 000 different bacterial genomes according to soil types (Torsvik *et al.*, 1990). They also

discovered that the majority of the diversity was present in the fraction of bacteria which cannot be cultured (Torsvik *et al.*, 1990).

- *Guanine plus cytosine (% G+C) content*

The base composition, expressed as mole percentage guanine and cytosine, was one of the first properties of DNA used for taxonomic purposes (Øvreås, 2000). The G+C content is the amount of G's and C's present in the genome of an organism. Organisms differ in their G+ C content, and in bacteria the value ranges from 25% up to 75% (Øvreås, 2000). Thus differences in the G+C content of DNA can be used to study the bacterial diversity. Organisms with close evolutionary relationships have similar % G+C in their genome. The G+C profile of taxonomically related groups only differs by between 3% and 5% (Kirk *et al.*, 2004). The G+C value is determined by the thermal denaturation of double-stranded DNA, the melting temperature is linearly correlated to % G+C (Øvreås, 2000).

2.11) Common shortfalls of molecular-based methods

During recent years molecular techniques based on PCR have been used to overcome the limitations of culture-based methods, however they are not without their own disadvantages and limitations (Wintzingerode *et al.*, 1997; Kirk *et al.*, 2004).

In PCR-mediated approaches, the manner in which cells are lysed is very crucial. If the method used for cell disruption is too gentle, then only gram-negative cells will be lysed. If it is too harsh, both gram-positive and gram-negative cells may be lysed but their DNA may become too sheared (Wintzingerode *et al.*, 1997). In this respect, the method of DNA and RNA extraction used can also bias diversity studies. The extraction of RNA from environmental samples requires great discretion as RNA is highly susceptible to degradation by Rnases. Harsh extraction methods, such as bead beating, can shear the nucleic acids leading to problems in subsequent PCR detection (Wintzingerode *et al.*,

1997). Repeated purification steps can lead to loss of DNA or RNA, especially when small samples of nucleic acids are available (Wintzingerode *et al.*, 1997).

When working on environmental samples, it is essential to remove substances such as humic acids, which can be co-extracted and interfere with subsequent PCR analysis. PCR reactions are sensitive to reaction conditions. Minor contamination may lead to false results. During PCR, sequences that are more abundant may be amplified more than the less abundant ones (Theron and Cloete, 2000).

The amplification of different target genes can also bias PCR-based diversity studies. Wintzingerode *et al.* (1997) discussed some issues surrounding differential PCR amplification including different affinities of primers to templates, different copy numbers of target genes, hybridisation efficiency and primer specificity. In addition, sequences with lower G+C content are thought to separate more efficiently in the denaturing step of PCR and could thus be preferentially amplified. These are just some of the limitations of molecular-based microbial community analysis. Further limitations and advantages are given in Table 2.4 below.

Table 2.4 Advantages and disadvantages of molecular-based methods used in the study of microbial diversity (Kirk *et al.*, 2004).

Method	Advantages	Disadvantages
G+C	<ul style="list-style-type: none"> - Not influenced by PCR bias - Includes all DNA extracted 	<ul style="list-style-type: none"> - Requires large quantities of DNA - Dependent on lysing and extraction efficiency
Nucleic acid Hybridisation and Reassociation	<ul style="list-style-type: none"> - Total DNA extracted - Not influenced by PCR bias - Studies DNA and RNA - Can be studied in situ 	<ul style="list-style-type: none"> - Lack of sensitivity - Sequences need to be in high copy number to be detected - Dependent on lysing and

extraction efficiency

DGGE and TGGE	<ul style="list-style-type: none"> - Large number of samples can be analysed simultaneously - Reliable, rapid 	<ul style="list-style-type: none"> - PCR bias - Dependent on lysing, extraction efficiency and sample handling
ARDRA or RFLP	<ul style="list-style-type: none"> - Detect structural changes in microbial community 	<ul style="list-style-type: none"> - PCR bias - Banding patterns too complex

2.12) The fate of lime-treated acid mine water (Gypsiferous mine water)

Gypsiferous mine water ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) is produced when acid mine water is treated with a neutralising agent such as calcium carbonate/limestone (CaCO_3). Due to high levels of salinity and presence of precipitated metals, the direct disposal of the water to the environment poses a serious risk from an ecological point of view (Hilgsmann *et al.*, 1996; Pulles *et al.*, 1996; Jovanoic *et al.*, 1998). If the water is freely discharged into the environment, it can cause salinisation of soil and surrounding catchment areas (Jovanoic *et al.*, 1998). To avoid contamination of water resources, the lime-treated water has previously been used for dust alleviation on gravel roads and for irrigation of lawns. Possible utilisation of this water for the irrigation of agricultural crops has also showed promise (Jovanoic *et al.*, 1998). Du Plessis (1983) first evaluated the potential use of gypsiferous mine water for crop irrigation to predict the amount of leached salts which would contaminate the groundwater. Subsequent to the study by Du Plessis (1983), a substantial amount of work was focused on investigating the feasibility of using gypsiferous mine water for irrigation purposes (Jovanoic *et al.*, 1998; Annandale *et al.*, 2002). The use of gypsiferous mine water not only enabled for the production of crops during dry seasons but also provided a cost-effective method of minimising excess mine drainage (Annandale *et al.*, 2007).

2.12.1) Application of Gypsiferous mine water in agriculture

Anthropogenic activities as well as agricultural practices often disturb the soil chemical properties by making it either too acidic or alkali and/or saline. In most cases, the application of fertilisers and/or amendments is necessary to reclaim the soil nutrient supply and balance (Baligar *et al.*, 2001).

Gypsum can be viewed as feasible option to ameliorate soils with high levels of salinity (Carter *et al.*, 1986; Suhayda *et al.*, 1997). Gypsum has been used to reclaim/ameliorate both alkali and acidic soils with high levels of salinity (Suhayda *et al.*, 1997; Sun *et al.*, 2000). Sun *et al.* (2000) discovered that the application of gypsum to acidic soils was able to ameliorate the soils by increasing the calcium ions and significantly reducing the levels of toxic aluminium. They found that the addition of gypsum to the soils led to an increase in the amount of exchangeable calcium and sulphate and reduced exchangeable aluminium. It has been noted that high levels of aluminium and/or reduced amounts of calcium restrict root elongation and thus hinder on the ability of crops to access adequate water. In another study, Suhayda *et al.* (1997) discovered that gypsum amendment significantly improved the chemical properties of saline-alkaline soils by reducing the pH and replacing the sodium with calcium. They found that replacing sodium with calcium resulted in the flocculation of soil particles and improved the porous structure and water permeability of the soil.

In South Africa, large amounts of gypsiferous mine water are made available to the farming community and utilised for irrigation of high-potential soils in the coalfields of Mpumalanga Province, where water resources for irrigation are already under extreme pressure. Contamination of downstream water resources is reduced, and additional income achieved through farming (Annandale *et al.*, 2002).

2.12.2) Microbial utilisation of gypsum

The amendment of soil with gypsum introduces among others, elevated level of exchangeable sulphate and calcium (Suhayda *et al.*, 1997; Sun *et al.*, 2000). Dissimilatory

microbial sulphate reduction is a process in which certain bacteria are able to use sulphate as an electron acceptor in the oxidation of organic matter. During the process, sulphate reducing bacteria reduce sulphate (sulphur +6) to sulphide (sulphur -2) and oxidize organic substrate into carbonates (Hilgsmann *et al.*, 1996). Bajpai *et al.* (1976) established that in the presence of organic matter, the addition of gypsum lead to improved microbial activity. Ehrlich (1990) discovered that sulphate reducing bacteria were able to utilise the sulphate in gypsum for metabolic activities. Later, Ehrlich (1996) described soil microorganisms as opportunist as they were able to use minerals in soil to favour their own growth and survival. He explained that microorganisms are able to utilise the minerals in one of the following ways:

- As an energy source;
- As a trace element requirement;
- As an electron acceptor during respiration or;
- To enhance competitiveness in a community.

CHAPTER 3

3. MATERIALS AND METHODS

3.1) Study area and sampling

The Soil samples were collected from three plots, namely Major, Four and Tweefontein, at the Kleinkopjè Colliery (Anglo Coal Mine), situated near Witbank in the Mpumalanga province (South Africa). For the purpose of this study, the plots are referred to as ‘pivots’ based on the mode of irrigation, since center-pivot irrigation was used. The pivots were planted with maize (*Zea Mays*) at the time of sampling. The soils samples were collected using simple random sampling procedure as described by Tan (2005) and each random sample was presumed to be a representative of the environment from which it was collected. The samples were collected every 50 m and at depths of 0-10 cm and 40-60 cm, both inside and outside of each pivot. Descriptions of samples collected from each pivot are given in Table 3.1. The samples were taken using a stainless steel auger which was sterilised with 95% ethanol between sampling to avoid cross-contamination. Samples were stored in sterile Petri dishes and transported on ice to the refrigerator, where they were stored at 4 °C before total genomic DNA extraction within 48 hours.

Pivot characteristics

Major: 30 ha of under-mined soil, irrigated with water from Jacuzzi dam since 1997. It has a loamy sand soil type made up of clay 12%, silt 5% and sand 83%.

Tweefontein: 20 ha rehabilitated open mine, irrigated with water from Tweefontein dam since 1997. It has a sandy loam soil type made up of clay 17%, silt 10% and sand 73%.

Four: 30 ha of un-mined (virgin) soil, irrigated with water from Tweefontein dam since 1999. It has a sandy loam soil type made up of clay 14%, silt 3% and sand 83%.

Soil chemical analyses

Soil samples were collected along a down slope transect across all three pivots. The samples were collected randomly from 3 locations inside each pivot and 2 random

locations outside each pivot (Table 3.1). The samples were collected every 50 m, starting from outside the pivot and ending at the other side (Figure 3.1 to Figure 3.3).

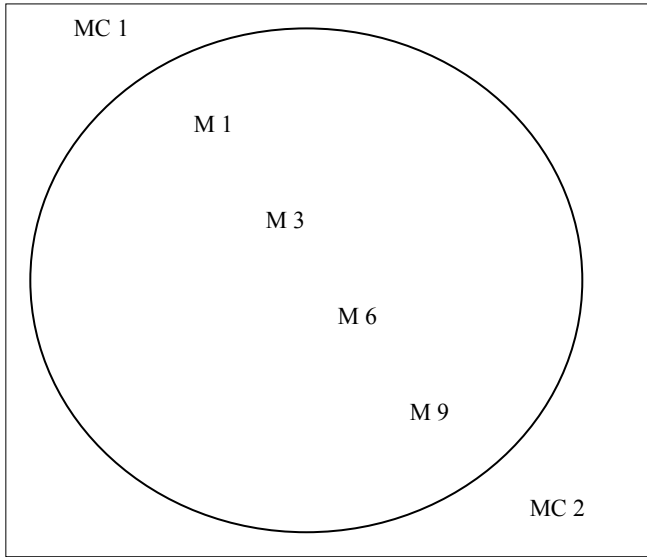


Figure 3.1 Location of sampling points at pivot Major.

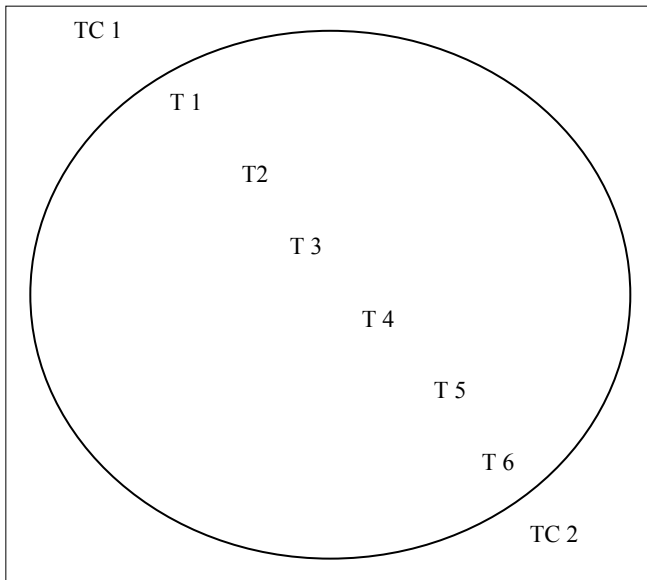


Figure 3.2 Location of sampling points at pivot Tweefontein.

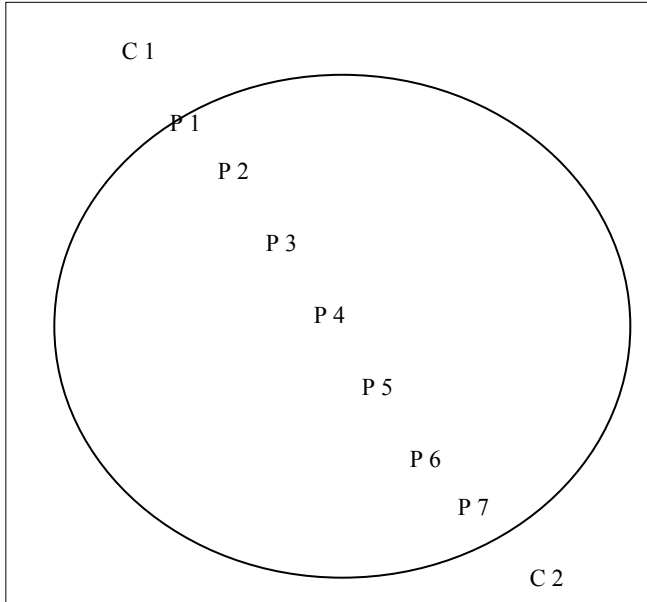


Figure 3.3 Location of sampling points at pivot Four.

Irrigation water quality

The pivots were irrigated with water from two sources, namely Jacuzzi and Tweefontein. Jacuzzi water was pumped into a large storage dam and released under controlled conditions under licence of the Department of Water Affairs and Forestry, Pretoria. The water was used to irrigate pivot Major. Tweefontein water was pumped from an active opencast pit and stored in the Tweefontein pan (Table 3.2). The water was used to irrigate both pivot Tweefontein and Four.

Table 3.1 Soil sample descriptions and soil chemical analyses. Soil Science laboratory, University of Pretoria (2006).

Sites	Sample descriptions	pH	EC (mS m ⁻¹)	Exchangeable cations			
				Ca ²⁺ (cmol kg ⁻¹)	Mg ²⁺ (cmol kg ⁻¹)	Na ⁺ (cmol kg ⁻¹)	SO ₄ ²⁻ (me 100 g ⁻¹)
M1-1	Major 0-10 cm, gypsum-irrigated	5.1	317	10.02	0.78	0.06	0.93
M1-2	Major 40-60 cm, gypsum-irrigated	5.2	284	8.95	0.62	0.05	1.13
M6-1	Major 0-10 cm, gypsum-irrigated	5.4	369	7.86	0.49	0.04	0.88
M6-2	Major 40-60 cm, gypsum-irrigated	4.8	374	6.44	0.56	0.05	1.15
M9-1	Major 0-10 cm, gypsum-irrigated	4.5	264	6.28	0.46	0.02	1.02
M9-2	Major 40-60 cm, gypsum-irrigated	5.0	259	6.79	0.51	0.03	0.94
MC1-1	Major 0-10 cm, (non gypsum-irrigated)	6.8	106	4.09	0.22	0.10	0.23
MC1-2	Major 40-60 cm, (non gypsum-irrigated)	6.0	123	4.23	0.19	0.09	0.19
MC2-1	Major 0-10 cm, (non gypsum-irrigated)	5.9	83	3.31	0.26	0.87	0.08
MC2-2	Major 40-60 cm, (non gypsum-irrigated)	6.1	112	3.62	0.20	1.21	0.22
T1-1	TWF 0-10 cm, gypsum-irrigated	5.6	375	5.23	0.41	0.09	1.15
T1-2	TWF 40-60 cm, gypsum-irrigated	5.5	297	4.85	0.39	0.08	0.96
T3-1	TWF 0-10 cm, gypsum-irrigated	4.9	386	4.67	0.34	0.07	1.82
T3-2	TWF 40-60 cm, gypsum-irrigated	5.2	412	5.48	0.47	0.06	1.94
T5-1	TWF 0-10 cm, gypsum-irrigated	5.7	445	6.01	0.52	0.05	1.58
T5-2	TWF 40-60 cm, gypsum-irrigated	5.9	423	6.09	0.63	0.06	1.63
TC1-1	TWF 0-10 cm, (non gypsum-irrigated)	6.3	164	4.07	0.20	1.29	0.52
TC1-2	TWF 40-60 cm, (non gypsum-irrigated)	6.0	180	4.18	0.27	1.52	0.43
TC2-1	TWF 0-10 cm, (non gypsum-irrigated)	6.4	169	3.74	0.19	1.97	0.64

Sites	Sample descriptions	pH	EC (mS m ⁻¹)	Exchangeable cations			
				Ca ²⁺ (cmol kg ⁻¹)	Mg ²⁺ (cmol kg ⁻¹)	Na ⁺ (cmol kg ⁻¹)	SO ₄ ²⁻ (me 100 g ⁻¹)
TC2-2	TWF 40-60 cm, (non gypsum -irrigated)	5.9	177	2.99	0.20	2.01	0.72
P1-1	Four 0-10 cm, gypsum-irrigated	5.0	324	6.15	0.92	0.06	1.07
P1-2	Four 40-60 cm, gypsum-irrigated	4.8	301	6.68	0.85	0.05	1.94
P4-1	Four 0-10 cm, gypsum-irrigated	5.3	304	5.59	0.71	0.07	1.09
P4-2	Four 40-60 cm, gypsum-irrigated	4.6	354	5.78	0.80	0.06	1.50
P7-1	Four 0-10 cm, gypsum-irrigated	5.2	449	7.24	1.09	0.08	1.44
P7-2	Four 40-60 cm, gypsum-irrigated	4.9	420	7.39	0.99	0.08	1.82
C1-1	Four 0-10 cm, (non gypsum -irrigated)	5.3	95	4.32	0.69	0.09	0.53
C1-2	Four 40-60 cm, (non gypsum -irrigated)	5.4	101	4.09	0.61	1.12	0.67
C2-1	Four 0-10 cm, (non gypsum -irrigated)	5.8	116	3.85	0.59	1.20	0.32
C2-2	Four 40-60 cm, (non gypsum -irrigated)	5.9	87	3.62	0.64	1.05	0.29

At each pivot, soil samples were collected randomly at 3 locations inside the pivot and 2 locations outside the pivot. Each random sample was presumed to be a representative of the environment from which it was collected.

Table 3.2 Water chemical analyses from Jacuzzi (underground) and Tweefontein pan, as supplied by Kleinkopjè Colliery (2006).

Pivots	pH	EC (mS m ⁻¹)	TDS (mg l ⁻¹)	Ca ²⁺ (mg l ⁻¹)	Mg ²⁺ (mg l ⁻¹)	Na ⁺ (mg l ⁻¹)	K ⁺ (mg l ⁻¹)	SO ₄ ²⁻ (mg l ⁻¹)	Cl ⁻ (mg l ⁻¹)
Jacuzzi	6.2	344	5256	578	242	52	13	2550	12
Tweefontein	8.2	454	6132	712	448	132	31	3660	64

3.2) DNA extraction and purification

Total DNA was extracted from the soil using the Bio101-fast DNA spin kit (soil) (Qbiogene molecular biology products, IESA, Pretoria, S.A). Approximately 0.5 g of soil was used for DNA extraction. Successful DNA extraction was confirmed by gel electrophoresis on a 1.5% TAE agarose gel (20 mM Tris, 10 mM acetate, 0.5 mM Na-EDTA, pH 7.4), with ethidium bromide as an intercalating agent to allow fluorescence under UV light.

3.3) Polymerase chain reaction amplification

A portion of the 16S rRNA gene was amplified by PCR from total genomic DNA using primers PRUN518r (5'-ATT-ACC-GCG-GCT-GG-3') (Øvreås *et al.*, 1997) and pA8f-GC (5'-CGC-CCG-CCG-CGC-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3') (Fjellbirkeland *et al.*, 2001). These primers have been found to be useful in determining 16S rRNA gene diversity in ecological and systematic studies (Øvreås and Torsvik, 1998). A 1-µl aliquot (ca 10ng) of the extracted DNA was amplified by PCR with a gradient thermal cycler (Bio-rad laboratories, Hercules, CA). The PCR mixture used contained 1 µl of each primer (10 pmol), 2 µl of deoxy-nucleotide triphosphates (2.5 mmol), 2.5 µl buffer without MgCl₂, 2 µl 10 x MgCl₂, 0.2 µl Taq polymerase and 10.3 µl sterile distilled water. The 16S rRNA genes from the soil microbial communities were amplified in the thermal cycler as follows: initial denaturation at 95 °C for 10 min followed by 35 cycles of denaturation for 30 sec at 94 °C, annealing for 30 sec at 58°C, and extension for 1 min at 72 °C, a final extension

at 72 °C for 10 min was included. Amplification was confirmed by electrophoresis on a 1.5 % TAE agarose gel.

3.4) Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed according to Muyzer *et al.* (1993) and carried out in a BioRad Dcode vertical gel system (Bio-rad laboratories, Hercules, CA). Briefly, PCR products were resolved on 8% (w/v) polyacrylamide denaturing gradient gels in 1.5X TAE (20 mM Tris, 10 mM acetate, 0.5mM Na-EDTA, pH 7.4). The denaturing gels were prepared with a 35-65% gradient of denaturants (urea and formamide) (Table 3.3). Electrophoresis was run at 60 °C, first for 10 min at 20 V and then for 17 h at 70 V. The gels were stained with SYBR gold to detect dsDNA and the resulting bands were viewed under a UV transilluminator.

Statistical comparison of DGGE patterns was performed using Gel2K software program (Norland, 2004). Similarities among the band patterns were calculated using the Jaccard co-efficient based on band positions. The jaccard co-efficient divides the number of corresponding bands by the total number of bands.

Table 3.3 Volumes of denaturants DSSA (denaturing stock solution A: 8% acrylamide in 0.5% TAE (40Mm Tris, 20Mm acetic acid, 1nM EDTA buffer (pH 8.3)) and DSSB (denaturing stock solution B: 8% acrylamide, 7M urea, 40% formamide in 0.5% TAE buffer).

Denaturing gradient	DSSA (ml)	DSSB (ml)
15	12.3	2.2
20	11.6	2.9
25	10.9	3.6
30	10.2	4.4
35	9.4	5.1
40	8.7	5.8
45	8.0	6.5
50	7.3	7.3
55	6.5	8.0
60	5.8	8.7
65	5.1	9.4
70	4.4	10.2

3.5) Direct plate counts

Viable bacterial cell counts were determined by the spread plate method. From each composite sample, 10 g of each soil sample, in duplicate, was added to 90 ml of 0.9 % (w/v) sterile saline solution and the suspension shaken on a rotatory shaker for 10 min at 280 rpm. The resulting suspension was used to prepare a tenfold serial dilution in sterile saline solution. Appropriate decimal dilutions (10 µl) were spread onto R2A agar plates amended with 200 µg ml⁻¹ cycloheximide to suppress fungal growth. Plates were incubated at 25 °C for a period of 3 d and the colony counts recorded regularly over that period. The colonies were expressed as cfu g⁻¹.

3.5.1) Diversity of colony-forming units

Diversity analysis based on colony morphology was determined by grouping colonies appearing on agar plates according to distinct visual differences, e.g., colour, shape, size (Balkwill and Chiorse, 1985). Plates containing approximately 100 colonies after the 3 d were used. Shannon-Weaver indices, $H = -(\sum p_i \ln p_i)$, where p_i is the ratio between the number of colonies in a specific group and the total number of colonies, were calculated on the basis of the groupings (Shannon and Weaver, 1949). Statistical analysis of the results was performed using non-parametric testing.

3.6) Community-level physiological profiles

Biolog™ Ecoplates (Biolog Inc. Hayward, CA) were used to analyse the soil microbial structure through substrate utilisation patterns. Biolog Ecoplates contain 31 replicates of different carbon sources and 3 control wells, making up a total of 96 wells. Each of the 31 wells contain various carbon sources, nutrients and a tetrazolium dye, which acts as an electron acceptor and is reduced by NADH during respiration. The reduced product undergoes a colour change and can be quantified spectrophotometrically (Konopka *et al.*, 1998). The rate of colour development depends upon the rate of respiration in the wells.

To obtain a bacterial suspension, 10 g of each soil sample was suspended into 90 ml of sterile distilled water. The flasks were shaken on a rotatory shaker for 1 h and allowed to stand for 30 min after which the supernatant was used for further dilutions. Each well was inoculated with 150 μ l of 1:100 dilution using an 8-channel multipipette. The plates were incubated in the dark at 20 °C and the optical density at 590 nm (OD_{590}) measured with a Multiscan Ex microplate reader (Thermo Fischer Scientific Inc. MA, USA) every 8 h for a period of 9 d. In order to minimise the effects of different inoculation densities, the plates were read when the average well colour development (AWCD) reached 0.25 (Garland, 1996). The data was transformed by subtracting the reading of the control well from the reading of each substrate-containing well and then dividing the value by the AWCD. Only wells with an OD higher than the control were used for further analysis.

Data was analysed by multivariate statistical analysis and calculation of Shannon-Weaver indices. For multivariate statistical analysis, the data was analysed by principal component analysis (PCA) (Garland and Mills, 1991) using SPSS and NCSS statistical programs (Department of Statistics, University of Pretoria). PCA reduces complex multidimensional data into a smaller number of variables or principal components (PCs) which represent a subset of the original data (Garland and Mills, 1991, Garland, 1996). PCA is an eigenvalue-eigenvector procedure that attempts to maximize the amount of variance in the data set that can be explained by the first few principal components. The differences observed between samples in coordination space can be related to the differences in carbon source utilisation capabilities.

Shannon-Weaver indices were calculated as follows: $H' = \sum p_i \ln p_i$; where p_i is the ratio between the optical density in the single well and the total optical density summed from all the well (Zak *et al.*, 1994). The results were analysed statistically using non-parametric testing.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1) Bacterial community diversity

Bacterial community diversity was investigated by total genomic DNA extraction from soil, PCR amplification of partial 16S rDNA, followed by species separation using DGGE.

Suitable yields of high-molecular weight DNA were successfully extracted (usually 5-15 $\mu\text{g g}^{-1}$ soil) for all soil samples using the BIO 101 soil kit (Qbiogene molecular biology products, IESA, Pretoria, South Africa). Extraction was confirmed by electrophoresis on 1.5% TAE (20 mM Tris, 10 mM acetate, 0.5mM Na-EDTA, pH 7.4) agarose gel (Figure 4.1). The extracted DNA showed no protein and/or RNA contamination.

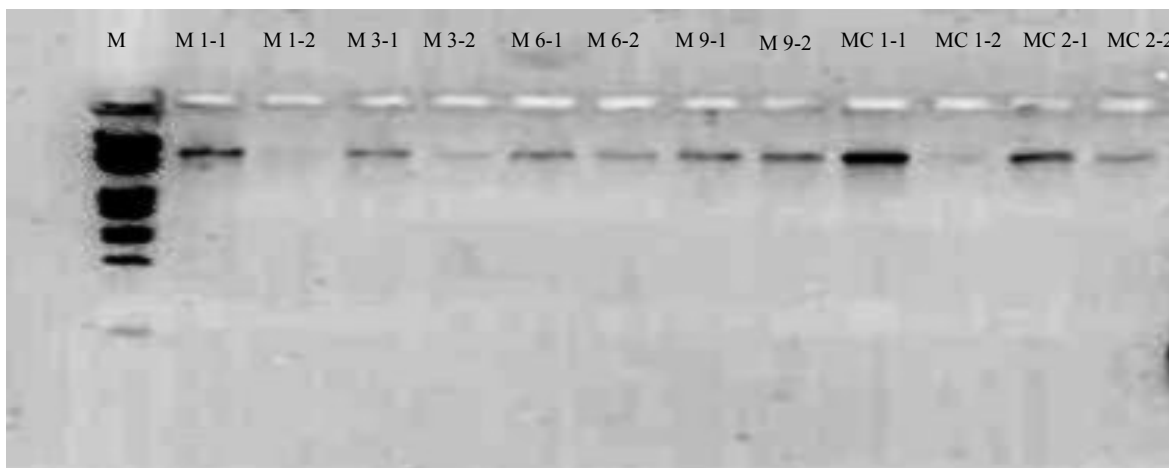


Figure 4.1 DNA extracted from soil samples collected from pivot Major , run on a 1.5 % TAE agarose gel. (M = 100 bp marker; M1-1 to M 9-2 = gypsum-irrigated soils; MC1-1 to MC 2-2 = control soils).

Partial 16S rDNA from soil microbial communities was amplified by PCR using primers PRUN518r and pA8f-GC (Øvreås *et al.*, 1997; Fjellbirkeland *et al.*, 2001). These primers have been found to be useful in ecological studies since they are able to amplify partial length 16S rDNA products as well as 23S rDNA (256f-1930r) sequences (Øvreås and

Torsvik, 1998). PCR product was confirmed by electrophoresis on a 1.5% TAE agarose gel (Figure 4.2).

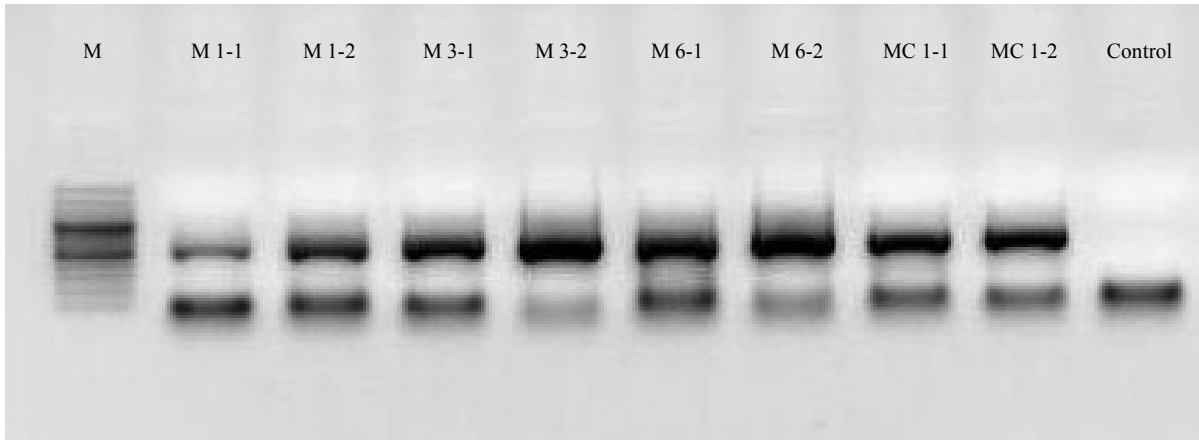


Figure 4.2 1.5% TAE agarose gel showing amplified 16S rDNA from soil samples collected from pivot Major.

Following PCR-amplification, DGGE was optimised by varying concentrations and ratios of denaturants (formamide and urea) to obtain the best separation of PCR amplicons throughout the gel (Figure 4.3). DGGE was performed according to Muyzer *et al.* (1993) by loading 10 μ l of amplified PCR product on 8% (w/v) polyacrylamide denaturing gradient gels in 1.5xTAE using a gradient of 35-65% denaturants and run on 70 V at 60 °C for 17h. As the fragments moved through the increasing chemical gradient, they began to partially melt (Helms, 1990). This partial melting reduces the mobility of DNA fragments; thus PCR fragments of the same length were separated along the gel according to differences in their base-pair sequences (Muyzer *et al.*, 1993).

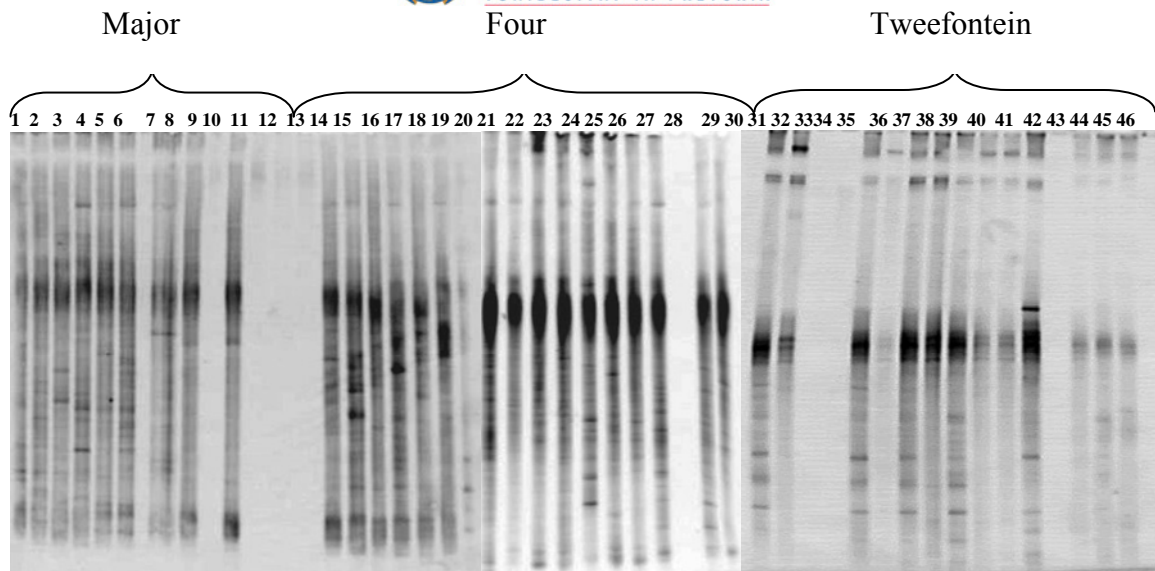


Figure 4.3 DGGE fingerprint patterns of PCR-amplified partial bacterial 16S rDNA genes. PCR products were separated using a denaturing gradient of 35-65% according to differences in base-pair sequences. Lanes 1-12: Pivot Major; lanes 13-30: Pivot Four; and lanes 31-46: Pivot Tweefontein.

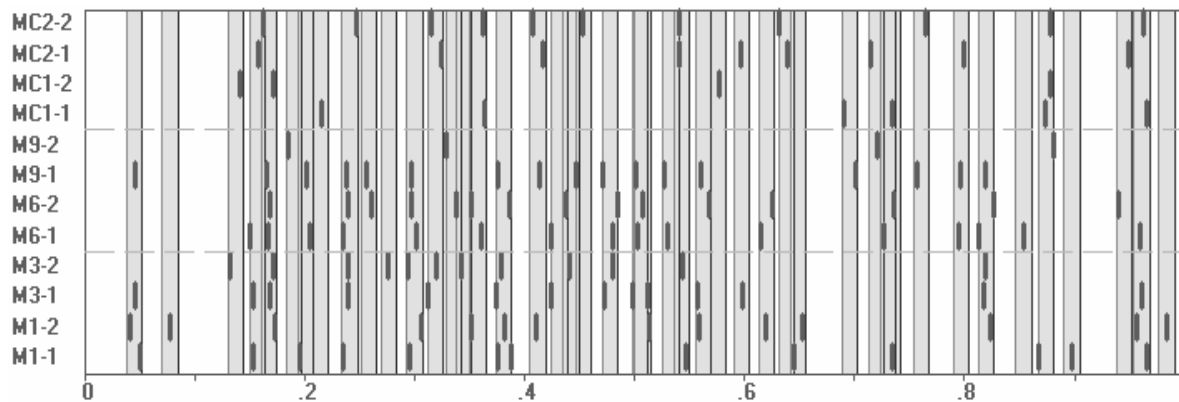
Separation of PCR amplicons produced distinct and reproducible patterns comprising of between 15-25 dominant and faint bands for each soil sample (Figure 4.3). Each band was presumed to represent a specific bacterial species present in the particular soil community being studied. Several of these species appeared to be present within multiple samples although there were some which were unique within a particular sample site. These unique species could be representing those selected cosmopolitan species with specialised abilities to adapt and take advantage of the new environment. Kozdrój and van Elsas (2001) explained that often after a disturbance, a selected group of bacteria, with specialised capabilities, are ready to take advantage of the new conditions.

Soil samples collected from pivot Four (lanes 13-30) displayed more numerous bands, and thus a higher species diversity, than those from pivot Major (lanes 1-12) and pivot Tweefontein (lanes 31-46). In addition to this, control samples from pivot Major (lanes 9-12) and pivot Tweefontein (lanes 43-46) displayed marginally lower species diversity than their gypsum-irrigated counterparts. The increase in species diversity following the introduction of gypsum may be attributed to the increase in growth of selected groups of

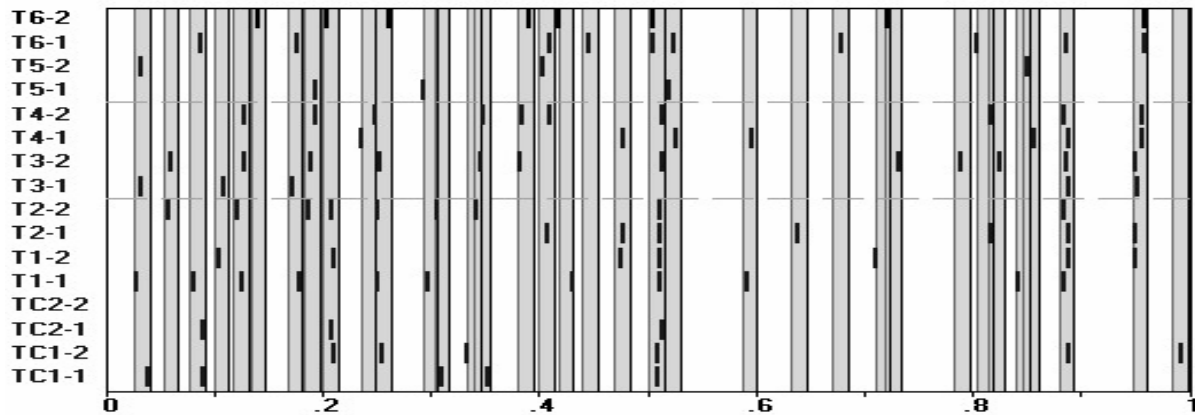
bacteria able to utilise gypsum for their growth and metabolic activities (Ehrlich, 1990). Atlas (1991) established that microbial communities within disturbed environments demonstrated enhanced capabilities for generalised adaptations. Similarly, Rasmussen *et al.* (2001) and Evans *et al.* (2004) observed an increase in genetic diversity following the introduction of mercury and oil, respectively, into soils.

Graphic representations of the DGGE band patterns were drawn using the Gel2K software program (Norland, 2004) (Figure 4.4). This program estimates the presence and intensity of band peaks in each lane and then displays the dominant species present in the community as dark bands. The graphic representations revealed that control samples from both pivots Major (Fig. 4.4 *a*) and Tweefontein (Fig. 4.4 *b*) had lower species diversity than gypsum-irrigated samples. Species diversity of soil samples from pivot Four appeared to not have been affected by the gypsum as there was no distinct difference between gypsum-irrigated and control samples (Figure 4.4 *c*).

(a)



(b)



(c)

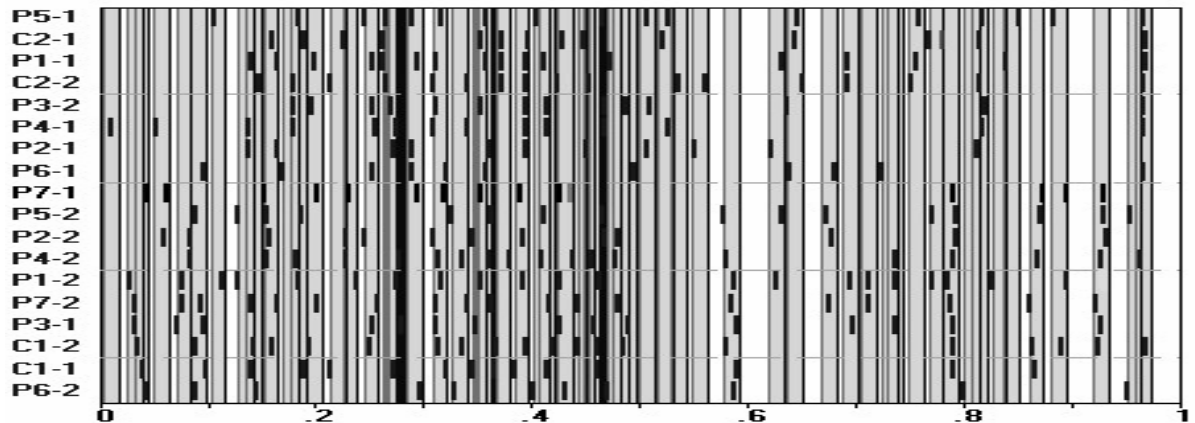


Figure 4.4 Graphical band patterns produced on DGGE profiles from three pivots: (a) soil samples from pivot Major; (b) soil samples from pivot Tweefontein; (c) soil samples from pivot Four (dark bars represent dominant bands present in each sample). All samples with the letter ‘C’ denote control samples.

DGGE banding patterns for each soil sample were further used in a cluster analysis for the construction of dendrograms. A dendrogram demonstrates the arrangement of species into clades produced by hierarchical clustering. Comparison of DGGE fingerprints was done using Gel2K (Norland, 2004), and calculated by Jaccard co-efficient over a complete link setting.

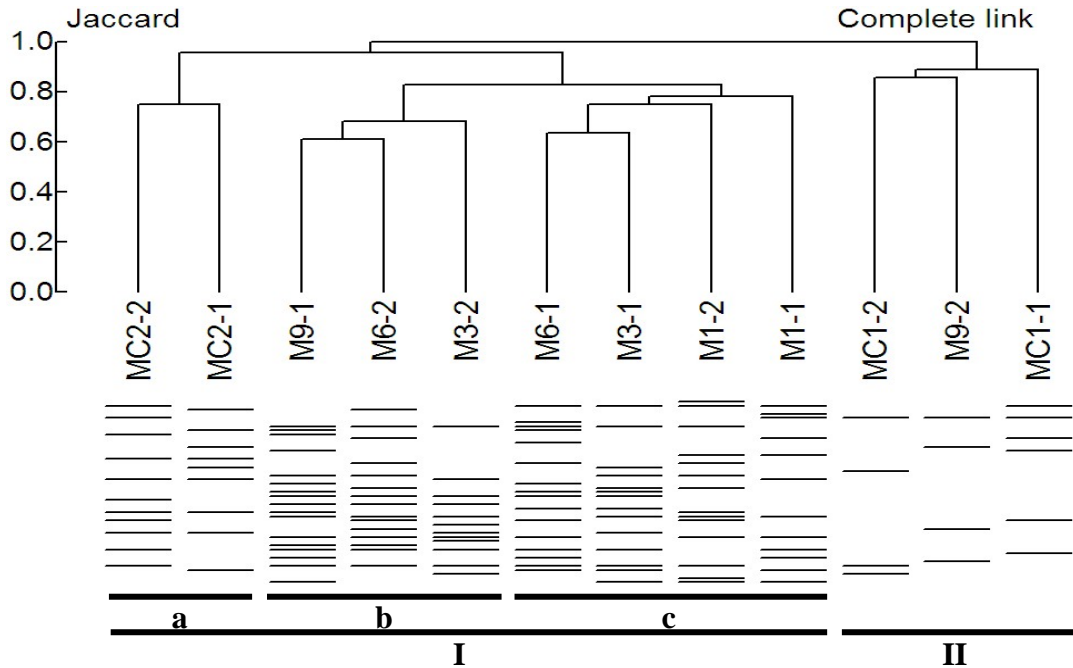


Figure 4.5 Cluster analysis of soil samples from pivot Major showing two distinct clades.

The Jaccard, complete link cluster analysis revealed distinct differences in banding patterns between the gypsum-irrigated and control samples, indicative of the differences in their bacterial community structures (Figure 4.5). The dendrogram displayed two distinct clades. Clade I consisted mainly of gypsum-irrigated soils, with the exception of subclade *a*, which contained control samples MC 2-1 and MC 2-2. The other set of control samples were clustered under clade II, along with gypsum-irrigated sample M9-2. These samples displayed the least number of bands, and thus lowest species diversity, when compared with other samples within clade I.

On average, the gypsum-irrigated samples displayed a greater number of bands, representative of higher species diversity, when compared with the control samples. This could be because the addition of gypsum lead to the selection of those bacteria which were able to adapt to the new conditions. This is in accordance with Kozdrój and van Elsas (2001) who witnessed a shift in microbial community structure, with those species capable of adapting to the new conditions taking dominance. Øvreås (2000) explained

that often after a disturbance or stress, the bacterial communities consist of members ready to take advantage of the new situation. This observation is considered to be an accurate representation of community diversity changes within the gypsum irrigated soils, since the apparent increase in genetic diversity of a microbial community following the introduction of contaminant has been observed by others.

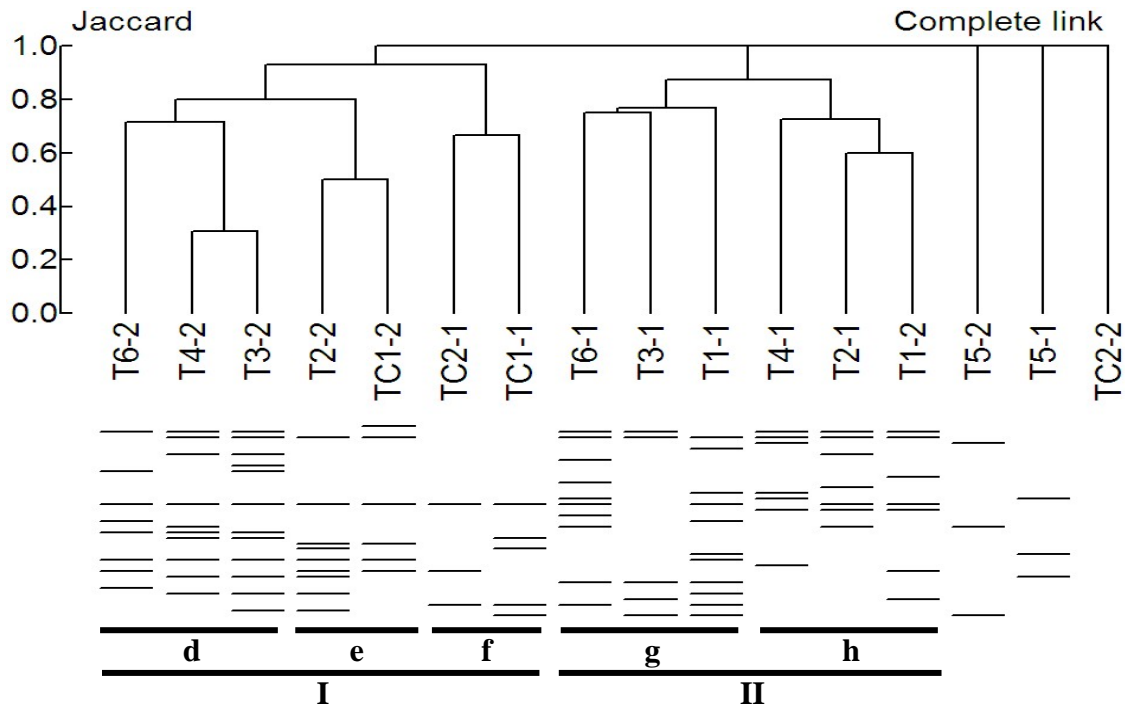


Figure 4.6 Cluster analysis of soil samples from pivot Tweefontein.

The cluster analysis of pivot Tweefontein was divided into two distinct clades, with three other samples falling basal (Figure 4.6). On average, gypsum-irrigated samples displayed the most number of bands as compared to their control counterparts. The control samples had the least number of bands, with control sample TC2-2 giving no bands. The gypsum-irrigated samples exhibited higher bacterial species diversity than that found in control samples. This elevated genetic diversity noted in the gypsum-irrigated samples could also be due to the selection of those cosmopolitan bacterial species with capabilities to survive and take dominance under the conditions (Kozdrój and van Elsas, 2001). Ehrlich (1996) explained that soil microorganisms displayed capabilities to utilise minerals, such as

gypsum, competitively for their own growth and survival. An increase in the number of bands, indicative of high species diversity, was also observed by Evans *et al.* (2004), following the introduction of oil to soil microcosms. Atlas (1991) concluded that microbial communities within disturbed environments demonstrated enhanced capabilities for generalised adaptations.

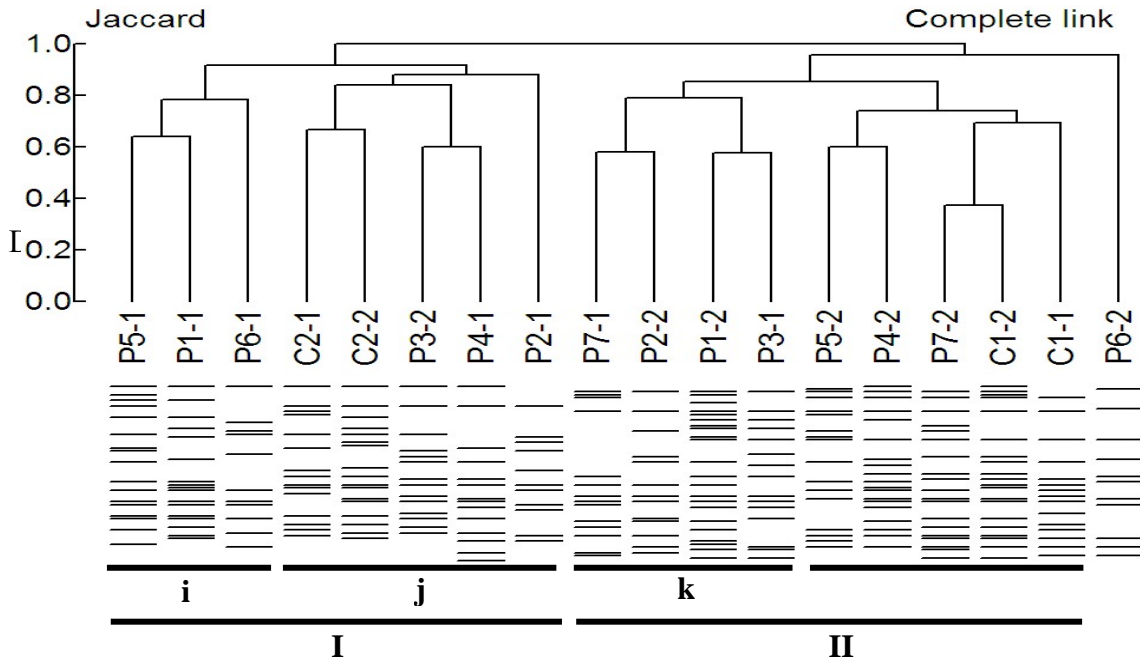


Figure 4.7 Cluster analysis of soil samples from pivot Four showing two distinct clades.

There was no noteworthy difference in the number of dominant bands between the gypsum-irrigated and control samples from pivot Four (Figure 4.7). Although the number of dominant bands present in control samples ranged between 15 and 20 and those from gypsum-irrigated samples ranged between 11 and 22, this observed difference was not significant. Once again, a group of selected species with specialised capabilities was able to adapt to the new conditions and utilise the gypsum for their competitive survival (Atlas, 1991; Ehrlich, 1996). However, the differences in diversities between the gypsum-irrigated and control samples were not as distinct as in the other two pivots (Major and Tweefontein). This could be attributed to previous land-use histories of the pivots.

Gorbena *et al.* (2005) found land use history as one of the major determinant of microbial diversity. Both pivot Major and Tweefontein were once mined, whereas pivot Four was never mined. The previous mining activities could have affected the soil bacterial community structures in advance causing an adaptation of the community to an already stressed soil.

The response of bacterial community structures along different soil profiles has been well investigated (Fierer *et al.*, 2003; Gorbena *et al.*, 2005). It has been found that the microbial community composition changes with soil depth due to differences in spatial and resource factors (Zhou *et al.*, 2002; Fierer *et al.*, 2003). Maila *et al.* (2005) later confirmed that different soil layers harbour different numbers and species of bacteria owing to a decline in the availability of carbon substrates with increasing soil depths.

It was therefore expected that the bacterial profiles present at 0–10 cm would harbour more bacterial diversity than those collected at 40–60 cm. However, in all samples, DGGE patterns revealed no distinct difference in species diversity between the two soil depths. Both soil depth profiles displayed a uniform number of dominant bands within each sample type as illustrated by the clusters presented in Figures 4.5, 4.6 and 4.7 as well as table 4.1 below. The observed similarities in bacterial species diversity between the two soil depth profiles may be attributed to the presence of plants (*Zea Mays*) as well as soil chemical properties. In all three pivots, the pH values did not differ significantly with soil depth (as tabulated in Table 3.1 under section 3.1). It is well known that plants can influence the biodiversity of bacteria through the release of amino acids, sugars and growth factors in root exudates, and these are known to stimulate both microbial activity and growth (Dunfield and Germida, 2001). Gelomino *et al.* (1999) observed similar molecular profiles to be present in topsoil and along the soil gradient. Their soil profiles were stable with respect to the presence of dominant species, irrespective of soil depth.

Table 4.1 Number of dominant DGGE bands per soil sample.

Samples	Description	Number of bands
M1-1	Major 0-10 cm, gypsum-irrigated	12
M1-2	Major 40-60 cm, gypsum-irrigated	14
M3-1	Major 0-10 cm, gypsum-irrigated	14
M3-2	Major 40-60 cm, gypsum-irrigated	12
M6-1	Major 0-10 cm, gypsum-irrigated	16
M6-2	Major 40-60 cm, gypsum-irrigated	15
M9-1	Major 0-10 cm, gypsum-irrigated	17
M9-2	Major 40-60 cm, gypsum-irrigated	4
MC1-1	Major 0-10 cm, control	6
MC1-2	Major 40-60 cm, control	4
MC2-1	Major 0-10 cm, control	9
MC2-2	Major 40-60 cm, control	11
T1-1	Twefontein 0-10 cm, gypsum-irrigated	11
T1-2	Twefontein 40-60cm, gypsum-irrigated	7
T2-1	Twefontein 0-10 cm, gypsum-irrigated	7
T2-2	Twefontein 40-60 cm, gypsum-irrigated	9
T3-1	Twefontein 0-10 cm, gypsum-irrigated	5
T3-2	Twefontein 40-60 cm, gypsum-irrigated	12
T4-1	Twefontein 0-10 cm, gypsum-irrigated	7
T4-2	Twefontein 0-10 cm, gypsum-irrigated	10
T5-1	Twefontein 0-10 cm, gypsum-irrigated	3
T5-2	Twefontein 0-10 cm, gypsum-irrigated	3
T6-1	Twefontein 0-10 cm, gypsum-irrigated	10
T6-2	Twefontein 0-10 cm, gypsum-irrigated	8
TC1-1	Twefontein 0-10 cm, control	5
TC1-2	Twefontein 40-60 cm, control	6
TC2-1	Twefontein 0-10 cm, control	3
TC2-2	Twefontein 40-60 cm, control	0
P1-1	Four 0-10 cm, gypsum-irrigated	17
P1-2	Four 0-10 cm, gypsum-irrigated	22
P2-1	Four 0-10 cm, gypsum-irrigated	11
P2-2	Four 0-10 cm, gypsum-irrigated	13
P3-1	Four 0-10 cm, gypsum-irrigated	15

Samples	Description	Number of bands
P3-2	Four 0-10 cm, gypsum-irrigated	14
P4-1	Four 0-10 cm, gypsum-irrigated	14
P4-2	Four 0-10 cm, gypsum-irrigated	19
P5-1	Four 0-10 cm, gypsum-irrigated	17
P5-2	Four 0-10 cm, gypsum-irrigated	16
P6-1	Four 0-10 cm, gypsum-irrigated	11
P6-2	Four 0-10 cm, gypsum-irrigated	11
P7-1	Four 0-10 cm, gypsum-irrigated	14
P7-2	Four 0-10 cm, gypsum-irrigated	19
C1-1	Four 0-10 cm, control	15
C1-2	Four 0-10 cm, control	20
C2-1	Four 0-10 cm, control	15
C2-2	Four 0-10 cm, control	17

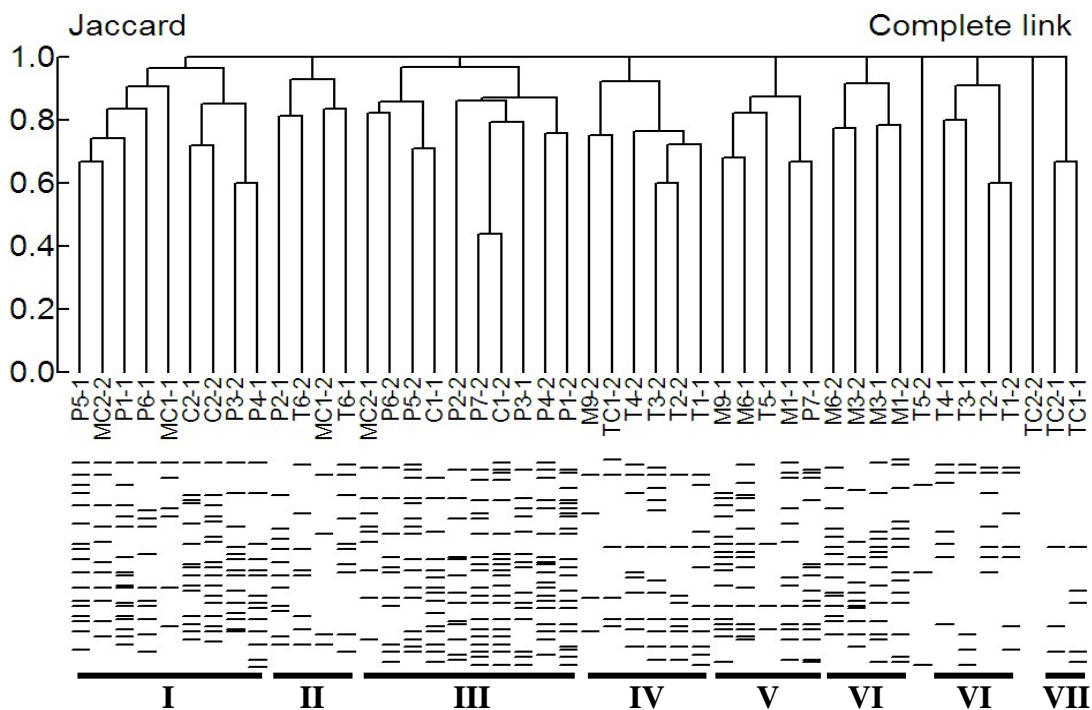


Figure 4.8 Combined cluster analysis of the DGGE profiles of bacterial species in soil samples from three pivots.

All the fingerprint profiles obtained were combined and subjected to a Jaccard complete link analysis to compare the differences and/or similarities in bacterial diversity between soils collected from all three pivots (Figure 4.8). The cluster was divided into eight distinct clades. There was a notable separation in terms of species diversity between soil samples from pivot Four and Tweefontein. The majority of soil samples from pivot Tweefontein were clustered under clades IV, V, VI, VII and VII,I whereas those from pivot Four grouped together under clades I, II and III (Figure 4.8). Of the two pivots, pivot Four displayed the highest bacterial species diversity when compared to pivot Tweefontein. The observed differences in bacterial diversities between these two pivots could be due to differences in their soil histories. The low species diversity in pivot Tweefontein could be because the pivot was previously mined for coal and had undergone rehabilitation. These mining activities could have had a negative impact on the soil's chemical and physical properties. Baligar *et al.* (2001) explained that anthropogenic activities often lead to soil degradation, also Kozdrój and van Elsas (2001) noted that these anthropogenic activities may have a negative effect on soil microbial communities. On the other hand, soil communities from pivot Four were the most diverse, the soil was never mined and had never been subjected to any industrial or environmental stresses; which could have affected the soil properties. This conclusion is supported by Goberna *et al.* (2005), who discovered that land use history was one of the major determinants of microbial community structure. They found the highest number of DGGE bands, and therefore species diversity, present in undisturbed soils as compared to the least diversity present in undisturbed soils.

4.2) Enumeration of total heterotrophic bacteria

An estimate of the relative total heterotrophic bacteria present in each pivot was determined by viable plate counts performed on R2A agar plates. Culturable bacteria were streaked out and incubated at 25 °C and bacterial counts documented every day for a period of 3d. In each pivot, the average culturable bacterial count was in the range of 10^6 and 10^8 cfu g⁻¹ soil (Table 4.2).

Table 4.2 Enumeration of total heterotrophic bacterial populations.

Samples	Bacterial counts (10^6 cfu g ⁻¹)	Samples	Bacterial counts (10^6 cfu g ⁻¹)
M1-1	460	TC2-2	4.6
M1-2	620	P1-1	88
M3-1	510	P1-2	63
M3-2	45	P2-1	65
M6-1	420	P2-2	40
M6-2	300	P3-1	760
M9-1	630	P3-2	550
M9-2	44	P4-1	320
MC1-1	61	P4-2	750
MC1-2	72	P5-1	110
MC2-1	42	P5-2	380
MC2-2	60	P6-1	40
T1-1	44	P6-2	38
T1-2	31	P7-1	310
T2-1	57	P7-2	490
T2-2	58	C1-1	360
T3-1	7.6	C1-2	440
T3-2	6.1	C2-1	320
T4-1	10	C2-2	400
T4-2	36		
T5-1	4.9		
T5-2	6.9		
T6-1	65		
T6-2	42		
TC1-1	6.8		
TC1-2	7.9		
TC2-1	7.7		

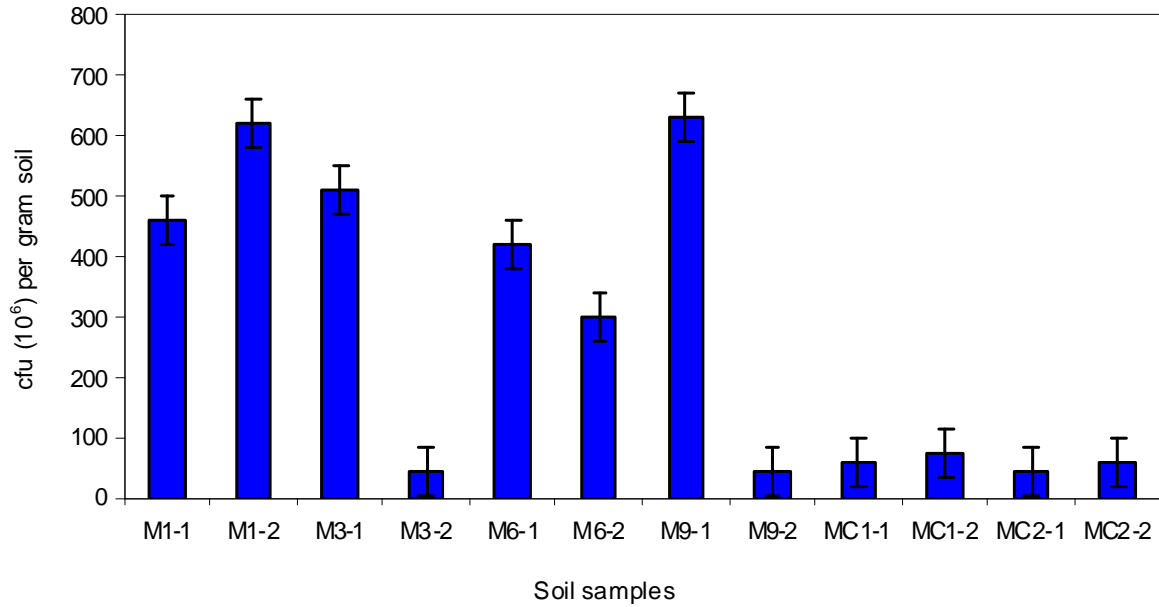


Figure 4.9 Total heterotrophic bacterial counts of gypsum-irrigated (M) and control (MC) soil samples from pivot Major.

The introduction of gypsum seemed to increase the total heterotrophic population present in pivot Major. Bacterial colonies in gypsum-irrigated soils yielded an average of $3,76 \times 10^8$ cfu g⁻¹ soil; as compared to control soils with an average of $5,9 \times 10^7$ cfu g⁻¹ soil (Figure 4.9). This observed increase in bacterial population may be due to the presence of the selected cosmopolitan bacterial species better adapted to the new conditions (Kozdrój and van Elsas, 2001). Bajpai *et al.* (1976) witnessed enhanced microbial activity and increased bacterial populations following the application of gypsum. Similar findings have been observed by Lindsay *et al.* (2009) who found higher counts of sulphur reducing bacteria present in solid tailings rich in sulphide minerals.

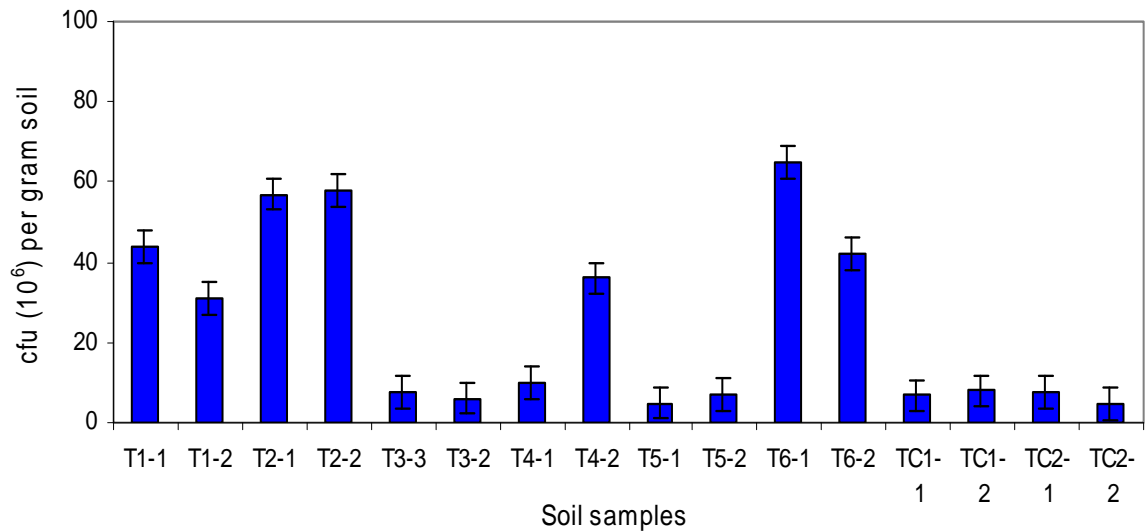


Figure 4.10 Total heterotrophic bacterial counts of gypsum-irrigated (T) and control (TC) soil samples from pivot Tweefontein.

In pivot Tweefontein, the heterotrophic counts were higher in gypsum-irrigated soils than in control soils, with averages of $3,7 \times 10^7$ and 7×10^6 cfu g^{-1} soil respectively (Figure 4.10). Bacterial diversities are known to decrease in response to environmental stress resulting in the survival of limited a number of microorganisms with specific capabilities to survive under the conditions (Atlas, 1991). The observed increase in bacterial counts could be due to the growth of selected groups of bacteria with specialized capabilities to survive under the new environment and utilise gypsum for their own metabolic activities. Ehrlich (1996) highlighted that microbial communities were able to utilise gypsum competitively for their growth.

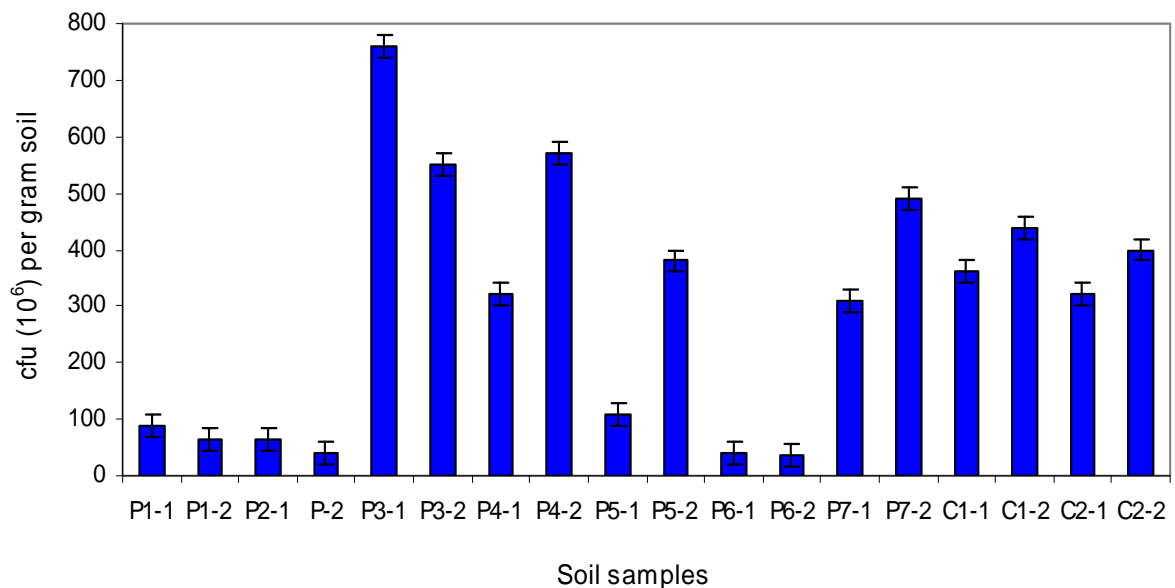


Figure 4.11 Total heterotrophic bacterial counts of gypsum-irrigated (P) and control (C) soil samples from pivot Four.

There were no observed differences between the number of total heterotrophic bacteria present in gypsum-irrigated and control samples from pivot Four (Figure 4.11). Heterotrophic bacterial counts in gypsum-irrigated soils had an average of $2,73 \times 10^8$ cfu g^{-1} soil and those present in the control soils were slightly higher at $3,80 \times 10^8$ cfu g^{-1} soil. The application of gypsum lead to a selection of those bacteria with specialised skills to utilise gypsum for the own competitive growth (Ehrlich, 1996). However, the lack of difference in total heterotrophic counts between the gypsum-irrigated and control samples, as previously mentioned, could be due to the pivot's previous land-use history. Since the pivot never underwent any mining activities, its soil composition was undisturbed. Previous land-use history has been identified as one of major factors influencing microbial diversity (Gornena *et al.*, 2005).

4.3) Community-level physiological profiles (CLPP)

Community-level physiological profiles (CLPP) of soil bacterial communities were investigated based on each community's potential to utilise carbon substrates found on

Biolog™ Ecoplates (Zak *et al.*, 1994). The results were analysed using both principal component analysis (PCA) and Simpson's diversity index.

4.3.1) Principal component analysis

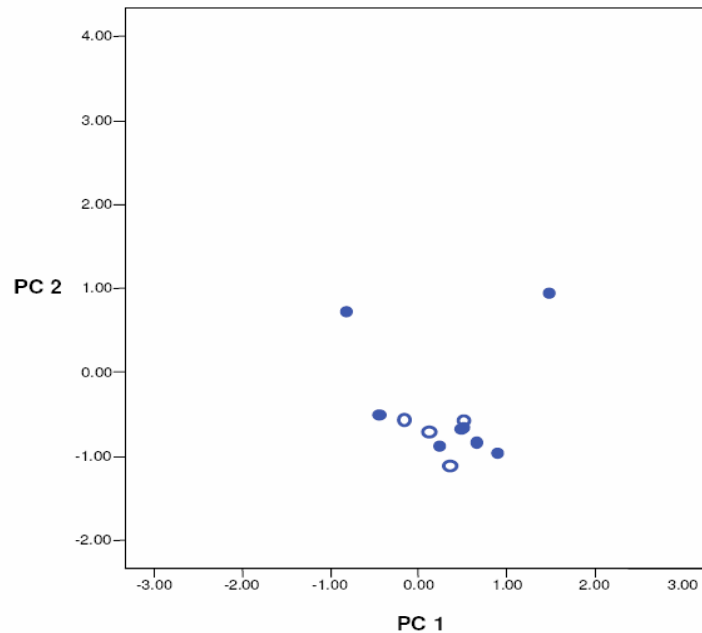


Figure 4.12 Principal component analysis of sole carbon source utilisation (SCSU) patterns of soils from pivot Major showing gypsum-irrigated ● and control ○ samples.

In pivot Major, soil samples demonstrated no dissimilarities in carbon utilisation patterns, based on their substrate utilisation abilities between the gypsum-irrigated and control bacterial communities. However, this was with the exception of two gypsum-irrigated samples which were clearly separated from others (Figure 4.12). These samples exhibited varied carbon utilisation patterns which could be indicative of diverse bacterial species capable of utilising a broader spectrum of carbon sources. Figure 4.13 agreed with figure 4.12, revealing the two outliers (M1-2 and M9-2) which were clustered further away from the other samples.

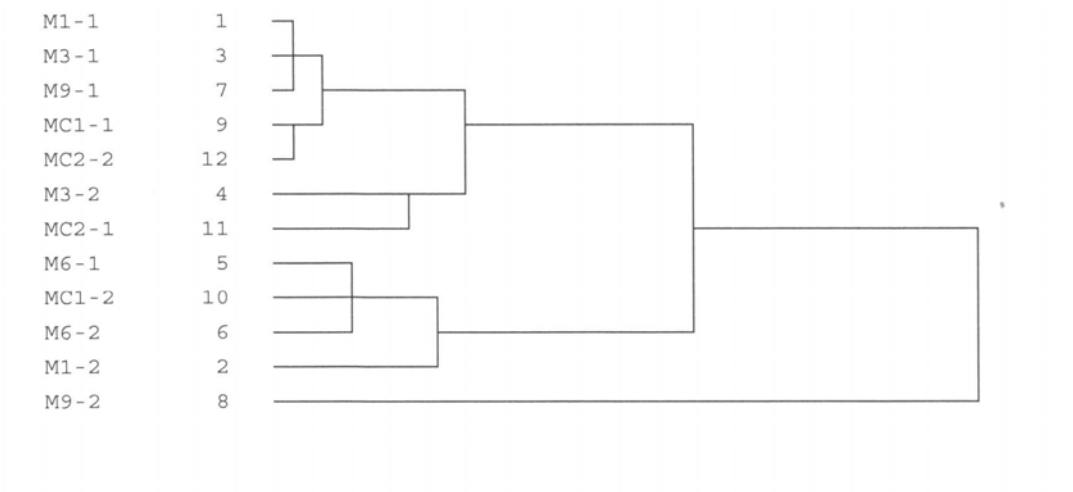


Figure 4.13 Cluster analysis of SCSU patterns of gypsum-irrigated and control soil samples from pivot Major. Cluster was constructed using Wards and squared Euclidean algorithms.

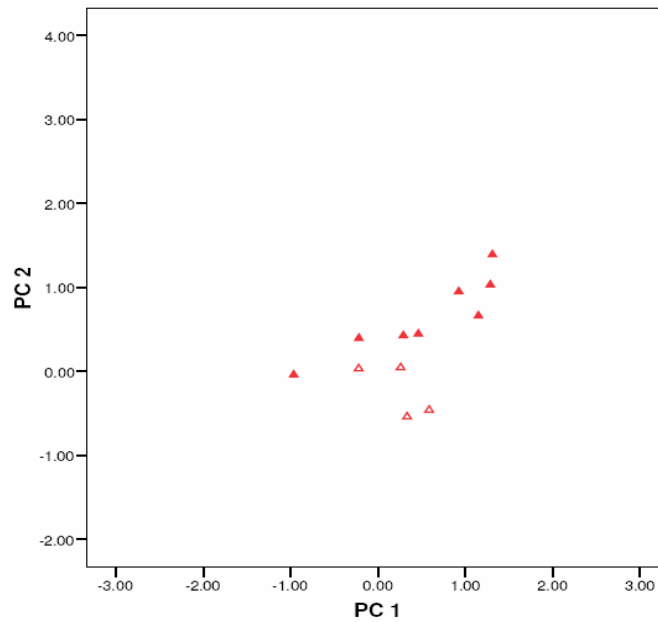


Figure 4.14 Principal component analysis of SCSU patterns of soils from pivot Tweefontein showing gypsum- irrigated ▲ and control △ samples.

There was a clear separation, in terms of carbon utilisation capabilities, observed between most the gypsum-irrigated and control samples from pivot Tweefontein. The control samples were grouped ‘closely’ together and exhibited uniform substrate utilisation patterns (Figure 4.14). On the other hand, the gypsum-irrigated samples displayed a more diverse carbon utilisation pattern indicative of higher species diversity. Cluster analysis, also revealed a relationship among the control samples (TC1-1, TC 1-2 and TC2-1) as these were clustered closely together, indicative of similar and uniform substrate utilisation capabilities (Figure 4.15). These findings are contrary to those of Rasmussen *et al.* (2001) who discovered similar substrate utilisation patterns to be present in the mercury-contaminated soils. The difference between these findings and those of Rasmussen *et al.* (2001) could be related to the length of exposure to the treatment. The soils in their study were only exposed to the mercury for a period of 3 months whereas in this study the soils were exposed to gypsum over a much longer period (i.e., 12-14 years). During that period, the selected bacterial communities adapted specialised properties to thrive under the new environment (Atlas, 1991).

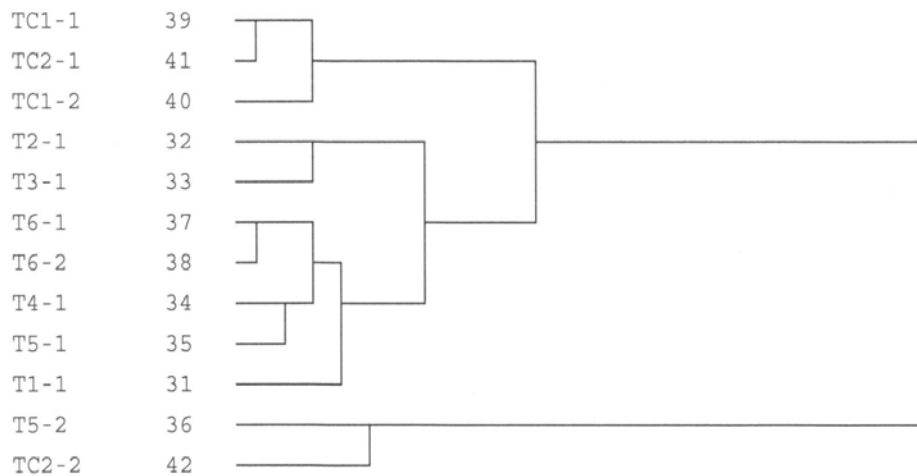


Figure 4.15 Cluster analysis of SCSU patterns of gypsum-irrigated and control soil samples from pivot Tweefontein. Cluster was constructed using Wards and squared Euclidean algorithms.

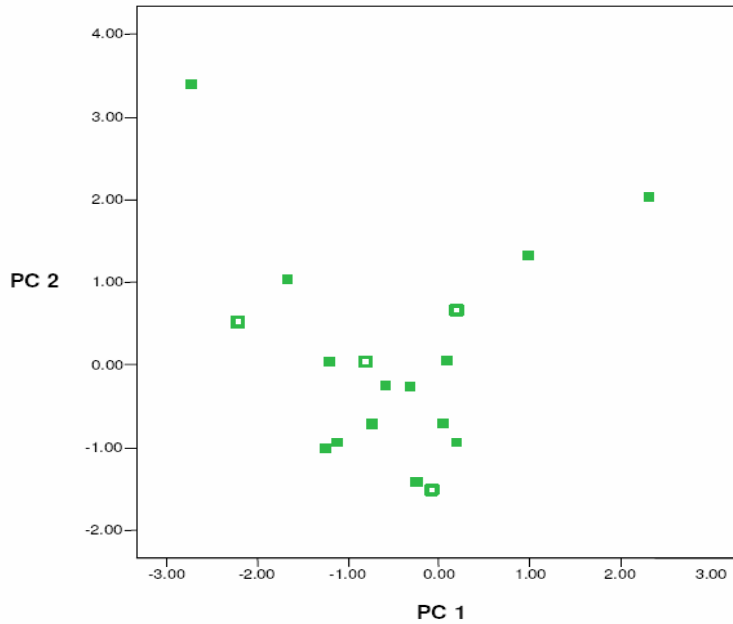


Figure 4.16 Principal component analysis of SCSU patterns of soils from pivot Four showing the gypsum-irrigated ■ and control □ samples.

Figure 4.16 illustrates the carbon utilisation patterns among soil bacterial communities from pivot Four. There was no clear separation between gypsum-irrigated soil and control soil in terms of substrate utilisation patterns. Both the gypsum-irrigated samples and control samples exhibited diverse carbon utilisation patterns. This is related to the high species diversity present in both communities. The cluster analysis also revealed no similarities in utilisation patterns among the samples (Figure 4.17). These findings are similar to those of Derry *et al.* (1998) who found similar substrate utilisation patterns present in both creosote-contaminated and uncontaminated soils.

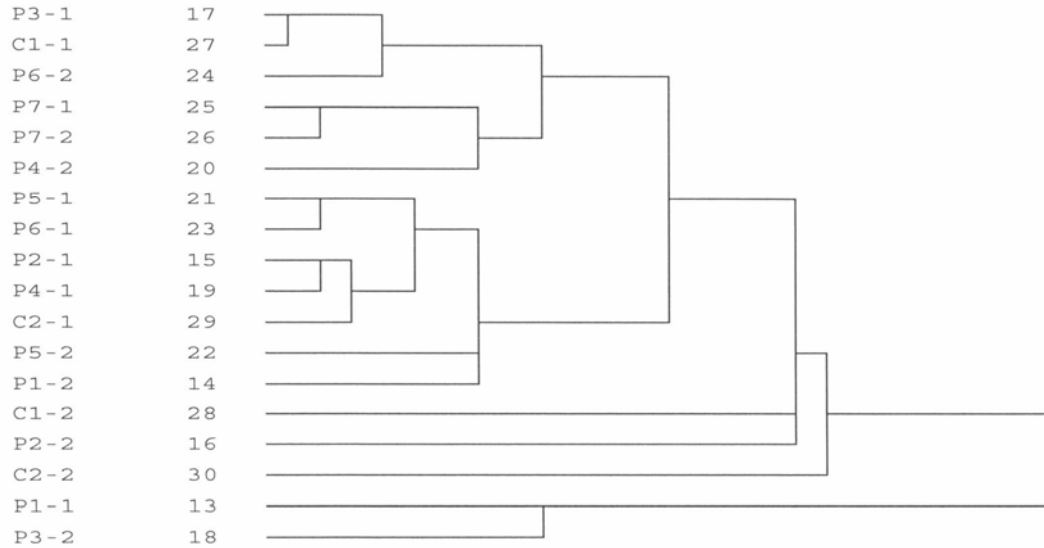


Figure 4.17 Cluster analysis of SCSU patterns of gypsum-irrigated and control soil samples from pivot Four. Cluster was constructed using Wards and squared Euclidean algorithms.

4.3.2) Shannon diversity index

Results obtained from substrate utilisation and colony counts were further evaluated by determining the Shannon diversity indices. The indices were calculated using $H' = -\sum p_i \ln p_i$; where p_i is (a) the ratio between the optical density in the single well and the total optical density summed from all the wells; or (b) the ratio between the number of colonies in a specific group and the total number of colonies. Firstly, the data was tested for normality using the Kolmogorov-Smirnov and Lilliefors test as well as the Shapiro Wilkes W-test. The p value from the Shapiro test was $p < 0.05$ and the data was thus assumed non-parametric and data analysed using the Mann-Whitney non-parametric test.

Table 4.3 Bacterial diversities (mean \pm SD) of soil samples from all pivots determined by the Shannon index based on substrate utilisation (Biolog TM Ecoplates) and colony morphology.

Pivot	Treatment	Substrate utilisation	Colony morphologies
Major	Gypsum-irrigated	2.82 \pm 0.19*	1.25 \pm 0.10**
	Control	2.77 \pm 0.07	1.10 \pm 0.09
Tweefontein	Gypsum-irrigated	2.72 \pm 0.10*	1.31 \pm 0.26**
	Control	2.51 \pm 0.13	1.12 \pm 0.16
Four	Gypsum-irrigated	2.62 \pm 0.13	1.42 \pm 0.24
	Control	2.64 \pm 0.18	1.44 \pm 0.15

Statistically significant (Mann-Whitney) effects of gypsum-irrigated and control soils are indicated as * $p < 0.05$ and ** $p < 0.5$.

The bacterial diversities of soils from the three pivots are presented in Table 4.3. Shannon index values are average values of replicates from each soil. In pivot Major, there was a significant difference between the gypsum-irrigated and the control soils on the basis of both substrate utilisation ($p = 0.024$) and colony morphologies ($p = 0.226$). The same was observed for pivot Tweefontein, where the gypsum-irrigated soils differed significantly from the control soils on the basis of substrate utilisation ($p = 0.000007$) and colony morphologies ($p = 0.461$). In both pivots, the bacterial diversities were higher in the gypsum-irrigated soils and lower in control soils. Sharma *et al.* (1998) found a significant increase in functional diversity in soils following maize litter amendment as compared to a rapid decline in control soils.

There was no significant difference in diversities between the gypsum-irrigated and control samples based on substrate utilisation ($p = 0.226$) and colony morphologies ($p = 0.925$) from soils collected from pivot Four. Although the application of gypsum encouraged the growth of those selected bacteria able to utilise gypsum (Bajpai *et al.*, 1976; Ehrlich, 1996); their growth did not supersede the growth of species found in the

control samples. Microbial communities in pivot Four were never affected by any anthropogenic practices prior to irrigation with gypsiferous mine water. This indicates that the application of gypsum did not have any affect on microbial community when applied to healthy soils. Majority of anthropogenic practices are known to have a negative effect on soil microbial communities (Kodzdrój and van Elsas, 2001).

In terms of the two soil depths, there was no significant difference in soils from all three pivots on the basis of substrate utilisation ($p = 1.00$) and colony morphologies ($p = 1.00$). (Table 4.4). These findings are in accordance with those from DGGE profiles and total heterotrophic bacterial counts. All three methods revealed no distinct difference in diversity between the soil profiles. The lack of difference in diversity prevalent between the two soil depths may be attributed to the presence of plants (*Zea Mays*). Their root systems release compounds which can enhance the growth of microorganisms below the soil surface. It is well documented that plants can enhance biodiversity of bacteria through the release of amino acids, sugars and growth factors in root exudates; and these are known to stimulate both microbial activity and growth (Dunfield and Germida, 2001).

Table 4.4 Bacterial diversities (mean \pm SD) of soils from all three pivots (at different soil depths) as estimated by the Shannon indices based on colony morphology and substrate utilisation (Biolog TM Ecoplates).

Pivot	Colony morphologies				Substrate utilisation			
	Gypsum-irrigated		Control		Gypsum-irrigated		Control	
	0-10	40-60	0-10	40-60	0-10	40-60	0-10	40-60
Major	1.32 \pm 0.23	1.17 \pm 0.25	1.06 \pm 0.08	1.14 \pm 0.10	2.68 \pm 0.18	2.57 \pm 0.20	2.75 \pm 0.04	2.80 \pm 0.09
Four	1.37 \pm 0.23	1.45 \pm 0.25	1.50 \pm 0.12	1.37 \pm 0.17	2.75 \pm 0.15	2.68 \pm 0.09	2.70 \pm 0.17	2.57 \pm 0.18
Twefontein	1.36 \pm 0.21	1.27 \pm 0.29	1.45 \pm 0.19	1.10 \pm 0.14	2.74 \pm 0.09	2.65 \pm 0.10	2.55 \pm 0.08	2.48 \pm 0.16

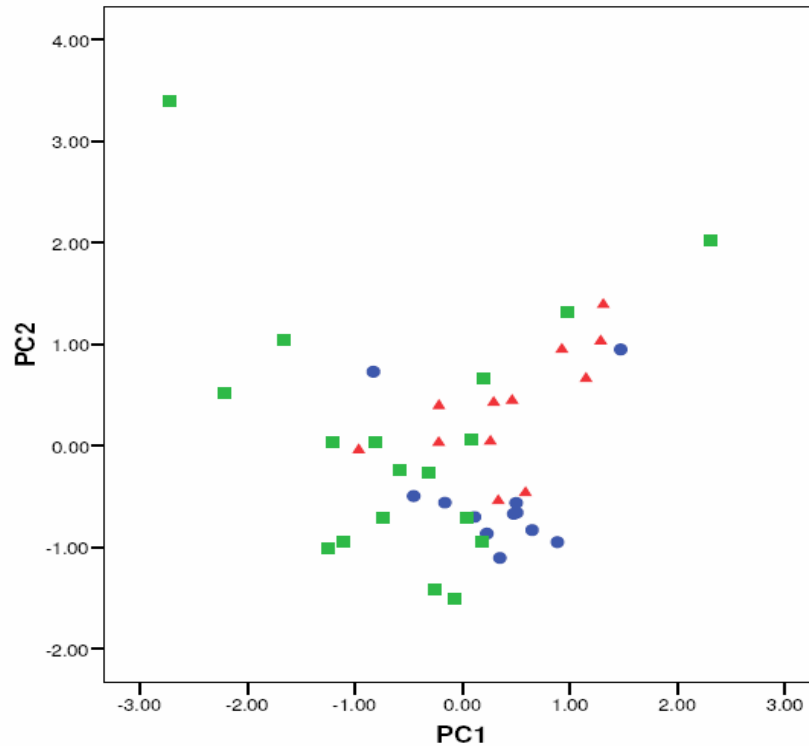


Figure 4.18 Principal component analysis of SCSU of soil samples from Major ●, Four ■ and Tweefontein ▲.

Substrate utilisation patterns of soil samples from all three pivots (Major, Four and Tweefontein) were compared. These revealed distinct patterns of sole carbon source utilisation on the basis of PCA transformed data (Figure 4.18). There was no clear resolution between soils samples from the different pivots. However, soil samples from pivot Four demonstrated abilities to utilise a much wider spectrum of carbon sources as these samples were distributed throughout the PC space. Garland and Mills (1991) and Zak *et al.* (1994) are among researchers that have used PCA to differentiate between samples from different environments. Similarly, they found that samples from the same environment/community displayed similar substrate utilisation patterns

Differences in substrate utilisation patterns between the different pivots were further analysed using hierarchical clustering (Figure 4.19). The cluster was divided into two distinct clusters. There was a distinct separation between soil samples from pivot Four

and pivot Tweefontein. Samples from pivot Four were grouped together under one cluster and those from pivot Tweefontein grouped together under a second cluster. This clustering pattern agrees with the cluster analysis of the combined DGGE profiles (Figure 4.8).

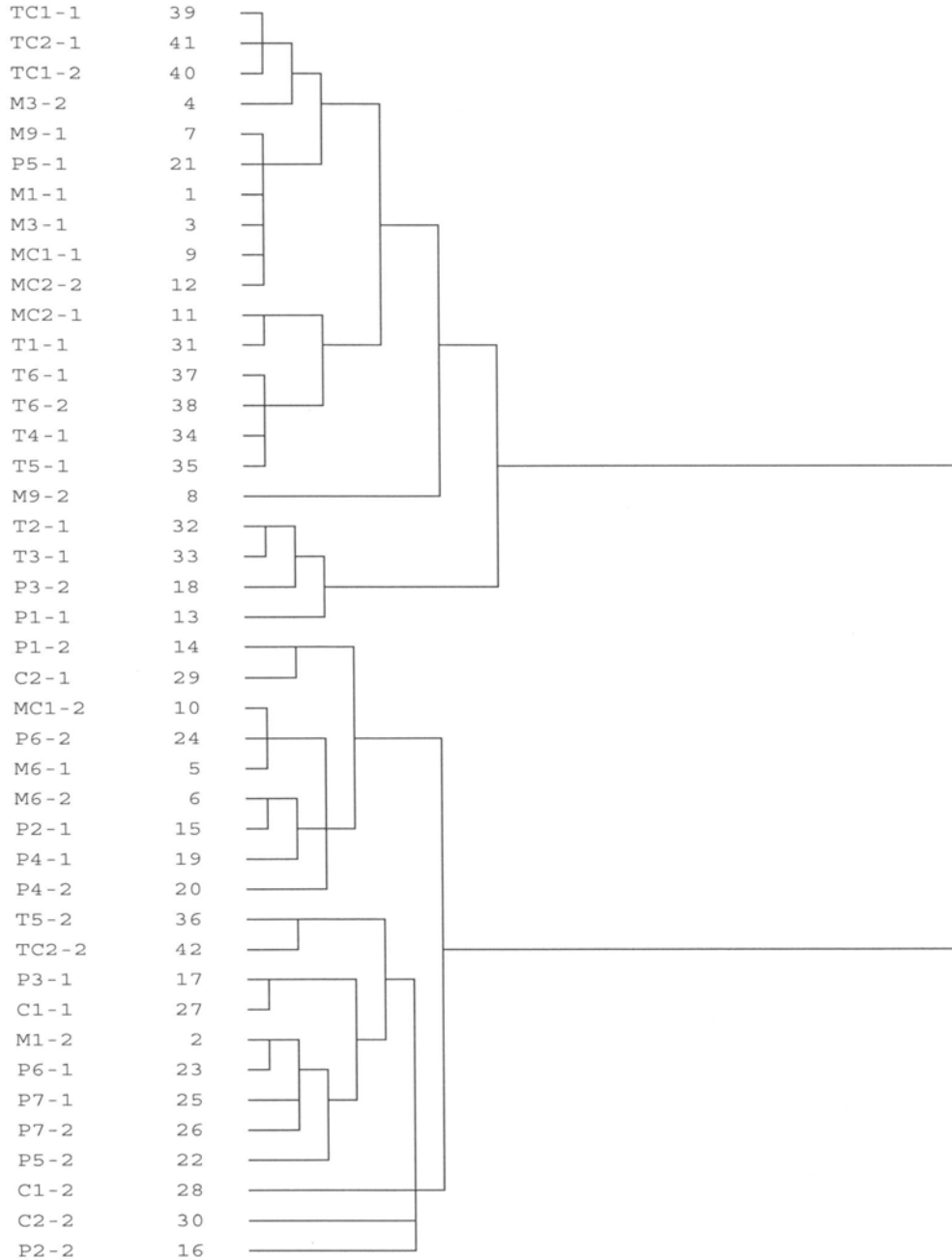


Figure 4.19 Cluster analysis of SCSU of all soil samples from pivots' Major, Four and Tweefontein. Cluster was constructed using the Ward and squared Euclidean algorithms.

4.4) General Discussion

In this study, a combination of culture-dependent methods (heterotrophic diversity and functional diversity) and culture-independent methods (PCR-DGGE) were used to gain better insight into the impact of gypsiferous mine water irrigation on soil bacterial communities. To achieve this, natural soil samples with a history of irrigation with gypsum rich water were used and compared with soil samples from the same location but which were not irrigated with gypsum rich water.

The application of gypsiferous mine water changed the soil chemical properties by decreasing the pH and increasing the concentrations of exchangeable calcium and sulphate. This supports findings observed by Suhayda *et al.* (1997) during their study of the response of saline-alkaline soil to gypsum. They observed a large decrease in pH as well as a significant increase in calcium and sulphate ions. Carter (1986) also used gypsum to reclaim sodic soils and observed an increase in calcium ions.

The introduction of gypsum not only affected the soil chemistry but also had an impact on the soil bacterial communities. A number of studies have been centred on the response of microbial communities to the gypsum (Bajpai *et al.*, 1976; Ehrlich 1990 and 1996). These studies revealed that the microbial communities exhibited enhanced activity and increased diversity in the presence of gypsum. Ehrlich (1996) explained that soil microorganisms were capable of utilising gypsum for their own growth and survival and to promote enhanced competitiveness in the community.

The results from this study showed that DGGE profiles of the gypsum-irrigated and control samples were dissimilar. This was most evident in soil samples from both pivot Major and Tweefontein. In these two pivots, higher species diversity was apparent in the gypsum-irrigated soils than in the control soils. The increase in diversity in the gypsum-irrigated soils could primarily be due to appearance of selected bacterial species with specialised capabilities taking advantage of the new conditions and not survive but grow therein (Ehrlich, 1996). Atlas (1991) established that bacterial communities under disturbed conditions exhibited enhanced capabilities to survive. During their study,

Rasmussen *et al.* (2001) observed an instant increase in genetic diversity in the mercury-contaminated soils. Genetic diversity in the mercury-contaminated soil continued to increase with time whereas the control samples remained constant. Evans *et al.* (2004) also found an increase in microbial diversity following oil contamination.

Likewise in this study, the number of total culturable heterotrophic bacteria was found to be higher in the gypsum-irrigated soil than in control soils. The increase in bacterial population could be owing to the growth of specific groups of bacteria capable of utilising the minerals in gypsum to enhance their own growth (Ehrlich, 1990 and 1996). These findings are in agreement with those of Lindsay *et al.* (2009) who found higher counts of sulphur reducing bacteria present in solid tailings rich in sulphide minerals.

The results from DGGE profiles and total heterotrophic bacteria corroborated with those of community-level physiological profiles, calculated on the basis of Principal Component Analysis (PCA) and Shannon diversity index. PCA revealed differences in substrate utilisation patterns between the gypsum-irrigated samples and control samples. The majority of control samples displayed uniform utilisation patterns whereas the gypsum-irrigated samples were able to utilise a diverse range of carbon substrates. The diverse substrate utilisation patterns observed in the gypsum-irrigated samples is indicative of high species diversity.

Furthermore, Shannon diversity indices were calculated on the basis of colony morphology and community-level physiological profiles. There were significant differences between the gypsum-irrigated samples and control samples from pivot Major and Tweefontein. In both pivots, the gypsum-irrigated samples gave higher indices (H') for both colony morphology and substrate utilisation. However this was not the case for pivot Four, as there were no significant differences in either colony morphology and substrate utilisation evident among the gypsum-irrigated samples and control samples.

The three pivots were compared among each other in terms of their functional, culturable heterotrophic bacteria and genetic diversity. Distinct differences were observed between

the three pivots in terms of the three diversity measures. Pivot Four was the most diverse as it displayed the highest number of DGGE bands, the most counts of heterotrophic bacteria, as well as most diverse substrate utilisation capabilities. On the other hand, pivot Tweefontein showed the least diversity in terms of culturable heterotrophic and genetic diversity. Cluster analysis from both DGGE profiles and community-level physiological profiles revealed distinct differences between pivot Four and Tweefontein as samples from each pivot were clustered under two separate clusters.

Pivot Four and Tweefontein received irrigation water from the same source. The distinct differences in their bacterial diversity patterns could be due to their previous land-use histories. The reason for the low bacterial diversity observed in Tweefontein may be explained by the previous land use history of the pivot. The soil in Tweefontein was previously mined for coal and had undergone rehabilitation. However, the soil in pivot Four never underwent any mining activities and the soil properties were uninterrupted. Goberna *et al.* (2005) discovered that land use history was one of the major determinants of microbial community structures as they uncovered high genetic diversity present in undisturbed soils as compared to undisturbed soils. Microbial communities are known to be sensitive indicators of soil health as there is a clear relation between microbial diversity and soil health and/or quality (Coleman and Crossley, 1996).

CHAPTER 5

5. CONCLUSIONS

- Application of the gypsiferous mine water did not have an adverse effect on soil microbial communities. In fact, the gypsum favoured the growth of soil bacterial communities as seen by an increase in genetic, heterotrophic and functional diversity.
- Bacterial species diversity, determined by the number of DGGE bands in each soil sample, was on average higher among the gypsum-irrigated soil than in the control soils.
- The number of culturable heterotrophic microbial populations was observed to be greater in the gypsum-irrigated than in the control soils. Bacterial diversity, calculated on the basis of colony morphology, was also found to be higher in the gypsum-irrigated soils in than control soils.
- Principal component analysis (PCA) revealed that the gypsum-irrigated soils were able to utilise a broader range of carbon sources. This was indicative of the high species diversity present in these soils. Control soils displayed uniform substrate utilisation patterns, thus lower species diversity.
- Functional diversity, calculated by the Shannon index of diversity, was higher in the gypsum-irrigated soils and lower in the control soils. This indicates the ability of diverse bacterial communities present in the gypsum-irrigated to utilise a broader range of carbon sources.
- Bacterial communities remained constant with increasing soil depths. In both the gypsum-irrigated and control soils, the bacterial diversities prevalent in the bulk soil (0–10 cm) were the same as those in the subsurface (40-60 cm). The similarities in bacterial communities with increasing soil depth could be attributed to the presence of crops. Plants are known to stimulate bacterial growth by providing nutrients such as amino acids and sugars.
- From this study, it appeared that the gypsiferous mine water ameliorated the soil bacterial communities. This was most evident in those pivots with a history of mining. Furthermore, the use of gypsiferous mine water for irrigation purposes

proved to be a sustainable option, especially in semi-arid areas experiencing a shortage of rainfall. This study spanned soil samples collected in 2006 and 2007; however a more comprehensive study over a longer term is required to reveal the long-term impacts. Furthermore, the dominant bacterial species, visualised on DGGE profiles found in soils, should be sequenced to determine the species composition inherent in the gypsum-irrigated soils.

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SUMMARY

In the past, the response of microbial populations to anthropogenic disturbances was studied using conventional methods based on cultivation of microorganisms and on measurement of their metabolic activities (Fantroussi *et al.*, 1999). However, these culturing methods often account for a small proportion of the total microbial community (Ibekwe and Kennedy, 1998; Hill *et al.*, 2000). To overcome this, molecular techniques were developed and these allowed for the analyses of microorganisms in their natural habitats. Analysis of the 16S rRNA molecule and its corresponding gene (16S rDNA) has been the most widely used approach in the last decade (Amman *et al.*, 1995). Although molecular techniques based on PCR have been used to eliminate the bias of culturing methods, they also have their drawbacks (Wintzingerode *et al.*, 1997; Kirk *et al.*, 2004). As another alternative, Garland and Mills (1991) developed a rapid community-level physiological approach to study microbial communities. The use of the community-level approach to microorganisms provided an accurate and meaningful measure of the heterotrophic microbial community by measuring the community's metabolic abilities (Garland and Mills, 1991). Zak *et al.* (1994) used the method to study the functional diversity of microbial communities. The approach has been used to study the soil functional diversities in polluted or disturbed environments.

Over the years, the application of gypsum in agriculture has received much attention. The gypsum has been used to ameliorate both acidic and alkali soils with elevated amounts of salinity (Suhayda *et al.*, 1997; Sun *et al.*, 2000). In these studies, the application of gypsum lead to changes in the soil chemical properties by causing a drastic increase in the amount of exchangeable calcium and sulphate and reduced the levels of exchangeable aluminium. It has been noted that high levels of aluminium and/or reduced amounts of calcium restrict root elongation and thus hindered the plants ability to access adequate water (Sun *et al.*, 2000). Also, the replacement of sodium ions with calcium ions resulted in the flocculation of soil particles and improved the porous structure and water permeability of the soil (Suhayda *et al.*, 1997).

This study revealed that the application of the gypsiferous mine water did not have any negative impact on the bacterial communities. In fact, on average, the bacterial diversities were found to be higher in the gypsum-irrigated soils. This was most evident in pivot Major and Tweefontein, where the gypsum-irrigated soils were more diverse than the control soils. DGGE results from pivot Major and Tweefontein revealed a high level of bacterial diversity in gypsum-irrigated soils, as estimated by the number of dominant bands. Also, the number of heterotrophic bacteria in the gypsum-irrigated soils was one to two orders of magnitude higher than in the control soils. Principal component analysis performed on BIOLOG data showed that in both pivot Major and Tweefontein, the gypsum-irrigated soils were able to utilise a wider range of carbon sources as compared to their control counterparts. The bacterial communities in pivot Four appeared to be steady in both the gypsum-irrigated soils and the control soils. The number of visible DGGE bands was consistent between the gypsum-irrigated and the control soils. The heterotrophic bacterial counts in the gypsum-irrigated soils had an average of 273×10^6 cfu g⁻¹ soil and those present in the control soils were slightly higher at 380×10^6 cfu g⁻¹ soil. Principal component analysis revealed no differences in terms of substrate utilisation capabilities among the gypsum-irrigated soils and the control soils.

All three techniques revealed no significant difference in community structures between soil profiles at 0-10 cm and 40-60 cm. The lack of difference could be attributed to the crops planted in all three pivots during sampling. The root system of *Zea Mays* plants enhanced microbial growth by exuding nutrients such as amino acids and sugars

In conclusion, the application of polyphasic approach proved successful in studying the response of soil bacterial communities to gypsiferous mine water. The use of both culture-dependent and culture-independent methods is recommended as the methods compensate each other's limitations and therefore provide a more detailed description of the community.

In this study, the application of gypsiferous mine water did not have an adverse effect on the soil bacterial communities. In fact, the addition of gypsiferous mine water seemed to

ameliorate the soil bacterial communities. However, further comprehensive study is needed to determine the response of bacterial communities to gypsiferous mine water over longer periods of time. 16S rDNA sequencing and analysis of DGGE bands should also be done to identify the bacterial species present in the gypsum-irrigated samples.