

The Effects of Land Use and Management Practices on Soil Microbial Diversity as Determined by PCR-DGGE and CLPP

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

By

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March 2011

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Age is a question of mind over matter.....if you don't mind it does n't matter.

Satchel Paige

ABSTRACT

The environmental impact of anthropogenic disturbances such as agriculture, on the soil ecosystem, and particularly on soil microbial structural and functional diversity, is of great importance to soil health, conservation and remediation. Therefore, this study assessed the effects of various land use and management practices on both the structural (genetic) and functional (catabolic) diversity of the soil bacterial and fungal communities, at two long-term sites in KwaZulu-Natal. The first site is situated at Baynesfield Estate, and the second at Mount Edgecombe Sugarcane Research Institute. At site 1, the land uses investigated included soils under pre-harvest burnt sugarcane (*Saccharum officinarum*, Linn.) (SC); maize (*Zea mays*, Linn.) under conventional tillage (M); permanent kikuyu (*Pennisetum clandestinum*, Chiov) pasture (KIK); pine (*Pinus patula*, Schiede) plantation (PF); and wattle (*Acacia mearnsii*, De Wild) plantation (W), all fertilized; and undisturbed native grassland (NAT) that had never been cultivated or fertilized. At site 2, a sugarcane (*Saccharum officinarum* × *S. spontaneum* var. N27) pre-harvest burning and crop residue retention trial was investigated. The treatments studied included conventional pre-harvest burning of sugarcane with the tops removed (Bto), and green cane harvesting with retention of crop residues on the soil surface as a trash blanket (T). Each of these treatments was either fertilized (F) or unfertilized (Fo).

The polymerase chain reaction (PCR), followed by denaturing gradient gel electrophoresis (DGGE) were used to determine the structural diversity, and community level physiological profiling (CLPP) using BIOLOG plates, the catabolic diversity. In addition, the soils were analysed with respect to selected physicochemical variables, and the effects of these on the soil microbial communities were determined. Replicate soil samples (0–5 cm) were randomly collected from three independent locations within each land use and management, at both sites. Soil suspensions for the CLPP analyses were prepared from fresh soil subsamples (within 24 h of collection) for the bacterial community analyses, and from 8-day-old soil subsamples (incubated at 4°C to allow for spore germination) for the fungal community analyses. BIOLOG EcoPlates™ were used for the bacterial CLPP study and SF-N2 MicroPlates™ for the fungal analysis, the protocols being adapted and

optimized for local conditions. This data was log [X+1]-transformed and analysed by principal component analysis (PCA) and redundancy analysis (RDA). For PCR-DGGE, total genomic DNA was isolated directly from each soil subsample, and purified using the MO BIO UltraClean™ soil DNA Isolation kit. Protocols were developed and optimized, and fragments of 16S rDNA from soil bacterial communities were PCR-amplified, using the universal bacterial primer pair 341fGC/534r. Different size 18S rDNA sequences were amplified from soil fungal communities, using the universal fungus-specific primer pairs NS1/FR1GC and FF390/FR1GC. Amplicons from both the bacterial and fungal communities were fingerprinted by DGGE, and bands in the fungal DGGE gels were excised and sequenced. The DGGE profiles were analysed by Bio-Rad Quantity One™ Image analysis software, with respect to band number, position, and relative intensity. Statistical analyses of this data then followed.

Soil properties [organic C; pH (KCl); exchangeable acidity; total cations (ECEC); exchangeable K, Ca and Mg; and extractable P] were determined by PCA and were shown to have affected the structural and catabolic diversity of the resident microbial communities. At Baynesfield, canonical correspondence analysis (CCA) relating the selected soil variables to bacterial community structural diversity, indicated that ECEC, K, P and acidity were correlated with CCA1, accounting for 33.3% of the variance, whereas Mg and organic C were correlated with CCA2 and accounted for 22.9% of the variance. In the fungal structural diversity study, pH was correlated with CCA1, accounting for 43.8% of the variance, whereas P, ECEC and organic C were correlated with CCA2, and accounted for 30.4% of the variance. The RDA of the catabolic diversity data showed that the same soil variables affecting fungal structural diversity (organic C, P, ECEC and pH) had influenced both the bacterial and fungal catabolic diversity. In both the bacterial and fungal RDAs, organic C, P and ECEC were aligned with RDA1, and pH with RDA2. However in the bacterial analysis, RDA1 accounted for 46.0%, and RDA2 for 27.5% of the variance, whereas in the fungal RDA, RDA1 accounted for only 21.7%, and RDA2 for only 15.0% of the variance.

The higher extractable P and exchangeable K concentrations under SC and M, were important in differentiating the structural diversity of these soil bacterial and fungal

communities from those under the other land uses. High P concentrations under M were also associated with bacterial catabolic diversity and to a lesser extent with that of the soil fungal communities under M. Similarly, the higher organic C and exchangeable Mg concentrations under KIK and NAT, possibly contributed to the differentiation of these soil bacterial and fungal communities from those under the other land uses, whereas under PF, the high exchangeable acidity and low pH were possibly influencing factors. Under W, low concentrations of P and K were noted. Other factors, such as the presence/absence and frequency of tillage and irrigation, and the diversity of organic inputs due to the diversity of the above-ground plant community, (in NAT, for example) were considered potentially important influences on the nature and diversity of the various land use bacterial and fungal communities.

At Mount Edgecombe, CCA showed that organic C and Mg had a significant effect on soil bacterial structural diversity. Organic C was closely correlated with CCA1, accounting for 58.7% of the variance, whereas Mg was associated with CCA2, and accounted for 41.3% of the variance. In the fungal structural diversity study, ECEC and pH were strongly correlated with CCA1 and accounted for 49.1% of the variance, while organic C was associated with CCA2, accounting for 29.6% of the variance. In the functional diversity studies, RDA showed that both bacterial and fungal community catabolic diversity was influenced by soil organic C, pH, and ECEC. In the bacterial analysis, RDA1 was associated with organic C and pH, and accounted for 43.1% of the variance, whereas ECEC was correlated with RDA2, accounting for 36.9% of the variance. In the fungal analysis, RDA1 was correlated with ECEC and accounted for 47.1% of the variance, while RDA2 was associated with pH and organic C, accounting for 35.8% of the variance. The retention of sugarcane harvest residues on the soil surface in the trashed treatments caused an accumulation of organic matter in the surface soil, which did not occur in the pre-harvest burnt sugarcane. This difference in organic C content was a factor in differentiating both bacterial and fungal communities between the trashed and the burnt treatments. Soil acidification under long-term N fertilizer applications caused an increase in exchangeable acidity and a loss of exchangeable Mg and Ca. Thus, as shown by CCA, a considerably lower exchangeable Mg concentration under F compared to Fo plots resulted, which was influential in differentiating the bacterial and fungal communities under these two treatments.

In the structural diversity study at Baynesfield, differences were found in bacterial community species richness and diversity but not in evenness, whereas in the fungal analysis, differences in community species richness, evenness and diversity were shown. The soil bacterial and fungal communities associated with each land use were clearly differentiated. Trends for bacterial and fungal diversity followed the same order, namely: M < SC < KIK < NAT < PF < W. At Mount Edgecombe, no significant difference ($p > 0.05$) in bacterial structural diversity was found with one-way analysis of variance (ANOVA), but two-way ANOVA showed a slight significant difference in bacterial community species richness ($p = 0.05$), as an effect of fertilizer applications. A significant difference in fungal species richness ($p = 0.02$) as a result of management effects was detected, with the highest values recorded for the burnt/fertilized plots and the lowest for the burnt/unfertilized treatments. No significant difference was shown in species evenness, or diversity ($p > 0.05$), in either the bacterial or the fungal communities.

In the catabolic diversity study at site 1, the non-parametric Kruskal-Wallis ANOVA showed that land use had not affected bacterial catabolic richness, evenness, or diversity. In contrast, while fungal catabolic richness had not been affected by land use, the soil fungal community catabolic evenness and diversity had. At site 2, the land treatments had a significant effect on soil bacterial community catabolic richness ($p = 0.046$), but not on evenness ($p = 0.74$) or diversity ($p = 0.135$). In the fungal study, land management had no significant effect on the catabolic richness ($p = 0.706$), evenness ($p = 0.536$) or diversity ($p = 0.826$).

It was concluded, that the microbial communities under the different land use and trash management regimes had been successfully differentiated, using the optimized protocols for the PCR-DGGE of 16S rDNA (bacteria) and 18S rDNA (fungi). Sequencing bands produced in the 18S rDNA DGGE, enabled some of the soil fungal communities to be identified. CLPP of the soil microbial communities using BIOLOG plates showed that, on the basis of C substrate utilization, the soil bacterial and fungal communities' catabolic profiles differed markedly. Thus, it was shown that the different land use and management practices had indeed influenced the structural and catabolic diversity of both the bacterial and fungal populations in the soil.

DECLARATION

I hereby certify that this research, unless specifically indicated in the text, is the result of my own investigations and has not been submitted for a higher degree at any other institution.

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ACKNOWLEDGEMENTS

My sincere gratitude and appreciation go to the following people and organizations:

My supervisor, Dr L. Titshall for his continued interest, assistance and advice throughout this study.

My co-supervisor, Mr. C. Hunter for his guidance, advice and support during this research.

My co-supervisor, Mr. C. Morris for his expertise in the statistical analysis of the data.

My MSc supervisor, Professor R.J. Haynes, for introducing me to soil microbial diversity.

Professor J. Hughes and the Soil Science staff and students, particularly Irene Bamé, for their help, support and friendship.

Mr. K. Govender for freely imparting his knowledge of DGGE to me and Dr G. Watson for his help and advice with some molecular aspects of this work.

Mrs. M. Brunkhorst (Genetics) for her technical assistance, the loan of equipment and for her friendship, encouragement and support.

Miss D. Fowlds (Microbiology), for her assistance, friendship and the loan of equipment.

The National Research Foundation of South Africa for partially funding this research.

The South African Sugarcane Research Institute at Mt Edgecombe for allowing me access to their experimental field trial.

The Management of Baynesfield Estate for the use of their facilities.

Finally, my deepest gratitude to my husband Mike for his forbearance, understanding, ongoing love and support and sense of humour and to my son Chris for all his help, advice and constant encouragement throughout the course of this investigation.

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LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|--------------------|--|
| AAOB | Autotrophic ammonia oxidizing bacteria |
| AM | Arbuscular mycorrhiza (fungi) |
| <i>amoA</i> | Gene coding for the subunit of ammonia monooxygenase |
| ANNs | Artificial neural networks |
| ANOVA | Analysis of variance |
| A + T | Adenine plus thymine |
| AWCD | Average well colour development |
| BAC | Bacterial artificial chromosome |
| BLAST | Basic Local Alignment Search Tool |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| CCA | Canonical correspondence analysis |
| CLPP | Community level physiological profiling |
| CT | Conventional tillage |
| DGGE | Denaturing gradient gel electrophoresis |
| c DNA | Complementary DNA |
| c rDNA | Complementary ribosomal DNA |
| 16S rDNA | Small subunit ribosomal DNA in prokaryotes |
| 18S rDNA | Small subunit ribosomal DNA in eukaryotes |
| dNTPs | deoxynucleotide triphosphates |
| DOC | Dissolved organic carbon |
| EDTA | Ethylenediaminetetra-acetic acid |
| EM | Ectomycorrhiza (fungi) |
| FAME | Fatty Acid Methyl Ester |
| FISH | Fluorescent <i>in situ</i> hybridisation |
| FYM | Farmyard manure |
| G + C | Guanine plus Cytosine |
| GE | Genetically engineered |
| <i>H'</i> | Shannon Weaver Diversity Index |
| I | Inosine |
| IGS | Intergenic spacers |
| ITS | Internally transcribed spacers |
| J | Pielou's evenness variate |
| Kb | Kilo base |

| | |
|--------------------|---|
| LSU | Large subunit |
| <i>mcrA</i> | Methyl-coenzyme M reductase gene |
| MLPs | Multilayer perceptrons |
| MPN | Most-probable-number |
| MRPP | Multi-Response Permutation Procedures |
| NCBI | National Centre for Biotechnology |
| NMS | Non-metric Multidimensional Scaling |
| NT | No-till |
| OD | Optical density |
| OM | Organic matter |
| OTU | Operational taxonomic unit |
| PCA | Principal Component Analysis |
| PCR | Polymerase Chain Reaction |
| PCR-DGGE | PCR amplification followed by DGGE fingerprinting |
| PLFA | Phospholipid Fatty Acid |
| qPCR | Quantitative PCR |
| RDA | Redundancy analysis |
| RDP | Ribosomal Database Project |
| RNA | Ribonucleic acid |
| mRNA | Messenger RNA |
| 16S rRNA | Small subunit ribosomal RNA in prokaryotes |
| 18S rRNA | Small subunit ribosomal RNA in eukaryotes |
| <i>rrn</i> | Ribosomal RNA gene |
| RT | Reverse transcription |
| RT-PCR | Reverse transcription PCR |
| S | Richness variate |
| SIR | Substrate Induced respiration |
| SOC | Soil organic carbon |
| SOM | Soil organic matter |
| SOMs | Self-organising maps |
| SSCP | Single Strand Conformation Polymorphism |
| SSU rDNA | Small subunit ribosomal DNA |
| SSU rRNA | Small subunit ribosomal RNA |
| TAE | Tris acetate EDTA (buffer) |
| TGGE | Thermal gradient gel electrophoresis |
| T-RFLP | Terminal Restriction Fragment Length Polymorphism |
| TTGE | Temporal temperature gradient electrophoresis |

LAND USE AND MANAGEMENT

ABBREVIATIONS

Site 1: Baynesfield Estate

| | |
|------------|-------------------------------|
| SC | Sugarcane (pre-harvest burnt) |
| M | Maize (conventional tillage) |
| KIK | Kikuyu pasture |
| NAT | Native grassland |
| PF | Pine plantation |
| W | Wattle plantation |

Site 2: Mt Edgecombe Sugarcane Research Institute

| | |
|--------------|--|
| TF | Green cane harvested with retention of crop residues, fertilized with N, P and K |
| TFo | Green cane harvested with retention of crop residues but unfertilized |
| BtoF | Pre-harvest burnt sugarcane with tops raked off, fertilized with N, P and K |
| BtoFo | Pre-harvest burnt sugarcane with tops raked off but unfertilized |

Chapter 1

GENERAL INTRODUCTION

Biodiversity, encompassing the variety and variability of plants, animals and microbes required to sustain an ecosystem, plays a central role in maintaining the Earth's equilibrium. Biodiversity may be evaluated at three fundamental levels of biological organisation, namely, at the genetic diversity, species biodiversity and ecosystem diversity levels (Benckiser and Schnell, 2007). Species biodiversity may be described as the number of species in a community and their relative abundance (Nannipieri *et al.*, 2003), with trophic and functional components of a community and their interrelationships being of great importance (Naeem, 2001; Tilman and Lehman, 2001). From a molecular point of view, biodiversity is the end result of evolution, which occurs when mutations become fixed in different populations (Madigan *et al.*, 2003).

A major concern worldwide is the loss of biodiversity, which has implications for the sustainability, preservation and conservation of the environment. In recent years, environmental objectives and landscape conservation have become prominent issues in response to this loss (Reidsma *et al.*, 2006). The impact of anthropogenic activities, such as agricultural practices, should also be assessed, as human activity has been identified as one of the primary driving forces of change in biodiversity (Wilson, 1988; Benckiser and Schnell, 2007). Modification and destruction of habitats inevitably result in biological impoverishment (Ehrlich, 1988). The relationship between soil biodiversity and soil function is part of a more general concern to conserve biodiversity and its role in maintaining a functional biosphere (Nannipieri *et al.*, 2003; Chen *et al.*, 2007).

Understanding the biodiversity in soil is difficult. Consequently, research on biodiversity has concentrated on above-ground ecosystems, with soil biota largely being neglected (Usher and Davidson, 2006). This is in spite of the fact that most of the Earth's undiscovered biodiversity is likely to be found in soil (Tiedje *et al.*, 1999;

Benckiser and Schnell, 2007), and of the importance of these below-ground systems to global nutrient cycling (Lynch *et al.*, 2004). Hollister *et al.* (2010) recently demonstrated the linkages between above- and below-ground communities, and showed that soil microbial communities could be altered by changes in the above-ground plant cover. Globally, research has suggested that a strong interdependence exists between the soil biota and plants, with soil microbial communities being important drivers of terrestrial ecosystem productivity (Cai *et al.*, 2010).

The classical ecological approach to describe an ecosystem is firstly to characterize community composition by identifying and enumerating the species present, and then to assign roles in ecosystem function to species or groups. Although this is appropriate for different above-ground ecosystems, it is not practical for microbial ecology because of the vast numbers of microorganisms involved (Kent and Triplett, 2002). It is now accepted that the great microbial variation responsible for a number of soil processes far exceeds previous estimates (Kelly, 2003). How this large genotypic diversity affects functional diversity and microbial ecology is still being established (Upchurch *et al.*, 2008). Consequently, accurate, reliable mechanisms to study soil microbes are required (Kirk *et al.*, 2004; Mitchell and Zuccaro, 2006).

The ultimate goal of the soil microbial ecologist is to understand the interactions of the microbiota with the environment so that predictions of the impact of change can be made, leading to better management of soil functions (Hirsch *et al.*, 2010). Traditional methods of soil analysis that involve measuring and modelling the distribution of chemical compounds and determining their transformation rates, have hitherto provided the basis for understanding soil biogeochemical processes. While this approach also demonstrates the regulatory role of microbes in these processes, it does not indicate their complex contribution to soil function (Kelly, 2003). Of necessity, therefore, new directions have evolved in soil microbiological research to provide information on both taxonomic and functional diversity within the resident microbial communities (Kennedy and Gewin, 1997; Nannipieri *et al.*, 2003; Hollister *et al.*, 2010).

There is a close relationship between ecosystem function and soil *health*, which may be defined as “the continued capacity of soil to function as a vital living system,

within ecosystem boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal and human health” (Pankhurst, 1997). Soil *quality* is essentially “the capacity of soil to function” and depends on a combination of physical, chemical and biological properties (Rahman *et al.*, 2008). Microbial and biochemical characteristics may be used as indicators of soil quality because of their central role in C and N cycling and their sensitivity to changes in the ecosystem (Nannipieri *et al.*, 2003). Changes in the structure and activity of soil microbial communities may be used as an early indicator of soil health and quality, as these communities play a vital role in the recovery of soil following a disturbance (Hill *et al.*, 2000; Yang *et al.*, 2003).

Studies of soil microorganisms have typically been conducted at the process level, examining biomass, respiration rates and enzyme activities, with less attention given to responses at community or organism level. While such approaches are important for understanding overall microbial processes and how they affect soil health, they provide little indication of qualitative community-level changes, as these processes are carried out by diverse taxa. They are also limited when describing a particular microbial ecosystem. Soil microbial community analyses should ideally include determinations of microbial biomass and diversity; microbial growth, distribution and function; and the nature of interspecies interactions (Hill *et al.*, 2000).

Nutrient transformations in soil are most efficiently quantified by using a holistic approach based on the division of the systems into pools and the measurement of the fluxes linking them. However, the nature of pools such as microbial biomass needs to be identified using molecular methods (Nannipieri *et al.*, 2003). Therefore a deeper understanding of soil communities and their activities requires exploring biological processes at organism and molecular levels, and understanding how these are controlled by soil physical, chemical and climatic factors, and by the overlying vegetation (Tiedje *et al.*, 2001; Broeckling *et al.*, 2008).

In summary, it is now recognised that a multi-faceted approach using a variety of traditional, recently-developed and novel methods is required, to understand how the diversity of life in soil influences the various functions that soil fulfils (Usher and Davidson, 2006). Accordingly, the objectives of this study are:

- 1) to establish and optimise suitable protocols to study soil bacterial and fungal communities, using both molecular and community-level physiological profiling techniques in parallel. A dual approach of this kind has not been reported previously in South Africa;
- 2) to use these methods to determine how different land uses and different management practices within a single land use, have affected the structural (genetic) and functional (catabolic) diversity of the soil bacterial and fungal communities, and to relate these findings to selected soil physicochemical properties, at two long-term agricultural experimental sites in KwaZulu-Natal, South Africa, namely, Baynesfield Estate and the South African Sugarcane Research Institute (SASRI) at Mount Edgecombe;
- 3) to determine if the results obtained using these profiling techniques support those of previous research on the study soils, which involved only culture-dependent and biochemical techniques.

The remainder of this thesis is structured as follows:

Chapter 2 presents a review of the literature covering the nature and significance of biodiversity; land use and management factors affecting soil microbial diversity, including a brief discussion of soil pollution; and, finally, some of the techniques currently used for estimating soil microbial diversity, with emphasis on those to be used in this study. Chapters 3 and 4 present the initial research conducted from 2004 to 2006, to determine the effects of different land uses and management practices on the structural diversity of the resident soil bacterial and fungal communities at the two experimental sites. Chapters 5 to 7 relate to the follow-up study from 2008 to 2010. Chapter 5 is an extension of the initial study on fungal community structural diversity to address some of the shortcomings in the initial work. Chapters 6 and 7 cover the effects of land use and management practices, on the catabolic diversity of the soil bacterial and fungal communities, at these sites. Chapter 8 presents general conclusions based on the experimental results obtained over the duration of the entire project.

Chapter 2

LITERATURE REVIEW

2.1 INTRODUCTION

The advent of nucleic acid-based molecular techniques, particularly the polymerase chain reaction (PCR) has revolutionised the study of soil microbial ecology (Mitchell and Zuccaro, 2006; Hirsch *et al.*, 2010). In recent years, microbial ecologists have increasingly changed to molecular strategies to study the distribution and activity of environmental microorganisms (Liu *et al.*, 2006). Remarkable discoveries have been made in studies of microbial biodiversity, using techniques based on analyses of small subunit ribosomal RNA sequences (Forney *et al.*, 2004). These innovative molecular approaches have provided insight into the relationship between natural microbial communities and the functional genes responsible for soil biogeochemical processes (Hill *et al.*, 2000; Kelly, 2003). However, methodological limitations and a lack of taxonomic knowledge can cause problems when studying bacterial and fungal diversity in soil (Kirk *et al.*, 2004).

While DNA-based molecular methods can be used to determine microbial genetic diversity and biochemical methods such as phospholipid fatty acids (PLFA's) to assess microbial community structure, these techniques are complicated, time-consuming and not always suitable for routine analysis of large numbers of soil samples. A relatively simple technique to characterise potential microbial function is to generate community-level physiological profiles (CLPP) (Garland and Mills, 1991; Chen *et al.*, 2007). To understand the role of microbial communities in the soil it is vital to consider microbial functional diversity and also how soil disturbances may change this diversity (Calbrix *et al.*, 2005). Combined genetic and physiological assessments of the soil microbial community are needed for accurate determination of the effects of disturbances such as land use and management, on indigenous microbial systems (Cullings *et al.* 2005). However, as all the methods for investigating microbial activity and diversity have inherent biases, an understanding of the

underlying mechanisms is needed, before the strengths and weaknesses of each approach can be assessed (Hirsch *et al.*, 2010).

This chapter examines the nature and significance of biodiversity as well as current information on the influence of land use and soil management practices on soil microbial diversity. Some of the more important approaches for analyzing soil microbial diversity, including their advantages and shortcomings, are also reviewed. Emphasis is placed on the molecular and metabolic fingerprinting methods that are applied later in this study.

2.2 THE NATURE AND SIGNIFICANCE OF BIODIVERSITY

2.2.1 Numbers and interrelationships

Soil harbours high biodiversity with fertile surface soils typically containing several billion prokaryotes (bacteria and archaea) per gram of soil and often an equivalent fungal biomass (Tiedje *et al.*, 2001). Representatives of all groups of microorganisms, algae and almost all animal phyla are present in the soil biota and include macrofauna, mesofauna, microfauna and microflora (Pankhurst, 1997; Nannipieri *et al.*, 2003). Estimates of the number of species in some of the groups are (Pankhurst, 1997):

- bacteria: 30 000;
- fungi: 1 500 000;
- algae: 60 000;
- nematodes: 500 000; and
- earthworms: 3 000.

Other invertebrates commonly found in soil in large numbers include ants, termites, collembola and mites (Pankhurst, 1997; Benckiser and Schnell, 2007). The size of individual organisms in the soil varies greatly, ranging from the smallest bacterium with a diameter of $< 1 \mu\text{m}$ and a mass of 10^{-12} g, to the largest earthworm with a length of > 1 m, a diameter of > 20 mm and a mass of > 500 g. In fertile soils, organism biomass may exceed 20 t ha^{-1} (Pankhurst, 1997).

Species interactions at the community-level in soil are complex, with individual species relying on the presence, function and interaction of many other species (Hill *et al.*, 2000). The various interactions and linkages between the components of the soil-biota are vital for regulating nutrient cycling and energy flow and may be described as a detritus food web (Beare *et al.*, 1995; Haynes and Graham, 2004). From a functional standpoint, the soil detritus food web consists of primary decomposers (bacteria and fungi); herbivores (e.g. plant parasitic nematodes); consumers of bacteria and fungi (e.g. protozoa, bacterivorous and fungivorous nematodes and collembola); saprophagous mesofauna and macrofauna; and predators (e.g. predacious nematodes and mites) (Pankhurst, 1997). The base of the web is compartmentalised with bacteria and fungi as the primary decomposers at the first trophic level. The bacteria-based channel, involving bacteria with protozoa and bacterivorous nematodes from the second trophic level, requires a water film for growth. Conversely, the fungi-based channel, involving fungi, mites and collembola, does not require a continuous water film. The third trophic level contains common consumers such as predatory nematodes and mites (Rosenzweig, 1995; Haynes and Graham, 2004).

An example of the complex interactions that occur in simple bacteria-bacterivore food chains are changes in nitrogen (N) mineralization rates, which can be attributed to stimulatory and competitive interactions between bacterivorous fauna. Soil microfauna, such as protozoa and nematodes, directly affect biogeochemical cycling by feeding on and assimilating microbial tissue and excreting mineral nutrients. Soil bacterial communities are heavily grazed by bacteriophagous microfauna, with the composition of protozoan populations in soil significantly influencing the grazing of bacterial communities. Interacting mechanisms, such as selective feeding, differences in bacterial susceptibility to predation, and availability of bacterial nutrients and substrates, all have an effect on bacterial community structure (Rønn *et al.*, 2002). Similarly, fungal populations are grazed by mites, collembola, and in particular, fungivorous nematodes (Coleman and Crossley, 1996). Predation of bacteria and fungi by nematodes and protozoa is especially important in regulating microbial biomass, with bacterivorous protozoa and nematodes involved in the release of ammonium-N, with an associated improved N-uptake by plants. Meso- and macrofauna also have a direct effect on biogeochemical cycling by enhancing nutrient

mineralization, by comminution of plant debris, and via faecal deposition for subsequent microbial attack (Beare *et al.*, 1995).

2.2.2 Diversity of habitats

Soils are the most complex of microbial habitats as the characteristics of solid soil components are spatially and temporally variable. This influences the physical and chemical properties of soils, thus affecting both plant and microbial growth (Marshall, 1976). Soil differs from other habitats in that it possesses a solid phase comprising approximately half the soil's volume and consisting of particulate matter of varying size, which can bind biological molecules (Nannipieri *et al.*, 2003). The remainder of the soil volume consists of pores filled with air and water. The amount of pore space depends on the texture, structure and organic matter content of the soil, with individual pore size, total pore space and pore continuity affecting water movement and retention (Alexander, 1977).

The most important interfaces affecting microbial behaviour in natural habitats are those of the solid-liquid type. Moisture availability at this interface can limit the movement of soil microorganisms (Marshall, 1976), with water and air movement regulating the activities of the microflora. Bacteria are rarely free in the liquid phase of soil, with most cells (approximately 80–90%) adhering to solid surfaces such as clay particles and humus (Alexander, 1977). Soil microhabitats are dynamic systems since environmental factors are constantly changing (Nannipieri *et al.*, 2003). Within soil, several microhabitats exist such as the rhizoplane, the rhizosphere, aggregates, decaying organic matter and the bulk soil itself (Lynch *et al.*, 2004).

Nannipieri *et al.* (2003) described soil as a structured, heterogeneous, discontinuous system that is generally poor in energy sources and nutrients (compared with nutrient concentrations required for optimal *in vitro* microbial growth) where microbes occupy discrete microhabitats. Although available space in soil is extensive, less than 5% is generally occupied by living microorganisms (Alexander, 1977). The heterogeneous nature of soil results in the presence of so-called 'hot spots' (micro-sites) or areas of increased biological activity, where microflora and fauna are concentrated because

conditions in relatively few microhabitats are suitable to sustain microbial life (Giller *et al.*, 1997).

The concept of three levels of diversity, namely: α -diversity, which distinguishes between species within the community of a habitat; β -diversity, or the rate and extent of species change along habitat gradients; and γ -diversity, or species richness over a range of habitats, (i.e. total biodiversity in a landscape), was proposed by Whittaker (1972). Gamma-diversity is a function of α -diversity of habitats and the differences in β -diversity between them (Giller *et al.*, 1997; Lynch *et al.*, 2004). Although this approach may be useful for above-ground ecosystems, it is inadequate for interpreting soil diversity as the soil biota are also characterised by spatial diversity. This includes differences between bulk soil and the rhizosphere, macro- and microaggregates, macro- and micropores and different soil horizons (Lynch *et al.*, 2004).

Soil organisms can change the physical, chemical and biological properties of soil in innumerable ways, with the composition and structure of the biota at one hierarchical level influencing the spatial heterogeneity at another level. Consequently it was proposed that soil could be regarded as possessing at least five zones of concentrated activity (Beare *et al.*, 1995). These spheres of biotic activity with their various incumbent organisms interact in many different ways (Haynes and Graham, 2004). They include:

1. the *detritosphere*, or the zone of recognisable plant and animal detritus undergoing decay, where litter and humus above the soil surface show considerable saprophytic, mycorrhizal and root fungal activity and colonisation by fungivorous fauna;
2. the *drilosphere*, or the zone influenced by earthworms by means of their nitrogenous waste excretions and mucilage;
3. the *porosphere*, or the milieu occupied by organisms ranging from bacteria, protozoa, and nematodes inhabiting water films, to microarthropods, fungal mycelia and larger soil biota occupying channels between aggregates;

4. the *aggregatusphere*, or the area encompassing all the organic matter constituents, primary particles and voids, where microbial activity is concentrated in the interstices between and within macroaggregates often in association with particulate organic matter; and
5. the *rhizosphere*, or the zone of primary root influence by means of exudates and exfoliates (Beare *et al.*, 1995), where the numbers and types of microbes are greater than in the adjacent soil (Marshall, 1976). Rhizospheres are dynamic environments where microorganisms compete for resources such as plant-derived organic carbon/energy sources (Piceno and Lovell, 2000) and where plants have a strong influence on soil microbial communities by rhizodeposition and the decay of litter and roots (Nannipieri *et al.*, 2003; Maharning *et al.*, 2009). The population in the rhizosphere is composed primarily of non-pathogenic organisms. However, because of the density and increased microbial interactions (competitive, antagonistic and beneficial) in this zone, the elimination or suppression of pathogens may result (Alexander, 1977).

The composition of the microflora of any habitat is controlled by the biological equilibrium resulting from the association and interaction of all individuals within a population. Environmental changes may temporarily upset this equilibrium although it will be re-established as the population shifts to accommodate the new circumstances. In soil, many microbes occur in close proximity and interact in a unique way. The combination of all these individual interactions, establishes the equilibrium population in a given habitat (Alexander, 1977). As no single organism can respond to all environmental contingencies, the assemblage of microbial populations in a typical ecosystem most likely contains a mixture of 'generalists' and 'specialists'. This results in an overall community sensitivity to environmental conditions (Balser *et al.*, 2001).

Soil macrofauna, including earthworms and termites, have direct effects on nutrient cycling by vertical and horizontal redistribution of plant litter, which creates patches of organic matter that act as substrates for microbes and fauna. Some organisms reduce structural and functional diversity in soils by fragmentation of the mosaic of

patches into a relatively homogeneous mixture. By physically rearranging soil particles, the macrofauna change pore size and pore distribution, and consequently patterns of infiltration and gaseous emission (Beare *et al.*, 1995). It is apparent, therefore, that soil is both a heterogeneous medium and a dynamic, ever-changing habitat.

2.2.3 Biodiversity and productivity

The general link between biodiversity and productivity (the rate at which energy flows in an ecosystem, measured at various ecosystem levels) has long been disputed. Initially it was thought that increased productivity would raise diversity but it can have the opposite effect (Rosenzweig, 1995). Thus, loss of biodiversity can lead to changes in ecosystem functioning and a resultant loss of productivity, although no direct causal link has been shown in this frequently made assumption (Giller *et al.*, 1997). Many ecologists believe productivity to be one of the most pervasive influences on biodiversity (Rosenzweig, 1995). When the number of interacting species increases, the stability of the entire system decreases, unless special conditions are met (Lynch *et al.*, 2004). A key issue remains, however: is the loss of biodiversity in the environment of significance?

Most studies of the relationship between biodiversity and productivity are based on plant and animal communities (Rosenzweig, 1995). Little information is available regarding the contribution of microbial diversity to soil ecosystem functioning (Crecchio *et al.*, 2004). This is due to the difficulty of determining the functional role of microorganisms in soil (Nannipieri *et al.*, 2003). An example of productivity affecting plant/microbial interaction is seen in the rhizosphere, where changing plant productivity alters the availability of carbon/energy resources to rhizosphere microbiota. This intensifies competition for the remaining carbon, which can result in the loss of poor competitors from the assemblage (Piceno and Lovell, 2000). Earlier studies showed that, in above-ground systems, on a small scale, increasing nutrients increased productivity but decreased diversity, whereas on a large scale, diversity initially increased and then declined after productivity increased beyond a certain threshold (Rosenzweig, 1995). This contradicted the hypothesis that diversity always

grows with productivity. It is now recognised that in the increase phase of the productivity-diversity pattern, as productivity rises from very low to moderate levels, diversity also rises, whereas in the decrease phase, enhanced productivity tends to reduce the number of species. However, for species that have traits that allow them to coexist when exploiting and competing for limited resources, the competitive mechanisms that allow coexistence also cause greater diversity, leading to greater productivity, greater use of limiting resources and greater stability (Rosenzweig, 1995; Tilman and Lehman, 2001).

After a disturbance such as fire or agricultural practices, plant and animal species reoccupy the habitat and replace each other in a successional sequence. Succession is often associated with changes in the above-ground species diversity and in the first few years thereof, a rise in plant diversity (Rosenzweig, 1995). During grassland succession, secondary succession correlates with changes in community composition of the dominant soil bacteria (Kowalchuk *et al.*, 2000).

2.2.4 Microbial diversity and soil function

One of the roles of biodiversity is to ensure the continuation of the functions mediated by soil microbes, although it is uncertain whether links exist between the rate and efficiency of these functions and microbial biodiversity. Another role is to ensure the continuation of these functions during disturbances. It is assumed that the greater the degree of biodiversity (between or within species or functional groups), the greater the resistance or tolerance to stress or perturbations. This may not be indicative of taxonomic diversity, as a single organism may perform many functions, or alternatively, a large number of organisms may perform a particular function (Giller *et al.*, 1997). The assumption that a variety of microorganisms can perform a specific function implies that functional redundancy exists. However, functionally similar organisms have different microhabitat preferences, environmental tolerances and physiological requirements, and therefore probably play different roles (Beare *et al.*, 1995; Giller *et al.*, 1997). As previously stated, microbial diversity includes genetic diversity, diversity of bacterial and fungal communities and ecological diversity. In general, however, a reduction in a group of species has little effect on the overall

processes in soil because other microbes take over the discontinued function. As a result, no general relationship has been found between microbial diversity and soil function (Nannipieri *et al.*, 2003).

Numerous factors affect diversity, including negative effects such as stress (e.g. toxins, drought or limited nutrients), or positive effects such as resource and habitat diversity or biological interactions. The positive effects may be related to increased stability, resilience, productivity and resistance to stress (Nannipieri *et al.*, 2003; Lynch *et al.*, 2004). Those microorganisms that have greater resistance to a particular stress will become predominant in the presence of that stress. However, if the rate of genetic adaptation to the stress is rapid, then newly resistant individuals may arise that mask the effect (Giller *et al.*, 1998). Progressive extinction of sensitive species within a functional unit occurs as stress is imposed on the soil system, although no loss of overall function is apparent until a threshold is attained below which there are insufficient individuals to conduct the specific process. Accordingly, functions sensitive to disruption will be those carried out by a limited number of species (Giller *et al.*, 1997). However, Wertz *et al.* (2006), in a study on the maintenance of soil function following a reduction in microbial diversity, used a removal approach in which less abundant species were preferentially removed. They demonstrated that the vast diversity of the soil microbiota made soil ecosystem functioning largely insensitive to diminished biodiversity, even in the case of functions performed by specialised groups.

It was proposed by Schimel (1995) that ecosystem processes could be based on broad or narrow physiological groups of microbes, as the role played by the microbial community in ecosystem functioning was related to the characteristics of the responsible population. 'Broad' processes, such as soil respiration, simple carbon compound mineralization and nitrogen immobilization, require the metabolic capacities of many apparently redundant communities (e.g. most microbes utilize simple carbon substrates and all use nitrogen for biosynthesis). These processes hardly vary within or between systems and will be least influenced by stress. 'Narrow' processes, on the other hand, such as nitrification, the production and consumption of trace gases, degradation of lignin, and biological N₂ fixation, are performed by restricted groups of organisms. Here, changes in abundance or

characteristics of appropriate communities will markedly affect soil processes (Balser *et al.*, 2001).

Many anthropogenic activities can potentially affect soil microbial diversity (Kirk *et al.*, 2004). Investigations using molecular methods revealed that total microbial diversity in relatively undisturbed and unpolluted soil was high but after perturbation and pollution, the total soil microbial diversity was dramatically reduced. However, microcosm investigations indicated that some population types became numerically dominant under stress (Tiedje *et al.*, 2001). Lower levels of microbial diversity, biomass and altered community structure have been reported, due to disturbance caused primarily by pesticides, heavy metals and sludge amendments with strains of microorganisms showing differences in sensitivity to metal toxicity (Crecchio *et al.*, 2004). Studies of heavy metal pollution effects on soil microbial community diversity have shown that total microbial biomass decreases under chronic heavy metal stress, preceded by community structural changes, and a reduced ability to exploit all available niches. This may alter the potential of the soil microbial community to respond to new stresses. It has also been observed that under long-term metal pollution, the genetic structure of the soil microbial population changes (Giller *et al.*, 1998).

2.2.5 Indices of diversity

Information on diversity is often reduced by researchers into discrete, numerical measurements such as diversity indices (Kirk *et al.*, 2004), in order to describe the diversity, irrespective of how it was determined (Pankhurst, 1997). Biodiversity is a function of two components, namely: (i) the total number of species present (i.e. species richness or abundance); and (ii) the distribution of individuals among the species (i.e. species evenness or equitability) (Bohannan and Hughes, 2003; Kirk *et al.*, 2004). In consequence, many attempts have been made to devise a single numerical index that measures both these properties (Gotelli and Graves, 1996). However, any given diversity index is a single value, which cannot indicate the total composition of a community (Bohannan and Hughes, 2003). A major problem is that since both richness and evenness play a role in determining the index value, different

communities can have the same index. Therefore, richness, evenness and diversity values should all be calculated when assessing community structure (Pankhurst, 1997; Bohannan and Hughes, 2003). A further aspect of community diversity is the genetic relatedness of the operational taxonomic units (OTUs) present (Bohannan and Hughes, 2003). Accurate assessment of the level and patterns of genetic diversity is essential (Mohammadi and Prasanna, 2003). Several approaches to compare microbial diversity at the molecular level have been used, including parametric estimation, nonparametric estimation and community phylogenetics (Bohannan and Hughes, 2003).

2.2.5.1 Species richness

The easiest and simplest diversity index to interpret is species richness, as measured by a direct count of species. These counts are influenced by a combination of species richness, the total number of individuals counted and the size of the area sampled. Therefore, unless communities are exhaustively and identically sampled, comparison of simple species counts is not appropriate. For comparing communities of different sizes, algorithmic rarefaction of large samples that subsequently can be compared with smaller samples, is one solution to the problem. However, rarefaction can only be used for interpolation to a smaller sample size and not for extrapolation to a larger sample size (Gotelli and Graves, 1996). As an alternative, numerous species-individuals relationships have provided bases for richness indices, such as the logarithmic relationship suggested by Margalef in 1958:

$$R_1 = (S - 1) / \log N \dots\dots\dots \text{Equation 1}$$

where S is the number of species and N is the total number of organisms (Pielou, 1977).

2.2.5.2 Species evenness

As diversity depends on both the number of species in a community and the evenness of their representation, it is sometimes necessary to treat the two components

separately (Pielou, 1977). To calculate evenness, the most frequent approach is to scale a heterogeneity measure relative to its maximum possible value when both sample size and species number are fixed. These two formulations for large samples are:

$$\text{Evenness} = (D - D_{\min}) / (D_{\max} - D_{\min}) \dots \text{Equation 2}$$

$$\text{Evenness} = D/D_{\max} \dots \text{Equation 3}$$

where D is a heterogeneity value for the sampled population and D_{\min} and D_{\max} are the minimum and maximum values possible for the given species number and sample size (Peet, 1974).

2.2.5.3 Species diversity

Many indices have been developed, which are favoured by ecologists for measuring diversity (Pielou, 1977). To measure heterogeneity (dual concept diversity), two contributing components are required, the number of species, and the distribution of individuals among the species, or equitability (Peet, 1974). The first heterogeneity index used in ecology was that of Simpson (Pielou, 1977). This index measures the probability that two individuals, randomly selected from a sample, will belong to the same species. For an infinite sample the index is:

$$\lambda = \sum_{i=1}^s p_i^2 \dots \text{Equation 4}$$

For a finite sample the index is:

$$L = \sum [n_i(n_i - 1)]/[N(N - 1)] \dots \text{Equation 5}$$

where p_i is the proportion of individuals in species i , s is the number of species (species richness), n_i the number of individuals in species i , and N the total sample size. As originally formulated, Simpson's index varies inversely with heterogeneity (Peet, 1974). The Simpson index and the Shannon index are closely related but unlike

the Shannon index described below the former cannot be adapted for measuring hierarchical diversity (Pielou, 1977).

The Shannon diversity index is the most commonly used index for measuring species diversity or heterogeneity, with the most popular of the heterogeneity indices being those based on information theory, such as:

The Shannon-Wiener index:

$$H' = -\sum p_i \ln(p_i) \dots\dots\dots \text{Equation 6}$$

where p_i is the relative abundance of the i th species, ($\sum p_i = 1.0$) (Gotelli and Graves, 1996).

The Shannon-Weaver index:

$$H' = -\sum_{i=1}^s p_i \log p_i \dots\dots\dots \text{Equation 7}$$

where p_i is the relative abundance of each species, calculated as the proportion of individuals of a given species, to the total number of individuals in the community, and s is the number of species (species richness) (Peet, 1974).

The Shannon-Weaver index (H') is often used, in the form:

$$\begin{aligned} H' &= -\sum (n_i/N) \log (n_i/N) \\ &= -\sum p_i (\log p_i) \dots\dots\dots \text{Equation 8} \end{aligned}$$

where n_i is the importance value for each species, N is the total of importance values and p_i is the importance probability for each species (n_i/N). Thus H' takes into account both the 'richness' and 'evenness' component of diversity (Lynch *et al.*, 2004).

However both these indices have their limitations, which include serious conceptual and statistical problems (Gotelli and Graves, 1996; Kennedy and Gewin, 1997; Pankhurst, 1997).

The application of information theory to diversity measurement suggests that heterogeneity (or a combination of richness and equitability) can be equated with the amount of uncertainty regarding the species of an individual selected at random from a population. The more species there are and the more even their distribution, the greater the diversity (Peet, 1974; Pielou, 1977).

An alternative approach to conventional statistical methods for environmental studies is the use of artificial neural networks (ANNs) (Dollhopf *et al.*, 2001; Kim *et al.*, 2008). Two different ANN algorithms which are useful in ecological informatics are self-organizing maps (SOMs) and multilayer perceptrons (MLPs). These were used successfully to model convective flows and the associated oxygen transport in a wetland pond. These models were able to 'learn' the mechanism of convective transport, resulting in an ability to forecast oxygen saturation near the bottom of the wetland bed (Schramm *et al.*, 2003). In a different study, SOMs and MLPs were efficient at revealing community associations and environmental effects in an inter-taxa study of microbes and benthic macroinvertebrates subjected to different pollution levels in a stream (Kim *et al.*, 2008).

2.3 FACTORS AFFECTING SOIL MICROBIAL DIVERSITY

Many factors are believed to affect diversity. One of the most important anthropogenic activities having an impact on soil and consequently on soil microbial diversity, is agriculture (Upchurch *et al.*, 2008). Current information on the effects of land use and management practices on soil microbial diversity, determined using molecular methods is now reviewed.

2.3.1 The rhizosphere

The rhizosphere is an extremely favourable habitat for the metabolism and proliferation of numerous microbes. The influence of roots on these microorganisms is termed the *rhizosphere effect*, which is the enhancement of microbial numbers relative to the surrounding soil. Populations a short distance away from roots are not markedly affected by plants (Alexander, 1977). The species activity and composition

of the plant community critically determine the structural and functional diversity and biomass of the microbial species, as most soil microorganisms depend on autotrophic organisms, including bacteria and plants, for a carbon supply (Johnson *et al.*, 2003a). Plants in turn are dependent on the soil microbial community. This relationship is often highly specific and is mediated by chemical communication such as that in legume/rhizobial symbioses (Broeckling *et al.*, 2008).

2.3.1.1 Rhizosphere communities

As land use and management are known to affect the rhizosphere, Smalla *et al.* (2001) determined how dependent the rhizosphere effect was on plants and whether this effect increased by growing the same crop over two consecutive years. They found plant-dependent shifts in the abundance of bacterial rhizosphere communities that were more apparent in the second year, and seasonal shifts in the abundance and structure of these communities in both years. In bulk soils, *Bacillus megaterium* and *Arthrobacter* sp. predominated, whereas in the rhizosphere, the proportion of gram-positive bacteria increased. Evenness in the rhizosphere was reduced compared to the bulk soil.

Conflicting reports regarding the relative importance of the effects of soil type or plant species in determining rhizosphere bacterial community composition, have prompted several studies. Marschner *et al.* (2001) analysed soil- and plant-specific effects on the abundance and diversity of bacterial communities in the rhizospheres of chickpea (*Cicer arietinum*), rape (*Brassica napus*) and Sudan grass (*Sorghum bicolor*). Both soil type and nitrogen fertilization affected plant growth but nitrogen had no significant effect on the bacterial population. It was concluded that complex interactions of soil type, plant species and root zone location influenced rhizosphere bacterial population structure.

In contrast, Miethling *et al.* (2000) considered that crop species was the main determinant of microbial population characteristics with soil having only a minor effect. This conclusion was based on the comparison of the effects of alfalfa (*Medicago sativa*) and rye (*Secale cereale*), soil origin and inoculation with

Sinorhizobium meliloti strain L33, on the establishment of rhizobial communities. In a subsequent study, however, Miethling *et al.* (2003) found that both soil type and plant species affected structural diversity in the rhizosphere communities of three legumes, namely; alfalfa (*M. sativa*); common bean (*Phaseolus vulgaris*) and clover (*Trifolium pratense*). In the same soil, significant differences were found in the composition of leguminous rhizosphere communities and plant-specific organisms. Dominant alfalfa rhizosphere populations differed in two soils with distinct agricultural histories, and the three leguminous rhizosphere populations could be differentiated.

Anthropogenic disturbance has drastically altered the composition and productivity of plant communities in the arid land ecosystem of the Colorado (USA) plateau grasslands. Kuske *et al.* (2002) made comparisons at different depths of rhizosphere bacterial communities of the native bunchgrasses *Stipa hymenoides* and *Hilaria jamesii*, the invading annual grass *Bromus tectorum* and of interspaces colonised by cyanobacterial soil crusts. A significant difference was found in the total bacterial population structure and in the *Acidobacterium* division between the soil crust interspaces and the plant rhizospheres, with large differences also seen among the three rhizospheres, particularly in the *Acidobacterium* analysis. It was shown that soil depth in plant rhizospheres as well as in the interspaces affected both the total bacterial community and bacteria from the *Acidobacterium* division, with different members of this division occupying specific niches in the grassland soil.

The effects of genetically engineered (GE) crops on agricultural practice, human health and the environment were studied by Schmalenberger and Tebbe (2003). Bacterial community diversity in the rhizosphere of a transgenic, herbicide (glufosinate)-resistant sugar beet (*Beta vulgaris*) was compared with that of its non-engineered counterpart. Differences in community composition due to field and annual variability were evident but there was no detectable effect of transgenic herbicide resistance on the microbial community. In a similar study, Heuer *et al.* (2002) investigated the possibility that GE plants could change rhizosphere bacterial consortia through transgenic T4 lysozyme release or by a change in root exudate composition, and thereby change agroecosystems. Bacterial populations from transgenic potato rhizospheres were compared with those of wild-type plants and non-lysozyme producing transgenic controls. The authors found environmental factors

such as season, year and field site influenced the rhizosphere populations but T4 lysozyme expression by GE plants did not.

As little is known of the role of fungi in the rhizosphere, Gomes *et al.* (2003) investigated fungal communities in bulk and maize rhizosphere soil of two maize cultivars, differing in N utilization. A rhizosphere effect for fungal communities at all stages of plant development was observed, with marked changes in fungal population composition during plant growth. In young maize plant rhizospheres, ascomycetes of the order Pleosporales were selected for, whereas in senescent maize rhizospheres, different members of ascomycetes and basidiomycetic yeasts were found.

At the community level, fungal community response to plants is less well documented than that of bacteria. To clarify the role of plants and root exudates in the structuring of soil fungal communities, Broeckling *et al.* (2008) investigated the effect of a novel plant species on an existing soil fungal community and also the relative importance of root exudates in structuring this community. Their results showed that the two study plant species (*Arabidopsis thaliana* and *Medicago truncata*) could maintain resident soil fungal populations but not non-resident populations and that this was mediated largely through root exudates. They concluded that root exudates were a mechanism by which plants regulate soil fungal community composition.

The indigenous arbuscular mycorrhizal (AM) community in the rhizosphere of maize (*Zea mays*) genotypes with contrasting phosphorus uptake efficiency was investigated by Oliveira *et al.* (2009). They showed that the maize genotypes had a greater influence on the rhizosphere mycorrhizal community than soil P levels. Some mycorrhizal groups were found only in the rhizospheres of P-efficient maize genotypes cultivated in low P soils and more mycorrhizal OTUs were present in no-till (NT) maize than in maize under conventional tillage (CT).

2.3.2 Effects of land use

Changes in the soil environment resulting from agricultural land use or management practices, directly influence soil microbial diversity and activity (Jangid *et al.*, 2008).

Understanding the effects of these perturbations on soil physicochemical and microbiological properties can provide essential information for assessing sustainability and environmental impact (Rahman *et al.*, 2008). Changes in land use and land cover often alter ecosystem structure and function, which, in turn, affect above- and below-ground processes and contribute to global change (Hollister *et al.*, 2010). When several land use-biodiversity loss gradients were compared, it was shown that ecosystem quality decreases as agricultural practices intensify (Reidsma *et al.*, 2006).

Changes in land use, such as from forests to pasture, can have significant and long-lasting effects on soil nutrients, carbon content, soil texture and pH. These effects are largely as a result of changes in the composition of plant species and associated management practices across land use types (Lauber *et al.*, 2008). In addition, applications of chemical fertilizers have been reported to change soil bacterial community structure significantly (Wang *et al.*, 2008). It has also been observed that tillage, over-grazing and pollution, which reduce aboveground plant diversity, cause microbial variation to decrease (Kennedy and Gewin, 1997). Removal of crop plants, which results in plant-induced changes in the soil microbial community, has been shown to affect the follow-on crop (Heuer *et al.*, 2002).

Soil has a considerable capacity for diversity and hence a great buffering capacity before the effects of management practices on dominant community members are seen (Girvan *et al.*, 2003). By understanding how agricultural land use and management practices may alter soil quality and affect the diversity of soil microbial populations, lower-input sustainable systems may be developed (Purkhold *et al.*, 2000; Rahman *et al.*, 2008).

2.3.2.1 Arable agriculture

Land degradation reduces the productive capacity of arable agricultural soils causing a loss of biodiversity. However, land use management practices, such as tillage methods, crop residue management, application of fertilizers and manures, can help minimise this deterioration (Girvan *et al.*, 2003).

Research evidence suggests that the microbial community under long-term arable soils differs from that under grasslands. To verify this, Garbeva *et al.* (2003) studied the diversity of *Bacillus* spp and related taxa in agricultural soil under three different management regimes, namely, short- and long-term arable land, and permanent grassland. The effects on the structure and diversity of *Bacillus* communities were marked, with samples from permanent grassland and those from short-term arable land having similar profiles. Samples from long-term arable land showed significant differences to those from short-term arable land as some grass remained in the latter, enhancing diversity in the short-term. The authors concluded that the long-term presence of grass in soil supported and maintained greater numbers and a higher diversity of *Bacillus* and related taxa than did long-term arable land.

Other research has suggested that within arable soils, the main factor affecting community composition is soil type rather than crop management. In a study by Wakelin *et al.* (2008a), habitat selective factors in agricultural soils influencing the structural composition and functional capacity of the autochthonous microbial communities were investigated. The authors characterized soils from seven field sites differing in long-term agricultural management regimes, by assessing the relationship between soil physicochemical properties and community structure and potential catabolic functions of both the soil bacteria and fungi. They concluded that soil type and not agricultural management practice was the key determinant of microbial community structure and catabolic function, with pH a primary driver of both microbial diversity and function in these soils. Thus agricultural practices had the effect of selectively shifting microbial populations and functions.

The major sources of substrate for microbial growth and activity in arable soils are litter and crop plant residues. Dilly *et al.* (2004) investigated bacterial diversity in agricultural soils during litter (crop residue) decomposition. Rye and wheat litter were buried in comparable soil types under different vegetation and exposed to different climates. Bacterial diversity increased with advancing litter decomposition and with decreasing substrate quality resulting from the loss of readily available carbon and the accumulation of refractory compounds. At different locations containing the same buried litter, differences in bacterial diversity were observed, indicating that climate,

vegetation and indigenous soil microorganisms, in addition to litter type, affected bacterial community development.

Reports that plants are important in regulating net decomposition rates of litter by directly affecting the quality thereof, led to research by Trinder *et al.* (2009). They investigated how plant cover and litter type affected fungal community structure and litter decomposition in a cutover peatland. Results showed that plant species did not affect fungal community structure but most litter types had a significant effect. The quantities of carbon entering the soil via rhizodeposition were insignificant for regulating the activity and diversity of the litter-degrading soil microbes in the peatland. The chemistry of the litter produced by the peatlands had a strong and more varied effect on both decomposition and fungal community structure. They concluded that the initial decomposition of litter and also the structure of the soil fungal community were regulated by the litter type and not by the plant cover.

The effects of stubble retention and applications of nitrogen fertilizer on functional gene abundance and the structure of dominant soil microbial communities under irrigated maize, was studied by Wakelin *et al.* (2007). Both stubble-retention and N addition had significant and long-term effects on microbial community structure and size, with stubble-retention being the strongest driver affecting species composition, particularly in the case of the fungal community. However, diversity estimates were little affected. The authors concluded that the sustained shifts in the size and structure of the soil microbial communities and the overall changes in N-based functional genes could have an impact on ecosystem function and on the productivity of subsequent crops.

The addition of mulches is a management practice that can be used to protect and add organic matter to the soil surface, as well as to conserve soil water. Yang *et al.* (2003) investigated the influence of different organic mulches on soil bacterial communities a year after application. Results showed that the long-term effect of organic mulches on the activity and structure of soil microbial populations depended on the type of mulch and was evident only in the top few centimetres of the soil profile. None of the mulches affected the species composition of the chemolithoautotrophic ammonia-oxidizing bacteria (AAOB). The authors concluded that in the long-term, repeated

mulch applications might result in changes in the microbial community and in species diversity because of the increase in soil organic matter content.

To increase crop production, fertilizer applications are a common and important agricultural practice. However the effects of fertilizer on soil microbes, vital to agroecosystem health as residue decomposers and cyclers of nutrients, are not fully understood. Attempts to devise sound land management strategies led to a study by Ge *et al.* (2008) of the effects of long-term applications of inorganic and organic fertilizers on soil microbial communities under maize rotated with wheat. They showed that long-term fertilization regimes affected the structure and diversity of the microbial communities in the agricultural soils. The bacterial community structure in organic manure (OM) and phosphorus/potassium (PK) amended soils had a higher richness and diversity than those of the unfertilized control and the N-containing fertilizer combinations (NK, NP, NPK and $\frac{1}{2}$ NPKOM). They suggested that N-fertilizer could be a key factor that countered the effects of other fertilizers on soil microbial communities.

Inorganic fertilizers and organic manures are routinely applied to arable soils to replace nutrients removed by the harvested crop. Organic manures also add organic carbon which acts as a microbial substrate. Marschner *et al.* (2003) studied the effects of long-term applications of organic and inorganic fertilizer at low rates, on the chemical and biological properties of soil. They concluded that these amendments significantly changed soil chemical properties, and that ratios of Gram positive to Gram negative bacteria and of bacteria to fungi, were higher in organic treatments than in inorganic treatments. Different amendments influenced bacterial and eukaryotic community composition through their effect on the organic C content and C/N ratio in the soil. Dissolved organic carbon (DOC) concentration was also shown to influence bacterial community structure.

Several researchers have used the particle-size fractionation technique (Sessitsch *et al.*, 2001; Poll *et al.*, 2003) to investigate the effects of manures and fertilizers on microbial activity and diversity. Poll *et al.* (2003) studied the effect of long-term farmyard manure (FYM) additions in several particle-size fractions of soil, by comparing soil fertilized with FYM and an unfertilized control. Coarse sand fractions

were colonised by a fungal-dominated community, together with a simple bacterial community, but after applications of FYM, bacteria dominated. On the other hand, silt and clay fractions were colonised by complex bacterial communities. The addition of FYM increased organic matter content, total microbial biomass and enzyme activity in sand, whereas microbial communities in finer fractions were less influenced.

Concern over agricultural sustainability, arising from soil erosion and fertility decline, in the densely populated and intensively cropped subtropical highlands of the world, led to a study by Govaerts *et al.* (2007). They assessed the long-term effects of tillage/seeding practices, crop rotation and crop residue management on maize and wheat, grown under rainfed conditions. The authors concluded that, compared to common farming practices, cropping systems that included zero tillage, crop rotation and residue retention increased overall microbial biomass, activity and diversity. However, long-term zero tillage without residue retention was an unsustainable practice that led to poor soil health.

Most economically important field crops, such as maize (*Zea mays*) or wheat (*Triticum aestivum*) in agricultural soils, are associated with mycorrhiza. In contrast to these beneficial organisms, some of the most important plant pathogens are also found among the fungi. Therefore fungal diversity in agricultural soils, in particular of biocontrol strains of *Trichoderma* spp., was investigated by Hagn *et al.* (2003). Soils from high- and low-yield areas of a winter wheat field under two different farming management practices were analysed. Results showed that the dormant soil fungal community was minimally influenced by the factors investigated, whereas active populations exhibited a clear response to changes in the environment.

Concern over increased atmospheric concentrations of greenhouse gases (especially CO₂), agroecosystem transformation and sustaining land productivity, prompted Razafimbelo *et al.* (2006) to study the effect of sugarcane residue management on soil organic carbon (SOC). They reported that soils under pre-harvest burnt cane contained lower levels of SOC than those under green cane harvesting with trash retention. Soil bulk density was lower under residue mulching compared to pre-harvest burning. The authors hypothesized that the preferential enrichment of soil carbon observed in the fine (< 2 µm) fractions resulted in long-term storage of carbon.

Galdos *et al.* (2009), also working with sugarcane, evaluated the effects of trash management on the carbon dynamics of a sugarcane crop, but on land previously converted from native forest. They studied two chronosequences in plots which had been replanted to sugarcane, with or without pre-harvest burning, 2, 6 and 8 years prior to sampling. They concluded that soils from the area converted to the unburnt management for 8 years, had higher levels of total C, microbial biomass C and particulate organic matter C than those from plots under residue burning.

2.3.2.2 Grassland

Grassland ecosystems are important in terms of global C cycling and agricultural (particularly animal) production. Several studies, such as that by McCaig *et al.* (1999) compared microbial community structure under improved and unimproved agricultural pastures. The unimproved pastures were grazed by sheep and had never been fertilized, whereas the improved pastures were also grazed by sheep but received regular dressings of N, P and K. Substantial variation in the abundance of specific components of the bacterial populations in samples of unimproved, unfertilized pasture soil and improved, fertilized soil samples were found, but no significant difference in abundance of any phylogenetic group was observed. α -Proteobacteria were more diverse in unimproved pasture soil samples than in improved soils. As the improved plots were dominated by *Lolium perenne* and the leguminous *Trifolium repens*, whereas the unimproved plots were dominated by non-leguminous species such as *Agrostis capillaris*, selection for nitrogen-fixing bacteria may have occurred in the former plots.

As plant-derived carbon inputs from recent photosynthesis are important drivers of biological processes in soil, Hirsch *et al.* (2009) investigated whether biodiversity was lost in response to the removal of this source of carbon. They studied three cultivation regimes: permanent grassland, a long-term (60 years) arable rotation and a long-term (50 years) bare-fallow area, maintained by tilling. In soils from the latter area, organic matter reserves had degraded and fresh carbon inputs had been greatly reduced. In the arable soils compared to permanent grassland soils, organic matter had declined over time. Their results indicated that in the bare-fallow area, starving the soil of plant

inputs for 50 years reduced soil bacterial community abundance but not diversity. The bare-fallow soils supported a species-rich, metabolically active bacterial community similar in diversity to that under permanent grassland. This suggested that bacterial diversity was independent of plant inputs.

One of the most widespread changes in land use and land cover is that of woody plant encroachment on grassland ecosystems, which has contributed to extensive changes in the productivity and biogeochemistry of the affected areas (Hollister *et al.*, 2010). The authors characterised the structure and functional capacity of soil bacterial and fungal communities occurring in four vegetation types of a mesquite- (*Prosopis glandulosa*) encroached mixed grass prairie. Woody plant encroachment caused substantial changes to the structure and function of grasslands and grassland-like ecosystems, and a linkage between above- and below-ground communities was shown. They concluded that soil microbial communities, particularly the fungal component, might be altered by woody plant encroachment of grasslands.

In some parts of the world, agricultural overproduction has resulted in the release of land from agricultural management, and attempts are now being made to restore the former species-rich vegetation. The effect of grassland succession on bacterial populations was monitored by Felske *et al.* (2000) in experimental plots, which showed a constant decline in nutrient levels and changes in vegetation as soon as fertilization and agricultural production stopped. From this time, the grasslands showed specific shifts in bacterial community composition, where total bacterial numbers increased. This correlated with the collapse of the dominant *Lolium perenne* grass population and with an increase in the rate of organic matter mineralization. Although the vegetation clearly changed, no evidence of strong competition or major species replacement in the soil microbial community was found.

Fungi and bacteria are important degraders of chitin, thus contributing to the recycling of vital carbon and nitrogen resources in soil. Metcalfe *et al.* (2002) assessed a bacterial chitinolytic population in upland pasture, to establish how lime and sewage sludge applications affected microbial chitinolytic activity. Bacterial enrichment occurred in response to burying chitin-containing litter at the field site. Sludge application significantly reduced chitinase diversity but increased chitinolytic activity.

Levels of actinobacteria increased with sludge treatment and also with lime-plus-sludge treatment. Readily available C and N sources did not repress chitinases but promoted chitinolytic activity of specific groups of actinobacteria. The authors concluded that sludge amendment might adversely affect the presence and diversity of chitinase genes in soil so should be used with caution.

Pasture-based production systems are complex and subject to a wide range of management practices. Wakelin *et al.* (2009) determined the response of the soil microbial community to, and the effects of, various pasture-based land managements such as pasture type, grazing, liming, P fertilization and sampling date on the soil bacterial and fungal community structure. Results showed that liming of acidic soil was the strongest factor affecting soil microbial community structure, increasing fungal phylotype richness but not bacterial. In addition, microbial nitrogen fixing and nitrification capacity was increased. The effects of pasture type (annual or perennial) were minimal and denitrification capacity was not affected by pasture management. Additions of P fertilizer increased the intensity of pasture production and affected fungal and bacterial community structure, as did an increased stocking rate. Significant shifts in the soil biota occurred during the growing season. Overall results showed that soil biota, particularly soil fungi under pastures, were highly responsive to agricultural land use and management, which changed the dominant soil fungi and bacteria, and influenced the microbial groups involved in key processes.

Maharning *et al.* (2009) reviewed soil community changes during secondary succession to naturalised grasslands. Succession from former agricultural land and pastures to naturalised grassland was associated with changes in plant biodiversity and in the soil microbial communities. These changes resulted from a reduction in, or elimination of, management practices such as grazing, or applications of fertilizer. Naturalised grasslands differed from intensively-managed pasture, in that plant succession progressed in the former but was suppressed in the latter. Two factors influencing the soil community were nutrient availability and soil microhabitat diversity. Different plant species affected soil microbes differently as they varied in their chemistry and physiology, in the quality and quantity of their litter, and in rhizodeposition. Long-term effects of continuous, selective grazing led to a shift in the plant community to a sward dominated by plants of lower litter quality, which

negatively affected the soil biota. Nutrient returns in the form of urine and manure, also altered the quality of resources entering the system. Nutrients released into the rhizosphere by plants positively influenced microbial activity, with bacteria being more directly associated with root exudates than fungi, the latter mainly being associated with root turnover. Applications of fertilizer directly affected the quality and productivity of plants, and had both a positive and negative effect on the soil microbiota. During succession, fungal biomass was greater in older systems whereas bacterial biomass remained constant.

Potthast *et al.* (2010) examined the effect of a slash and burn approach for converting vast areas of rain forest into pastures. The converted pastures however, frequently became overgrown with tropical bracken fern, which led to abandonment of these sites, as bracken-dominated areas were extremely difficult to recultivate. The authors investigated the implications of invasive bracken on soil biogeochemical properties. A comparison of active and abandoned pasture sites, showed that displacement of grass by bracken in abandoned sites lowered the soil pH, reduced the amounts of readily available carbon and nitrogen, reduced microbial biomass and activity, and resulted in a higher relative abundance of actinomycetes. Readily available organic C from grass litter was preferentially utilized. They concluded that differences in litter quality between grass and bracken initiated changes in soil biogeochemical and microbial properties after pasture conversion.

2.3.2.3 Forests

Forestry is an important industry, with forest health and sustainability directly dependent on the extensive soil microbial populations (Torsvik *et al.*, 1990b; Axelrood *et al.*, 2002a)). Forest soils are heterogeneous, the forest floor being affected by woody debris, cycles of vegetation cover and organic matter decomposition. The structure of soil bacterial populations may change due to fluxes in their habitat's physical and chemical properties, such as temperature; soil nutrient levels and available oxygen and moisture. Tree harvesting, replanting tree seedlings, and deforestation (Borneman and Triplett, 1997) are severe disturbances to the forest ecosystem, causing perturbation of soil microbial communities (Axelrood *et al.*,

2002b). In terrestrial ecosystems, bacterial decomposition of the lignocellulosic component of wood is negligible in comparison with that performed by specialized lignocellulolytic fungi from the Basidiomycota and Ascomycota. However, Zhang *et al.* (2008) reported that an unexpectedly high bacterial diversity was to be found in decaying wood.

Deforestation of naturally occurring tropical forests, alters soil properties by increasing pH, NH_4^+ concentration and the bulk density of soil, and by decreasing NO_3^- and soil porosity, thus affecting soil microbial communities. The first description of the microbial diversity in two eastern Amazonian soils (mature forest and active pasture soils), using a culture-independent, phylogenetic approach, was published by Borneman and Triplett (1997). Microbial community shifts associated with deforestation were shown, and evidence of unusual microorganisms was obtained. No duplicate sequences were found and none had been described previously, with 18% of the bacterial sequences not classifiable in any known bacterial kingdom. Mature forest and pasture soils therefore contained unique communities. The authors concluded that their phylogenetic analysis of these Amazonian soils had provided evidence for novel organisms that could not be classified in any known kingdom of *Bacteria*.

Bacteria alter catabolic gene expression in response to differing environmental nutrient conditions. This led to a study by Eaton and Farrell (2004), on catabolic and genetic microbial indices, and levels of ammonium, nitrate and organic carbon in forest soils. Soils near black locust trees (*Robinia pseudo-acacia*, nodulated with N_2 -fixing bacteria) were richer in organic C and mineralized N, had a higher pH, and had a greater community diversity and microbial biomass but a lower community catabolic diversity than soils under tulip poplar trees (*Liriodendron tulipifera*). In contrast, the latter soils did not select for dominant catabolic pathways, instead permitting a less diverse microbial community of lower biomass to use a greater variety of metabolic pathways. The authors concluded that variations in functional diversity at the population level existed in soil from different habitats.

As agricultural land use has increased to a third of the total land area on earth, increases in erosion, loss of soil organic matter (SOM) and changes in the soil bacterial communities have occurred. Upchurch *et al.* (2008) studied the effects of

different land managements, namely tillage (both CT and NT) cropland compared to the effects of a successional forest (30 years regrowth) and an old forest (> 65 years old). A lower diversity of bacterial communities in forest than in cropland soils was observed, which was attributed to the presence of a few abundant taxa in forest soils that were either less abundant or absent from cropland soils. However, the composition of the soil bacterial community from the new forest, although similar to that of the old forest, was more diverse, suggesting that the soil bacterial communities changed slowly.

Nitrogen (N) plays a crucial role in plant nutrition and is the soil nutrient required in greatest quantity. The cycling of N in the soil-plant-atmosphere system involves many N transformations between inorganic and organic forms. Nitrogen is assimilated almost entirely in the inorganic state as nitrate or ammonium. However, most nitrogenous material found in soil or added as crop residues is organic and is largely directly unavailable. The biochemical heterogeneity of the N mineralizing soil microflora is critical in determining the influence of environmental factors on N transformation (Alexander, 1977). Many of these microbially mediated processes are directly affected by land management (Wakelin *et al.*, 2009).

Chemolithoautotrophic ammonia (NH₃)-oxidising bacteria (AAOB) aerobically transform ammonia to nitrite (NO₂⁻) and, therefore, play a central part in the natural cycling of N, which, from an anthropogenic perspective, may be regarded as both beneficial and detrimental (Purkhold *et al.*, 2000; Oved *et al.*, 2001). The AAOB are responsible for the first rate-limiting step in nitrification, where ammonia is transformed via nitrite to nitrate (NO₃⁻) (Oved *et al.*, 2001). AAOB oxidation of ammonia to nitrite frequently results in the evolution of the greenhouse gas N₂O that adversely affects the atmosphere, and indirectly causes additional loss of N through the denitrification of nitrate. Inhibition of ammonia oxidation in agricultural systems would reduce fertilizer costs and prevent considerable environmental pollution (Phillips *et al.*, 2000). The key enzyme in aerobic ammonia oxidation is ammonia monooxygenase and the gene coding for its subunit, *amoA*, is a useful target in environmental studies, as it reflects ammonia oxidiser phylogeny (Avrahami *et al.*, 2002).

Avrahami *et al.* (2002) studied the effects of ammonia-addition on community composition of soil ammonia oxidisers and denitrifiers, and also of soil ammonia concentration on N₂O release. N₂O emission rates were positively correlated with the ammonium concentrations in soil, with medium and high concentrations increasing N₂O release rates, thus providing an electron acceptor for denitrification. Both processes, therefore, led to higher overall emission rates. Shifts in the faster-growing denitrifiers were detected, due to the increased supply of oxidised N through nitrification, whereas only negligible shifts in both the total bacterial community and the AAOB were found. Thus, the addition of ammonium resulted in an increase in nitrification activity but not in a change in the community structure of ammonia oxidisers.

Deposition of acidifying compounds in soils of boreal forests in Northern Europe has occurred for decades, so some of the forests have been limed to improve tree vitality and to raise soil pH. Bäckman *et al.* (2003) investigated the AAOB population structure in acid spruce forest soils six years after liming, which had increased the organic horizon pH, but left the mineral soil unaffected. Limed soils contained a high genetic diversity of *Nitrospira*-like sequences, although no AAOB-like sequences were found in control soil, regardless of depth. Liming induced growth of a diverse AAOB flora, with the high lime dose having a more marked effect, and to a greater depth in the soil profile, than the low dose.

In boreal and temperate forests, above-ground production is usually restricted by N availability. Consequently, N fertilization has been widely practised, although it has often been reported to result in a decrease in soil microbial activity and biomass (Demoling *et al.*, 2008). As little is known of the effects of N fertilization on the soil microbial community, the response of soil microorganisms to long-term (>10 years) N fertilization in three coniferous forest soils with different climatic and nutrient conditions, were investigated by Demoling *et al.* (2008). Bacterial growth rates were more negatively affected by fertilization than fungal growth rates, and microbial community structure differed at the three sites. In all of the forests, soil bacteria were limited by a lack of carbon, which became more evident in fertilized plots, particularly where the forests had previously been N-limited. They concluded that the effects of N fertilization differed depending on site conditions prior to fertilization.

The amount of fixed N annually entering the ecosystem due to anthropogenic disturbance has increased considerably. When N availability exceeds biological demand in forested ecosystems, N saturation may result, which leads to changes in soil pH, forest decline and N losses (Allison *et al.*, 2007). While anthropogenic N deposition is comparatively low in boreal forest and tundra, climate change may lead to alterations in N availability in these ecosystems. As fungi are known to play a major role in organic matter decomposition and mineralization in boreal ecosystems, Allison *et al.* (2007) studied the effects of N deposition on fungal communities in a boreal ecosystem. It was shown that N deposition reduced Basidiomycete diversity in litter and total fungal and ectomycorrhizal (EM) diversity in soil. Since wood-decaying fungi competed poorly under high N conditions, a reduction in decomposer fungal diversity was expected. It was concluded that N fertilization had reduced fungal taxonomic richness and altered the community structure.

Root-inhabiting fungi play a key role in forest ecosystems and are essential in forest biodiversity. Most studies of fungi inhabiting tree roots have concentrated on the mycorrhiza. Therefore, Kwaśna *et al.* (2008) determined the species diversity of microfungal communities in forest tree roots using a combination of traditional and molecular approaches. The authors compared the biodiversity of tree root microbiota by assessing the relationship between plants and microfungi, the type of association (active or passive), and the spatial heterogeneity of the microbiota. Their results showed that with the pure culture method, mainly saprotrophs and endophytes, particularly from the Ascomycota, were identified, whereas when using molecular methods, mainly Basidiomycota and Oomycota were identified. They concluded that a combination of morphological and molecular techniques was required to assess the diversity and density of the forest root biota.

2.3.2.4 Spatial heterogeneity

Considerable spatial heterogeneities in both chemical and physical properties characterize soil, which may partly be responsible for and/or result from, biological heterogeneity. Nicol *et al.* (2003) studied scale-associated differences in rhizosphere archaeal communities, in improved (managed) and unimproved (natural) grasslands,

using both a macroscale and a microscale analysis. At the macroscale level, heterogeneity over the transect was unrelated to grassland type but, in general, there was considerable heterogeneity across both improved and unimproved transects. At the microscale level, archaeal community heterogeneity varied with sample size. Among large soil samples, archaeal profiles were similar, whereas in small samples greater heterogeneity was found.

2.3.2.5 Soil depth

Subsurface and surface soil bacterial communities in a Californian grassland along two vertical transects, were compared by LaMontagne *et al.* (2003). Richness and diversity were lowest in samples from the greatest depth, probably due to the low organic matter content in the horizon. Bacterial communities differed between depths with the more abundant OTUs in the deepest samples related to *Pseudomonas* and *Variovorax*. These findings supported the hypothesis that surface and deep communities varied in composition and dominance, and that loss of diversity with depth was due to changes in community function resulting, in turn, from loss of physiological capabilities and changes in both available resources and the physical environment.

Bacterial community ecology in native grassland soil was studied by Griffiths *et al.* (2003). They assessed the influence of depth and sampling time on bacterial population structure. Changes in the bacterial community caused by temporal changes in the organic layers as well as by soil depth, were observed. Variation due to depth was attributed to a decrease in soil moisture, together with changes in nutrient availability and soil texture. Evidence of genetic diversity differences down the soil profile was obtained, particularly between the organic and mineral soils, while temporal changes were observed only in the organic layers.

2.3.3 Effects of soil pollution

Varied responses by microbial populations to environmental disturbance have been reported, with lower genetic and taxonomic diversity in chemically polluted soil

compared with undisturbed soil being recorded (Kennedy and Gewin, 1997). A brief discussion of these effects follows.

2.3.3.1 Heavy metals

Heavy metals form a group of approximately 40 elements having a specific gravity greater than five. Examples include Hg, Zn, Cu, Pb, Ni, V and Cd. In soil, heavy metal toxicity may be markedly influenced by environmental conditions. Metals bind strongly to organic materials, such as proteins, and fulvic and humic acids, with precipitation, complexation, and ionic interactions all being important phenomena. This limits their availability, resulting in deficiency symptoms in plants growing in such soils, as many metals are necessary for growth. However, in excess, they are highly toxic to cells because they denature proteins (Gadd and Griffiths, 1978).

Environmental pollution with heavy metals is a serious problem (Hu *et al.*, 2007). However, among microbes, many of which are resistant to heavy metals, a variety of detoxification mechanisms occur widely. They therefore play a vital role in polluted ecosystems, and in the reclamation of metal-contaminated natural habitats (Gadd and Griffiths, 1978). In the environment, heavy metals are highly persistent, and alter ecosystem diversity, structure and function (Müller *et al.*, 2002). For example, Hu *et al.* (2007) showed that heavy metal pollution with Pb and Cd reduced both the biomass and the diversity of the soil bacterial community.

2.3.3.2 Hydrocarbons

Contamination of soil sites with oil is problematic, as crude oil contains polycyclic hydrocarbons and approximately 5% organically bound sulphur. The presence of sulphurous oil in soil induces changes in bacterial community composition, by selecting for organisms that can grow under the new conditions and suppressing those that cannot (Duarte *et al.*, 2001).

2.3.3.3 Pesticides

The influence of pesticides on soil microbial communities is of great concern because of their persistence in soil, their toxicity to non-target organisms and their selection for resistant organisms. Although large quantities of herbicides have been released into soil, little is known of their effect on the abundance and structure of soil microbes (Crecchio *et al.*, 2001; Seghers *et al.*, 2003). Ratcliff *et al.* (2006) reported that the herbicide glyphosate, when applied at the recommended rate, had a benign effect on soil fungal community structure, while at high concentrations, non-specific short-term stimulation of bacteria occurred.

2.4 TECHNIQUES FOR ESTIMATING SOIL MICROBIAL DIVERSITY

Various methods have been devised to determine soil microbial diversity. An overview of some of the non-molecular and molecular methods currently in use is now presented.

2.4.1 Non-molecular techniques

2.4.1.1 Plating and microscopy

Traditionally, taxonomic classification of bacteria is based on morphology and metabolic and physiological traits. This involves the isolation and cultivation of individual bacterial species that are used to characterise the community. Direct microscopic examination of soil microbial suspensions usually follows (Kent and Triplett, 2002). Similarly, quantification of fungi, such as AMF, has relied on microscopic assessment of spores and hyphal density in soil (Gamper *et al.*, 2008).

Viable plate counts or most-probable-number (MPN) methods, quantify active cells in the environment but are limited and fail to describe community diversity, as less than 1% of environmental microorganisms are isolated (Amann *et al.*, 1995; Kent and Triplett, 2002; Kelly, 2003). Bacterial species abundant in soil are rarely, if ever,

successfully cultured, resulting in the isolation of bacteria that are only a minor component of the community (Joseph *et al.*, 2003). Similarly, of the estimated 1.5 million fungal species worldwide, especially those from soil, only a small percentage has been cultivated thus far (Hawksworth and Rossman, 1997; Aguilera *et al.*, 2006). Amann *et al.* (1995) reported that direct microscopic counts of microbes in soils and sediments exceed viable cell counts by several orders of magnitude. This is termed the 'great plate count anomaly' as the majority of cells visualised microscopically, although viable, do not form colonies on plates. Nonetheless, this method is a reliable, rapid and inexpensive way of obtaining data and provides species for DNA libraries, for bio control (Kennedy and Gewin, 1997) and for rapid assessment of biological responses to heavy metal or toxin pollution (Ellis *et al.*, 2003).

2.4.1.2 Fatty acid methyl ester (FAME) analysis

FAME analysis is a biochemical method that uses groupings of fatty acids to provide information on microbial community composition. The microbial cell biomass consists of a relatively constant proportion of fatty acids, including signature fatty acids that can differentiate major taxonomic groups in a community. Thus a change in the fatty acid profile reflects a change in the microbial community (Kirk *et al.*, 2004; Zhang *et al.*, 2008). Fatty acids, extracted directly from whole soil, are methylated to form fatty acid methyl esters and then analysed using gas chromatography (Pankhurst, 1997; Ritchie *et al.*, 2000; Kirk *et al.*, 2004). FAME analysis provides rapid fingerprinting of lipid profiles; similarity indices for communities; the identification of biomarkers (from individual peaks) (Kennedy and Gewin, 1997); and assessments of environmental risk (Zhang *et al.*, 2008). Disadvantages of FAME analyses are that both living and dead microorganisms are detected, as well as plant and humic materials in soil (Kennedy and Gewin, 1997).

2.4.1.3 Phospholipid fatty acid (PLFA) analysis

This technique, also termed chemotypic fingerprinting, uses gel chromatography to determine microbial membrane phospholipids, extracted directly from soil (Hartmann *et al.*, 2007). As PLFAs are found only in membranes of living cells and not as

microbial storage products, they can be used to estimate the living component of a soil microbial population (Kennedy and Gewin, 1997). Thus, by restricting soil fatty acid analysis only to PLFAs, the possibility of detecting differences in soil microbial community composition is increased (Pankhurst, 1997). Profiles of phospholipids give precise measures of total biomass numbers, and can identify biomarkers using differences in the relative abundance of PLFAs. General peaks identify Gram positive and Gram negative bacteria, as well as eukaryotes (Kennedy and Gewin, 1997). Specific PLFAs can be used as indicators (e.g. 18:2 ω 6,9) for fungal biomass in soil (Hartmann *et al.*, 2007). This method allows similarity indices for communities to be obtained and shifts in community structure to be monitored (Demoling *et al.*, 2008). As total PLFAs and microbial biomass are usually correlated with organic C and total N in soils, they are good indicators of soil fertility (Hartmann *et al.*, 2007). The disadvantages of this procedure are that it is tedious and does not provide taxonomic information on individual species (Kennedy and Gewin, 1997; White and MacNaughton, 1997).

2.4.1.4 Substrate utilization

Analysis of community functioning based on substrate utilization (metabolic) profiling, measures biodiversity of communities rather than of species (Pankhurst, 1997). Substrate utilization patterns provide reliable ‘fingerprints’ of microbial populations and may indicate metabolic potential or microbial community function (Kennedy and Gewin, 1997; Buyer *et al.*, 2001). A simple approach to studying microbial functional diversity is based on the number of C substrates utilized by the community, the two most commonly used methods being the BIOLOG[®] microplate identification system (BIOLOG Inc., Hayward, California, USA) and substrate induced respiration (SIR) (Pankhurst, 1997).

2.4.1.4.1 The BIOLOG method

Garland and Mills (1991) published a method for the functional characterization of heterotrophic microbial communities. This was based on sole-carbon source utilization as a community-level physiological profiling (CLPP) approach, using

commercially available BIOLOG GN 96-well microtitre plates. The BIOLOG system was originally devised to identify pure bacterial cultures, mainly Gram-negative (GN) species of clinical importance. The 95 different substrates in the GN plates were selected from a set of 500 carbon sources, for their ability to distinguish clinically important species from among 6000 bacterial strains (Konopka *et al.*, 1998; Hartmann *et al.*, 2007). To identify Gram-positive bacteria, BIOLOG GP plates have been developed. Sixty two substrates are common to both GN and GP plates, with a further 33 substrates unique to each (Konopka *et al.*, 1998). In addition, wells in the GN and GP plates contain tetrazolium violet dye. This is reduced to purple-coloured insoluble formazan when oxidation of the C compounds by microbial metabolic activity occurs, thus enabling catabolic diversity to be assessed (De Fede *et al.*, 2001; Kirk *et al.*, 2004). The production of formazan is directly related to the reducing equivalents formed by the inoculated community, and subsequently, by the growing cells within a well (Smalla *et al.*, 1998).

Although the system was designed to monitor respiratory activity rather than growth, bacteria can grow in the microplate wells. If the inocula are diluted, colour does not develop until the cell concentration per well has reached 10^8 cells ml^{-1} . Therefore, at an inoculum density of $< 10^8$ cells ml^{-1} , colour development will be a sigmoidal function of time, in which: (i) the initial lag phase is a period when the inoculum grows to a population of 10^8 cells ml^{-1} ; (ii) the linear phase is a period of microbial metabolic activity on a specific substrate; and (iii) the plateau represents the formazan yield produced by particular microbes from a specific substrate. All phases are therefore, affected by the microbes' physiology, with the initial lag phase being highly dependent on the inoculum cell density (Konopka *et al.*, 1998).

CLPP analysis usually requires extraction of microbial cells from environmental samples (Smalla *et al.*, 1998). However, as researchers found that non-culturable cells from mixed communities also responded to substrate supply, the method was adapted to characterize the functional potential of these communities. Numerous studies have shown that habitat-specific and reproducible patterns of carbon utilization are produced by such microbial communities (Hartmann *et al.*, 2007).

Prokaryote community analysis and ecological studies are now possible using BIOLOG EcoPlates™, which were created specifically for this purpose. Each microplate contains 31 of the most useful carbon sources, together with tetrazolium dye, for soil community analysis, replicated three times (Appendix D, Table D1) (Liu *et al.*, 2007a, b; Zhang *et al.*, 2008). The typical procedure when using EcoPlates involves inoculating environmental samples directly into the microplate wells, either as aqueous samples, or suspensions of soil, sludge or sediment. The microplates are then incubated and plate readings taken at defined time intervals, using a microplate reader, to give optical density (OD) values. CLPPs are then assessed for pattern development (similarity), the rate of colour change in each well, and the richness of well response (diversity) (BIOLOG, 2008).

When using CLPP, soil samples present the greatest challenge as microbes bind to soil particles (Konopka *et al.*, 1998). To overcome this problem, vigorous shaking or homogenisation of soils suspended in either sterile distilled water, phosphate buffer or saline, is used to release bacteria from particles (Calbrix *et al.*, 2005; Chen *et al.*, 2007; Liu *et al.*, 2007a). The suspension is then allowed to settle or, in some protocols, centrifuged, as the presence of soil particles in the inoculum causes inaccurate OD readings (Calbrix *et al.*, 2005). The supernatant is retained and usually diluted before use (Govaerts *et al.*, 2007; Liu *et al.*, 2007a, b).

As fungi cannot be detected by the GN and GP plates, because they do not reduce tetrazolium violet and the dye is toxic to many fungal species, fungus-specific microplates (namely, SF-N and SF-P MicroPlates™) have been developed. The SF-N plates (Appendix E, Table E1) are identical to the GN microplates and the SF-P plates to the GP microplates, except that they lack the tetrazolium redox dye (BIOLOG, 2008). Testing fungi in SF-N and SF-P microplates is very simple and uses a protocol similar to that for testing bacteria and yeast. Modifications include the use of special culture media to promote sporulation, preparation of lower density inocula in a gel-forming colloid rather than in water or saline, and inoculation of 100 µl per well instead of 150 µl. Plates are incubated at a low temperature (~26°C) for several days and growth is measured by an increase in turbidimetry at OD₆₅₀. BIOLOG FF microplates and a FF database have also been developed for fungal analyses (BIOLOG, 2008).

The advantages of CLPP assays include their ease of use for replicated large-scale studies. The microplates provide a fast, simple and accurate method for studying microorganisms that is an alternative, or is complementary, to traditional macroscopic and microscopic methods. Also, CLPP is more sensitive than PLFA analysis for monitoring microbial community and ecological changes. CLPP provides broad coverage of a variety of microbial samples, both bacterial and fungal, and has many different applications. For example, it can distinguish spatial and temporal changes in microbial communities: it has been used to detect population changes in soil, water, wastewater, activated sludge, compost and industrial waste: and it also has clinical applications (BIOLOG, 2008).

Limitations of CLPP are that culturable microbes are required, the system is sensitive to inoculum density, and favours fast-growing microorganisms (Buyer *et al.*, 2001; De Fede *et al.*, 2001; Kirk *et al.*, 2004). Culture conditions in BIOLOG microplates are thought to have a harmful effect on a large proportion of the inoculated bacteria, as microbial diversity decreases continuously during the first week of incubation. In addition, the relative proportion of species in a microbial community may change during microplate incubation, and a long incubation time induces cell lysis and the formation of extracellular storage polymers (Calbrix *et al.*, 2005). Thus CLPP analysis does not necessarily reflect the functional potential of the dominant community members but is biased towards those populations that grow best under the assay conditions (Ros *et al.*, 2008).

2.4.1.4.2 Substrate induced respiration (SIR)

To avoid some of the drawbacks of the BIOLOG method, Degens and Harris (1997) developed substrate induced respiration (SIR), an *in situ* approach that does not require prior extraction and cultivation of soil microorganisms. Short-term SIR responses to 36 organic substrates added directly to soil are measured by assessing CO₂ efflux from soil by gas chromatography, thus revealing catabolic diversity of active bacterial and fungal communities.

2.4.2 Molecular techniques

Molecular techniques, based on nucleic acids isolated directly from complex ecosystems such as soil, are being used increasingly to study microbial communities, as non-molecular methods are not entirely adequate (Roose-Amsaleg *et al.*, 2001; Kent and Triplett, 2002). These techniques are dependent on obtaining representative nucleic acid extracts from entire microbial communities (Miller *et al.*, 1999). Different methods provide coarse-, medium- and fine-scale levels of resolution of the structure and activity of microbial communities. At the coarse-scale level, the entire community is sampled whereas at the fine-scale level, only target populations are analysed (Tiedje *et al.*, 1999). Most microbiological methods provide medium-to-fine-scale resolution, namely, genus to species to subspecies (Vandamme *et al.*, 1996). However, individual steps in the procedures may be inefficient so these methods introduce biases of their own (Miller *et al.*, 1999; Sessitsch *et al.*, 2002).

An overview of some of the DNA-based approaches currently used for measuring soil microbial community diversity is presented below.

2.4.2.1 DNA extraction and purification

The collection of soil samples and DNA extraction methods must take account of the fact that cell density is low in certain environments (Kent and Triplett, 2002). Therefore, selection of a suitable extraction and purification procedure is of major importance (Kozdrój and van Elsas, 2000; Roose-Amsaleg *et al.*, 2001; He *et al.*, 2005). DNA extraction and its fragmentation during sample treatment, are affected by differences in microbial cell wall structure and adhesion behaviour, in conjunction with physical, chemical and biological soil characteristics, making unbiased extraction difficult (Niemi *et al.*, 2001). Also, naked DNA adsorbs to soil particles, leading to biased population profiles (Sessitsch *et al.*, 2002).

Initially, DNA extraction from soils involved cell recovery from the soil before lysis (the indirect method) but, currently, direct lysis within the soil matrix is increasingly being used, as samples are less biased and the DNA yield is higher. The procedure

usually includes one or all of the following elements: physical disruption, chemical lysis and enzymatic lysis of the cells (Miller *et al.*, 1999; Roose-Amsaleg *et al.*, 2001). The resultant DNA must be amplifiable by PCR (section 2.4.2.2). Consequently, efficient, unbiased extraction procedures are essential (Kozdrój and van Elsas, 2000; LaMontagne *et al.*, 2002) otherwise humic substances, clay minerals, proteins, polysaccharides, metals and other PCR-inhibitors will be co-extracted (Griffiths *et al.*, 2000; Kozdrój and van Elsas, 2000; Kent and Triplett, 2002). Samples containing such inhibitors require additional purification steps, before PCR amplification (Griffiths *et al.*, 2000). Purification efficiency is gauged by both the amount of DNA recovered and the successful removal of contaminants (Miller *et al.*, 1999). It has been shown that both the isolation and purification methods used affect the apparent structure of bacterial communities in soil samples (Niemi *et al.*, 2001; Kent and Triplett, 2002). In addition, He *et al.* (2005) reported that pre-lysis buffer washing of DNA extracted from recalcitrant environmental samples, such as forest soils containing high levels of organic matter and iron oxides, resulted in higher yields of improved quality DNA.

When isolating fungal DNA from soil, optimisation of the initial DNA extraction procedure may overcome some of the problems typically encountered (Bridge and Spooner, 2001). Pennanen *et al.* (2001) developed a DNA purification method for the extraction of high quality fungal DNA for use in subsequent PCR. Fungal spores from arbuscular mycorrhizal fungi (AMF) pose a particular challenge, requiring alternating sonication and washing steps, followed by crushing under liquid nitrogen. The resultant powder is then used for DNA extraction (Kowalchuk *et al.*, 2002).

An alternative method of extracting and purifying soil community DNA involves the use of purpose-designed kits, such as the UltraClean™ Soil DNA Isolation Kit from MO BIO Laboratories, Inc. CA, USA. The kit facilitates rapid isolation and purification of PCR-grade DNA from soil samples for further analysis (UltraClean™ Soil DNA Isolation Kit Instruction Manual, 2008). The basic procedure is to lyse the soil microbes by a combination of heat, detergent and mechanical force using specialised beads in a vortex. The released DNA is then purified in a spin filter tube (Roose-Amsaleg *et al.*, 2001). Eaton and Farrell (2004) isolated soil genomic DNA from a mixed stand forest, using an UltraClean™ Soil DNA Isolation kit. They

assessed the efficiency of isolation and quality of each soil DNA sample and, in all cases, the microbial genomic DNA appeared as a single band of high molecular weight. Similarly, Pennanen *et al.* (2001) using one of these kits, isolated and purified fungal DNA from a forest ecosystem, although a prior homogenisation step was required.

Aguilera *et al.* (2006) compared the efficiency of sixteen DNA extraction protocols, differing in cell lysis treatments, and also two commercial kits, the Fast DNA kit and the Fast DNA Spin kit for soil (Bio 101, Carlsbad, CA. USA). The methods that combined bead-mill homogenization with SDS (for cell lysis), treatment with chemical extractants, followed by phenol extraction, were most efficient. However, DNA recovery with the commercial kits, was three times higher than DNA yields using the above methods, and the procedures were easier to perform.

2.4.2.2 Polymerase chain reaction (PCR)

DNA amplification by PCR is a simple concept but has had a marked influence on molecular biology, as 'insufficient DNA' is no longer a limitation. PCR is an *in vitro* technique used to amplify a specific DNA region, situated between two areas of known sequence, using oligonucleotide primers (amplimers), which are short, single-stranded DNA molecules (Madigan *et al.*, 2003). The PCR procedure is carried out in a thermal cycler and is divided into three stages, together constituting a cycle of synthesis:

1. Denaturation: Double-stranded DNA is heated to 93–95°C causing it to denature or 'melt' and become single-stranded.
2. Primer Annealing: 'Primers' initiate synthesis of a new DNA strand after they have bound (annealed) to the complementary target DNA strand. Two primers are required, one on each side of the region to be amplified. The temperature is lowered to 55°C for about 30-60 seconds for annealing to occur.

3. Extension: Thermostable DNA polymerase (Taq) extends the primers on the single-stranded template, in the presence of deoxynucleotide triphosphates (dNTPs) under suitable reaction conditions, which results in the synthesis of new DNA strands complementary to the template strands.

By repeating a series of these cycles, the number of double-stranded DNA fragments increases exponentially (PCR Applications Manual, Boehringer Mannheim, 1995) (Figure 2.1).

Subsequent to the development of PCR, the introduction of thermostable Taq polymerase allowed thermal cycling to be automated. These enzymes play a vital role in DNA amplification and sequencing. Essential properties are: (i) thermostability, as each PCR amplification cycle includes a denaturing step at $\sim 95^{\circ}\text{C}$ (although some DNA requires a higher temperature); (ii) extension rate, which is important because it represents the number of dNTPs polymerized per second per molecule of enzyme; (iii) processivity, characterized by the average number of nucleotides added by a DNA polymerase in a single binding event; and (iv) fidelity, which represents the frequency of correct nucleotide insertion per incorrect insertion. The efficiency of insertion of a correct nucleotide by DNA polymerases varies 10^7 fold, therefore some DNA polymerases are low fidelity and others, high fidelity (Pavlov *et al.*, 2004).

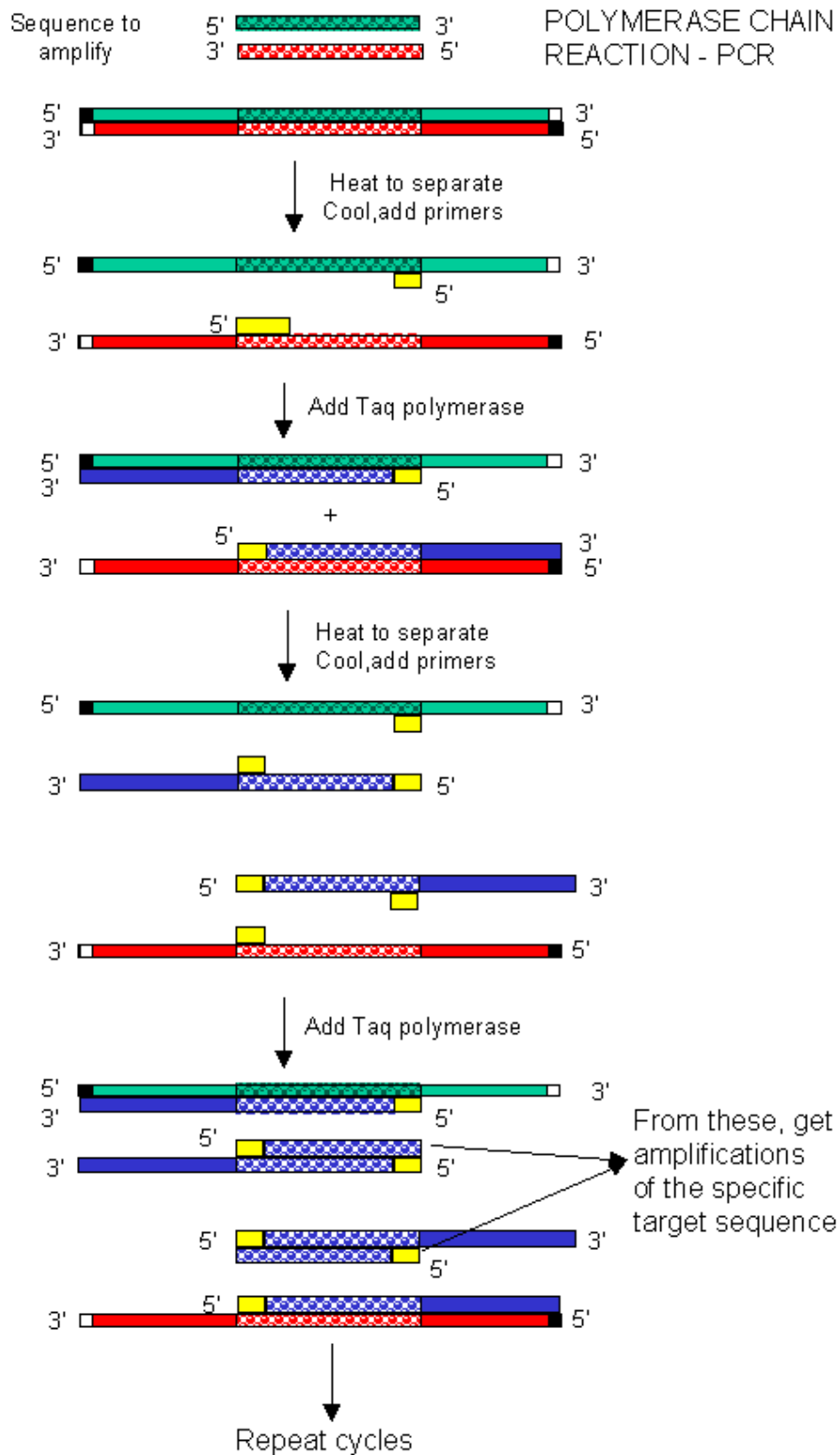


FIGURE 2.1 Schematic diagram of the PCR reaction (<http://people.uwec.edu/piercech/tox/Techniques.htm>).

Genes encoding ribosomal RNA are the most frequently used phylogenetic markers since the rDNA molecule is universal to all life forms (Hartmann *et al.*, 2007). In prokaryotes, small subunit ribosomal RNA (SSU rRNA), or 16S rRNA (Kennedy and Gewin, 1997; Kelly, 2003; Madigan *et al.*, 2003), consisting of approximately 1500 nucleotides, is the basis for most molecular studies of the soil microbiota. The diversity, richness and structure of microbial populations may be described using this marker (Sessitsch *et al.*, 2002; Kelly, 2003). It not only contains several regions of highly conserved sequences, to ensure proper sequence alignment, but also has sufficient sequence variability in other areas of the molecule, to serve as a good phylogenetic chronometer. In order to obtain actual 16S rDNA sequences, the genes encoding them are directly amplified from genomic DNA by PCR. For 16S rRNA sequences on the other hand, amplification by Reverse Transcription (RT)-PCR (section 2.4.2.2.3) is required (Nikolausz *et al.*, 2004). This may be followed by sequencing the PCR amplicons thus produced. Subsequent analyses may use DGGE (section 2.4.2.2.6), SSCP (section 2.4.2.2.7), T-RFLP (section 2.4.2.2.8) or chromatographic techniques (Suzuki and Giovannoni, 1996; Madigan *et al.*, 2003; Hartmann, *et al.*, 2007). Newly generated rDNA or rRNA sequences can then be compared with sequences in various databases such as the National Centre for Biotechnology (NCBI) or the Ribosomal Database Project (RDP) (Madigan *et al.*, 2003; Mitchell and Zuccaro, 2006).

Molecular techniques are also being applied increasingly to fungi, concentrating on the fungal ribosomal RNA gene cluster that consists of three rRNA subunits, internally transcribed spacers (ITS) and intergenic spacers (IGS) (Vainio and Hantula, 2000; Bridge and Spooner, 2001; Horton and Bruns, 2001; Gomes *et al.*, 2003; Anderson and Parkin, 2007). Broad-based studies can be performed using the nuclear large 28S subunit (LSU) or the small 18S rRNA subunit (SSU). These subunits are mainly used in phylogenetic studies to determine evolutionary relationships, and are the 'backbone' for identifying rDNA signals amplified from the environment (Mitchell and Zuccaro, 2006). In general, a lack of variation in the 18S rRNA gene often eliminates it as a target for studies (Kwańska *et al.*, 2008). However, de Souza *et al.* (2004) reported that while the V3–V4 region of the 18S rRNA gene contained insufficient variation to discriminate between different *Gigaspora* spp, heterogeneity

within the V9 region could be used to identify all recognised species within this genus.

The ITS regions one and two, display the greatest sequence and size variation in the fungal rRNA gene cluster (Mitchell and Zuccaro, 2006). Anderson and Parkin (2007) reported that the highly variable nature of rapidly evolving rDNA ITS spacer regions, has made them the most popular choice for species-level identification of fungal taxa in environmental pools. Analyses using the ITS region, while successful, may result in an overestimation of species diversity in the environment (Vainio and Hantula, 2000; van Elsas *et al.*, 2000; Hagn *et al.*, 2003; Öpik *et al.*, 2003).

Molecular strategies for the direct detection of the variation and fate of soil fungi are less well developed and lack specificity compared to those for soil bacteria (van Elsas *et al.*, 2000). Identification based on the 18S rRNA gene in fungi is problematic and is limited to genus or family level. This is due to the lack of variation in the conserved regions within the 18S rRNA as a result of the relatively short period of fungal evolution compared to that of bacteria (Kwaśna *et al.*, 2008). As a result, the eukaryotic 18S rDNA sequence database is less comprehensive than that of prokaryotic 16S rDNA (van Elsas *et al.*, 2000; Oros-Sichler *et al.*, 2006; Kwaśna *et al.*, 2008). Nonetheless, the electronic database is constantly expanding (Hirsch *et al.*, 2010).

2.4.2.2.1 PCR primers

To obtain unambiguous results after PCR amplification, careful primer design, and a strong specificity of these primers to the target genes are required (Madigan *et al.*, 2003; Marshall *et al.*, 2003). It is possible to tailor analyses to examine the entire community, particular organisms, or taxa of interest by using either universal or group-specific primers (Kent and Triplett, 2002). Universal primers may be used to produce a phylogenetic inventory of the whole prokaryotic component of a microbial community, as all the 16S rRNA genes in the sample will be amplified (Kelly, 2003). When using universal primers with PCR-DGGE (section 2.4.2.2.6), dominant species in soil bacterial consortia, can be detected over wide taxonomic distances (Niemi *et*

al., 2001). However, they often contain degeneracies that may influence the formation of primer-template hybrids (Suzuki and Giovannoni, 1996). Nikolausz *et al.* (2004) reported that universal primers designed for the domain Bacteria, also amplified plant chloroplast ribosomal genes. Consequently they devised a rapid RNA isolation and a RT-PCR method (section 2.4.2.2.3) to investigate rhizoplane bacteria which significantly reduced the abundance of chloroplast amplicons.

Unlike universal primers, those that are designed to target specific genes of a group, can differentially detect that particular group (Kelly, 2003). For example, Heuer *et al.* (2002) analysed the 16S rRNA genes of the rhizosphere communities of transgenic and wild type potato lines by PCR. They used primers specific for bacteria, Actinomycetales, and α - or β -Proteobacteria, which enabled a taxon level comparison to be made between the communities. In another example, by using specific primers that targeted the hypervariable regions of the 16S rRNA gene, Sabat *et al.* (2000) detected *Escherichia coli* in heterogeneous soil samples. Oligomer primers have been designed to study soil bacterial populations involved in the nitrogen cycle, as genes coding for the different enzymes of nitrification, N₂ fixation, and denitrification have all been sequenced (Rösch *et al.*, 2002).

In fungi, some highly conserved sequences in the ribosomal subunits may be used to produce broad-specificity PCR primers, including those having enhanced specificity for fungi in general, and others for asco- or basidiomycetes. This enables fungal sequences from mixed samples containing DNA from fungi and other organisms, to be selectively amplified from total soil extracts (Vainio and Hantula, 2000; Bridge and Spooner, 2001; Marshall *et al.*, 2003). Reliable amplification of fungal 18S rDNA fragments from soil requires primer systems that can amplify representatives from all four major fungal taxonomic groups, namely, Phycomycota, Zygomycota, Ascomycota and Basidiomycota (Deacon *et al.*, 2006; Oros-Sichler *et al.*, 2006)). In addition, the amplicons should contain sufficient phylogenetic information to differentiate between the fungal taxa. Furthermore, amplification should be highly reproducible for a wide range of different soil samples (Oros-Sichler *et al.*, 2006). As the fungal ITS regions are important for distinguishing fungal species, a complete set of primers was developed to discriminate between plant and fungal sequences in

environmental samples containing high levels of background plant DNA (Martin and Rygiewicz, 2005).

2.4.2.2.2 Limitations of PCR

While PCR-based community analysis is the molecular method most widely used to characterise soil microbial communities, and has substantially increased our knowledge of bacterial and, to a lesser extent, fungal structural diversity, it should be noted that it has several limitations (Kent and Triplett, 2002; Aguilera *et al.*, 2006; Mitchell and Zuccaro, 2006). These may be minimised by improving PCR conditions (Forney *et al.*, 2004; Mitchell and Zuccaro, 2006).

It is incorrect to assume that all DNA in a sample will be amplified with equal efficiency as, in some cases, DNA from certain organisms is preferentially amplified (Vainio and Hantula, 2000; Kelly, 2003). Differences in cell wall lysis, DNA extraction efficiency, differences in gene copy number and/or the efficiency of the PCR reaction itself, can cause biases in amplification (Miller *et al.*, 1999; Schweiger and Tebbe, 2000; Kelly, 2003). Another common problem encountered in PCR is contamination of the reaction mixture with foreign DNA (Amann *et al.*, 1995).

Leuders and Friedrich (2003) evaluated possible sources of PCR amplification bias, using terminal restriction fragment length polymorphism (T-RFLP) analysis of SSU rDNA. They also analysed methyl-coenzyme M reductase (*mcrA*) genes from soil DNA extracts, thus precisely quantifying the PCR product pool. This indicated that bias had occurred.

Several factors may bias relative frequencies of genes in PCR products from mixed-template reactions, namely, the guanine plus cytosine (G+C) content of template DNA, different binding energies resulting from primer degeneracy, and the influence of template folding (Suzuki and Giovannoni, 1996). Genome size and the copy number of rRNA (*rrn*) genes also influence PCR amplification (Farrelly *et al.*, 1995). Selective priming of certain sequences, biases during cloning and the production of chimeras (formed from sequences of different species during PCR amplification of

mixed culture DNA) are all disadvantages associated with this method (Kennedy and Gewin, 1997). The probability of chimera formation is increased by the availability of partial-length rDNA fragments in low molecular weight genomic DNA preparations; or by premature termination during the PCR elongation step; or due to the percentage of highly conserved stretches along the primary structure of rDNA. Fragmentation of soil DNA during extraction procedures may also result in the formation of chimeras. Once formed, chimeric sequence amplification proceeds with the same efficiency as nonchimeric fragments (Amann *et al.*, 1995; Niemi *et al.*, 2001; Aguilera *et al.*, 2006).

Another limitation of DNA-based community analysis is that both viable and non-viable bacteria and fungi, irrespective of their metabolic activity, are detected. In contrast, RNA-based analyses describe only the metabolically active members of a community, as the amount of rRNA produced can roughly be correlated with bacterial growth activity (Sessitsch *et al.*, 2002; Kelly, 2003). However, although methodologies are well established for analyses of a DNA-based phylogeny using the 16S rRNA gene, direct targeting of 16S rRNA as an indicator of activity is less common, as fewer suitable protocols for its extraction from natural environments are available. Consequently, Griffiths *et al.* (2000) developed a rapid method for co-extracting total DNA and RNA from natural environments. This facilitated concomitant assessment of microbial 16S rRNA diversity, by both PCR and Reverse Transcription (RT)-PCR (section 2.4.2.2.3) amplification from a single extraction. In another study, Sessitsch *et al.* (2002) evaluated different extraction and soil conservation protocols for the isolation of RNA from soil, for bacterial community and functional analysis. They found that different RNA isolation methods affected the recovery of mRNA, as well as community composition and the abundance of individual members.

2.4.2.2.3 Reverse transcription (RT) and reverse transcription-PCR (RT-PCR)

Two variations of PCR are RT, and RT-PCR. The enzyme, reverse transcriptase is used in RT to clone rDNA, transcribed from 16S rRNA that permits the selective retrieval of rRNA sequences. Once the complementary rDNA (c rDNA) clone has been constructed, the RNA may be amplified by RT-PCR, using rDNA-specific primers. This allows for more rigorous sequence retrieval from rRNA rather than from DNA, and permits the expression of functional genes. However, chimera formation is still problematic (Amann *et al.*, 1995; Kennedy and Gewin, 1997; Kelly, 2003). In a study by Anderson and Parkin (2007), RT-PCR amplification of fungal precursor rRNA molecules was used to detect fungal ITS regions in RNA pools. This method enabled active soil fungi to be detected.

2.4.2.2.4 Quantitative PCR (qPCR)

A further variation of PCR is quantitative PCR (qPCR) (Manter and Vivanco, 2007). This may be used to quantify the composition of functional guilds of diverse microorganisms, by analysing the abundance of functional genes. Whole-community DNA or RNA extracted from environmental samples such as soils, may thus be analysed (Sharma *et al.*, 2007). Purpose-designed primer sets target the conserved regions within functional gene sequences, and measure the abundance of each gene and its transcripts. While gene or transcript numbers per unit of soil DNA/cDNA can accurately be assessed by qPCR, converting these values to cell densities and biomass, and relating them directly to soil mass or volume are more difficult. This is due to the variability of both genome copy number and size (Sharma *et al.*, 2007). Specific qPCR assays have been developed to quantify particular AMF taxa under environmental conditions (Gamper *et al.*, 2008). qPCR can be performed using three different methods, namely, most probable number (MPN), competitive and real-time PCR. The latter is briefly outlined below.

2.4.2.2.5 Real-time PCR

The introduction of real-time PCR technology has improved and simplified nucleic acid quantification significantly, and now has wide applications (Klein, 2002). Real-time PCR enables specific organisms from complex, mixed DNA templates to be identified and quantified by measuring PCR product accumulation (Heid *et al.*, 1996). This technology employs either non-specific fluorogenic DNA binding dyes, such as SYBR[®] Green 1 (Klein, 2002), or sequence-specific, dual-labelled fluorogenic hybridization probes such as TaqMan[®] (Heid *et al.*, 1996). An increase in fluorescence during PCR reflects the increase of newly-generated DNA amplicons, and is used to estimate DNA content in samples. The main advantages of real-time over traditional endpoint quantitative PCR are its precision, greater accuracy (as high PCR cycle numbers are avoided after initial amplification) and reduced handling times, as amplification and detection are combined (Sharma *et al.*, 2007; Gamper *et al.*, 2008).

A number of community 'fingerprint' methods are currently used to assess differences in population composition between different samples or treatments, and to assess changes in microbial communities over time (Kent and Triplett, 2002), some of which are reviewed below.

2.4.2.2.6 Gradient gel electrophoresis

When a single set of primers is used for PCR, one band of amplified fragments of the same size usually results, although it may contain many closely-related but unidentical genes. Therefore, to resolve the different forms of the gene before further analysis, an additional step is required (Madigan *et al.*, 2003; Malik *et al.*, 2008). Denaturing gradient gel electrophoresis (DGGE) may be used for this purpose (Malik *et al.*, 2008).

Muyzer *et al.* (1993) described DGGE for directly estimating the genetic diversity of complex microbial communities. It involves the electrophoresis of 16S rDNA PCR amplicons (prokaryotes) or 18S rDNA amplicons (eukaryotes) in polyacrylamide gels

that contain a linearly increasing gradient of denaturants (urea and formamide). DNA fragments of the same size but differing in base sequence are separated on the basis of the electrophoretic mobility of a partially melted DNA molecule. Differential migration of DNA fragments occurs because more denaturant is required to separate sequences with a high G+C content, as there are three H bonds between the complementary nucleotides G and C but only two between adenine (A) and thymine (T), holding the DNA strands together (Nakatsu, 2007). The melting of fragments proceeds in discrete so-called 'melting domains', that is, stretches of base pairs with an identical melting temperature. Double-stranded DNA begins to 'melt' as soon as a region containing a high enough concentration of denaturant is reached. Migration then stops due to the transition of helical to partially melted molecules. Differences in base sequence control differences in melting properties. Therefore, individual bands in a DGGE gel represent different forms of a given gene, which may vary only very slightly in sequence (Muyzer *et al.*, 1993).

A variation of the method is to attach a GC-rich clamp to the end of one of the PCR primers so modifying the melting behaviour of the fragment thus generated (Muyzer *et al.*, 1993). The GC clamp ensures that part of the DNA fragment remains double-stranded during DGGE, which prevents it from denaturing completely into single strands (Kirk *et al.*, 2004). This allows for the detection of almost all possible sequence variations during subsequent DGGE (Myers *et al.*, 1985; Muyzer *et al.*, 1993) (Figure 2.2). Sharp bands are produced when a GC clamp is used, as migration of the fragments is stabilized (Nakatsu, 2007).

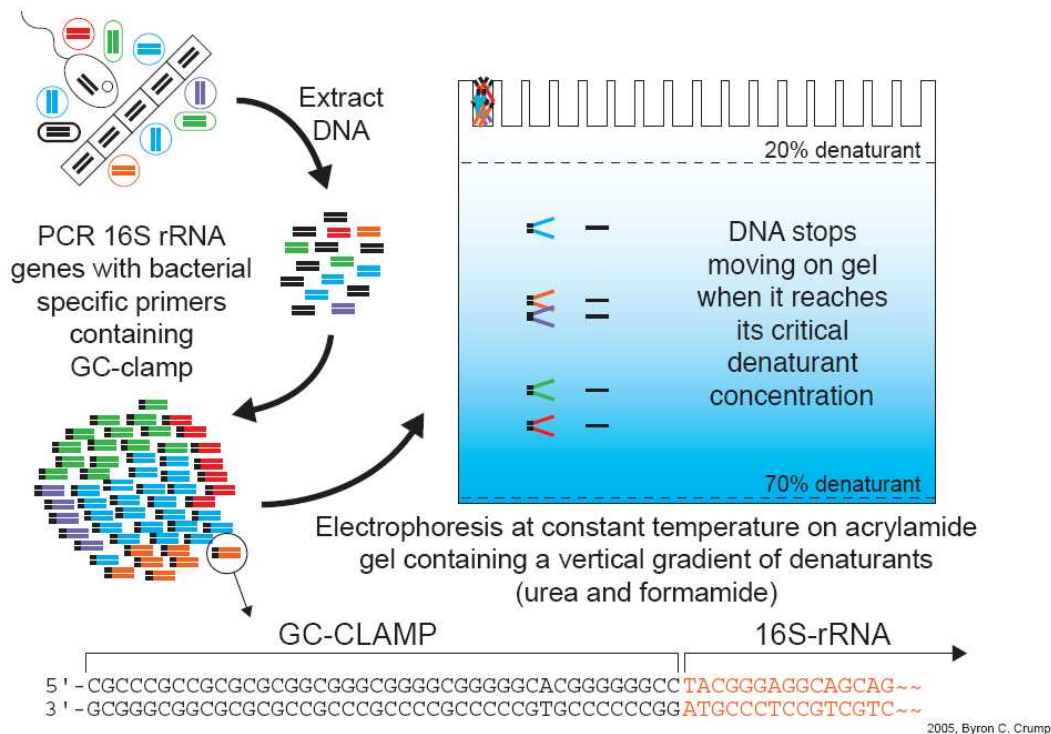


FIGURE 2.2 Schematic diagram of PCR-DGGE showing steps in the procedure from: (i) DNA extraction to (ii) PCR of rRNA genes to (iii) the separation of DNA fragments with a GC clamp in a denaturing gel (Crump, 2005).

Individual bands on the gel may be excised and sequenced, allowing the actual species present in the community to be determined, by comparing these sequences with those of known species in databases (Madigan *et al.*, 2003). Modern sequencing methods have formed the basis for analyses of species-specific habitat requirements, models of single-species and community dynamics, and assessments of the functional roles of species within an ecosystem (Ovaskainen *et al.*, 2010).

If specific genes, such as those coding for different metabolic processes are used in a DGGE analysis, information is obtained on the number of different types of organisms in a community that contain those genes, thus revealing the biocomplexity of a habitat (Madigan *et al.*, 2003; Lynch *et al.*, 2004). DGGE analysis has been used successfully to identify sequence variations in a number of genes from various different microorganisms; for direct genomic DNA analysis of organisms with large genomes (Muyzer *et al.*, 1993); to profile complex microbial communities, and to assess their phylogenetic and functional diversity (Kennedy and Gewin, 1997; Kelly, 2003). For example, Fjellbirkeland *et al.* (2001) characterised the diversity of

methanotrophic communities in an agricultural soil, using DGGE analysis of functional markers together with a phylogenetic marker. Wartainen *et al.* (2003) used PCR-DGGE and sequencing to show that only a low abundance of different methanotrophs occurred in different high Arctic wetland soils. Duineveld *et al.* (2001) used PCR-DGGE of 16S rRNA as well as of DNA fragments coding for 16S rRNA, in an analysis of bacterial communities in *Chrysanthemum* rhizospheres.

Fungal species profiles obtained directly from environmental samples have also been investigated using DGGE analysis of PCR amplified SSU rDNA molecules. These fungal groups would not normally have been detected as they are difficult to cultivate, but most of the PCR amplicons were successfully separated by DGGE (Vainio and Hantula, 2000). Sekiguchi *et al.* (2008) used PCR-DGGE in a comparative analysis of environmental variability and fungal community structure, in soils under conventional and organic farming systems. Gomes *et al.* (2003) showed that DGGE of fungal 18S rDNA fragments could discriminate between the fungal communities associated with the rhizospheres of young maize plants and those of senescent maize plants. In another study, various wood-inhabiting fungi were identified using PCR-DGGE of the ITS regions, followed by sequencing (Ovaskainen *et al.*, 2010).

The advantages of DGGE are that, in comparison to cloning, it is a relatively rapid, reliable procedure for evaluating the distribution of amplifiable sequence types, and also permits the separation of PCR products from different organisms. In addition, it detects populations in a sample that cannot be identified by cloning (Kennedy and Gewin, 1997). Concurrent analysis of multiple samples is also possible (Kirk *et al.*, 2004). It also enables spatial/temporal changes in microbial community structure to be monitored and provides a simple view of the dominant species in a sample (Malik *et al.*, 2008).

The disadvantages of DGGE are that the procedure is laborious (Kirk *et al.*, 2004), not all populations are detected and sequence data is limited. In addition, separation of fragments greater than 500 base pairs in length is reduced (Kennedy and Gewin, 1997; Malik *et al.*, 2008). This prevents adequate evaluation of phylogenetic relationships, as the limited fragment size may lack the required sequence specificity (Aguilera *et al.*, 2006; Malik *et al.*, 2008). Another disadvantage of DGGE is that as

denaturation characteristics and GC content separate bands, specific bands may contain more than one species, or several copies of the same amplified section, leading to an underestimation of species numbers (Marschner *et al.*, 2001; Porter, *et al.*, 2008). Also, most of the species found in soil are present only in relatively small numbers. Thus, if fewer than about one million of a bacterial species are present, they are not detectable by PCR-DGGE (Dilly *et al.*, 2004). Conversely, some species generate multiple bands, due to the heterogeneity of their 16S rDNA nucleotide sequences, thus leading to an overestimation of species numbers (Nakatsu *et al.*, 2000). Thus band intensity may not be a true reflection of microbial community abundance (Malik *et al.*, 2008).

Calábria de Araújo and Schneider (2008) reported that DGGE of genomic DNA was suitable for the detection of numerically important organisms but not for identification of the dominant organisms in a small community. The multiple bands they obtained for single organisms when targeting the V3 region, made interpretation of the DGGE profiles very difficult. In contrast, Zhong *et al.* (2007) found that PCR-DGGE could detect only the dominant species present in soil samples. Nakatsu *et al.* (2000), when evaluating microbial populations from soils under different agronomic treatments, reported that DGGE was useful as a preliminary step to differentiate between communities, and allowed for the rapid screening of multiple samples for ecological studies. A limitation was that quantitative analysis of all samples was not possible, as in highly diverse ecosystems, the large number of bands produced could not adequately be resolved.

Temporal temperature gradient electrophoresis (TTGE) and thermal gradient gel electrophoresis (TGGE) are two similar methods for studying diversity (Kirk *et al.*, 2004; Malik *et al.*, 2008). TTGE and TGGE are variations of DGGE where separation of PCR products in a polyacrylamide gel is achieved by means of a thermal gradient rather than a denaturing gradient (Heuer and Smalla, 1997a; Kent and Triplett, 2002). The number of bands produced during DGGE, TGGE and TTGE are proportional to the number of dominant species in the sample (Malik *et al.*, 2008).

2.4.2.2.7 Single strand conformation polymorphism (SSCP)

SSCP detects sequence variations between different PCR amplicons from 16S rRNA genes, and is based on the differential intra-molecular folding of DNA strands. Characteristic band patterns are formed in a polyacrylamide gel by bacterial strands and the relative diversity of bacterial communities is then measured, enabling the detection of a population making up only 1.6% of the community (Schweiger and Tebbe, 2000; Liu *et al.*, 2006). Spatial and temporal changes in a community are revealed by this method (Kennedy and Gewin, 1997; Smalla *et al.*, 2001; Lynch *et al.*, 2004). Limitations include PCR bias and the production of several bands by a bacterial species due to different conformations of the single-stranded PCR amplicons, or to the presence of several operons (Lynch *et al.*, 2004).

2.4.2.2.8 Terminal restriction fragment length polymorphisms (T-RFLP)

T-RFLP analysis involves restriction digestion (using endonucleases that cleave specific DNA sequences) of fluorescent end-labelled rRNA PCR amplicons from microbial community DNA, followed by electrophoresis. The complex community profiles thus obtained may be used for phylogenetic interpretation and provide quantitative estimates of diversity, as each fragment represents a single ribotype (Tiedje *et al.*, 1999; Dollhopf *et al.*, 2001; Lynch *et al.*, 2004). T-RFLP has been used to assess representivity of soil fungal community profiles in soil ecological studies (Schwarzenbach *et al.*, 2007). However, it is limited by DNA extraction, PCR biases, and the choice of universal primers. It may also underestimate true diversity (Kirk *et al.*, 2004). In bacterial analyses, community-level T-RFLP profiles are highly robust but not in fungal profiles, as sample size seems to have a greater effect (Schwarzenbach *et al.*, 2007).

2.4.2.3 PCR-independent techniques

2.4.2.3.1 DNA reassociation

An alternative to the phenotypic approach for measuring bacterial genetic diversity is DNA reassociation. By comparing the reassociation time of total community DNA with a standard curve of reassociation kinetics of a known number of cultured genomes, the first culture-independent estimate of 4600 genomes per gram of soil was determined. In this method, the total DNA extracted from the environment is thermally denatured and allowed to re-anneal. The rate of reassociation depends on the similarity of sequences present. With an increase in the DNA sequence diversity, the DNA association rate decreases (Torsvik *et al.*, 1990 a, b). Renaturation of homologous single-stranded DNA follows second-order reaction kinetics. The fraction of renatured DNA may be expressed as a function of the product (C_0^t) of DNA nucleotide concentration (C_0) in moles per litre and the reaction time (t) in seconds. Under specific conditions, C_0^t for a half-completed reaction ($C_0^{t/2}$) is proportional to the genome size or the complexity of the DNA present (defined as the number of nucleotides in the DNA of a haploid cell, without repetitive DNA). Similarly, bacterial community genetic diversity can be expressed using $C_0^{t/2}$ as a diversity index (Torsvik *et al.*, 1990a; Kirk *et al.*, 2004). Highly purified DNA is required to estimate prokaryotic diversity and as DNA from soil microbial communities is very complex, the reassociation rate is low. Good estimates of $C_0^{t/2}$ require long reassociation times to reach 50% reassociation (Lynch *et al.*, 2004).

2.4.2.3.2 Guanine plus cytosine (G+C) analysis

This method is based on the fact that prokaryotic DNA varies from about 24–76% G+C content vs A+T (adenine plus thymine) and organisms in specific taxonomic groups vary no more than 3–5% in G+C content. Separation by base composition is effected by the binding of bisbenzimidazole to adenine and thymidine, which changes the buoyant density of DNA. Centrifugation in a CsCl density gradient then establishes a gradient of DNA fragments of differing G+C concentrations. The analysis is not subject to PCR bias or ineffective hybridisation, and provides a coarse

level of resolution. It is a quantitative method separating low biomass fractions from the dominant biomass but a disadvantage is that large amounts of DNA (50 µg) are required (Tiedje *et al.*, 1999).

2.4.2.3.3 Fluorescence in situ hybridisation (FISH)

FISH involves direct microscopic examination of microorganisms and can be used to evaluate their function and distribution *in situ*, by means of oligonucleotide hybridisation probes, complementary to regions of the 16S rRNA gene. FISH may be used together with DAPI (4', 6'-diamidino-2-phenylindole), 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT)-formazan, or 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining, to evaluate total abundance or active cell count. FISH targets specific groups of organisms but has limited application because of low throughput (Kent and Triplett, 2002; Lynch *et al.*, 2004; Hartmann *et al.*, 2007).

2.4.2.3.4 Metagenomic libraries

Metagenomic libraries contain total genomic DNA isolated directly from soil microbial populations and are used to investigate genetic diversity of overall community structure. To access soil metagenomic DNA, large fragments of soil microbial DNA are cloned into bacterial artificial chromosome (BAC) vectors, which have the advantage of being able to maintain stable inserts as large as 100 Kb, in an *E. coli* host. These libraries are useful for investigating microbial diversity as they provide genetic information on uncultured soil microbes and may form the basis for new studies linking phylogenetic and functional information. As PCR amplification is not required, PCR bias is eliminated (Rondon *et al.*, 2000; Liles *et al.*, 2003; Lynch *et al.*, 2004).

2.4.2.3.5 Microarrays

Conventional hybridisation techniques involve base pairing between two complementary, single-stranded nucleic acid molecules to form a stable double-stranded molecule. Nucleic acid hybridisation can be used to identify a particular

clone if a DNA or RNA probe, complementary to the gene of interest, is used (Brown, 1980). This process is very time consuming, restricts the number of probes that can be applied, and allows only limited information to be obtained (Kelly, 2003).

Consequently, a powerful technique, microarrays, has been developed, which enables an entire genome to be analysed simultaneously (Madigan *et al.*, 2003; Hartmann *et al.*, 2007). This was made possible by developments in genomic sequencing, by advances in miniaturisation and in the high-density synthesis of nucleic acids on solid supports (usually glass or polymer slides) (Causton, 2002). A large number of nucleic acids can be spotted onto a very small surface area, and are then hybridised simultaneously (Kelly, 2003; Hartmann *et al.*, 2007). Two types of microarrays are available: cDNA arrays (using PCR products) and oligonucleotide arrays (oligoarrays), where the spots consist of oligonucleotides (Causton, 2002).

Microarrays can characterise environmental microbial populations and their function, as the expression of thousands of genes can be compared both qualitatively and quantitatively (Kelly, 2003; Madigan *et al.*, 2003; Hartmann *et al.*, 2007). They facilitate the analysis of functional assemblages or guilds, by differentiating among a broad and partly unknown variety of DNA targets of high sequence identity (Taroncher-Oldenburg *et al.*, 2003). Küster *et al.* (2007) used target microarrays and an *in silico* approach to profile transcriptomes in mycorrhizal symbioses.

Microarray technology avoids some of the limitations of 16S rRNA-based protocols as functional genes are analysed (Kelly, 2003) and the method is relatively quick (Tiedje *et al.*, 2001).

2.5 CONCLUSIONS

The loss of biodiversity, both above- and below-ground, and the effects of human activities on this diversity, are growing concerns. At the same time, the search for cost-effective, sustainable, low-input agricultural systems is ongoing. Furthermore, there is an increasing awareness of the important role of soil microbes in ecosystem processes and of the concept of soil quality as an indicator of soil health. The study of

these concepts has largely been made possible by the advent of powerful new molecular approaches. These techniques are invaluable since traditional methods of determining soil microbial community composition revealed only a very small proportion of the microorganisms present, leading to soil being described as a 'black box'. However, no single method is without its drawbacks. As such, if the contents of the 'black box' are to be determined, the best approach is most likely multifaceted, including traditional, CLPP and molecular techniques. The remainder of this thesis describes such an approach.

Chapter 3

EFFECTS OF LAND USE AND MANAGEMENT ON SOIL BACTERIAL DIVERSITY AS MEASURED BY PCR-DGGE*

3.1 INTRODUCTION

It is well known that anthropogenic activities such as changes in land use and management practices influence structural and functional diversity of soil microbes (Kennedy and Gewin, 1997; Mendum and Hirsch, 2002). In KwaZulu-Natal, South Africa, previous studies have shown, that agricultural land use, such as arable cropping, pasture and forestry, affects both soil organic matter status and the size and activity of the soil microbial biomass (Dominy and Haynes, 2002; Haynes *et al.*, 2003). It was demonstrated that soils under arable crops (sugarcane and maize) and annual ryegrass pasture had lost organic matter when compared with undisturbed native grassland, whereas under commercial forestry, organic matter was conserved. Conversely, under permanent kikuyu dairy pasture, soil organic matter content had increased (Haynes *et al.*, 2003). Microbial biomass C concentrations and microbial quotient biomass C (as a percentage of organic C) showed the same general trend as soil organic C (Dominy and Haynes, 2002).

Nsabimana *et al.* (2004) and Graham and Haynes (2005) determined the catabolic diversity of soil microbial communities under the above-mentioned managements, and found large differences in catabolic capability among the microbial communities inhabiting the different land use types. Nsabimana *et al.* (2004) concluded that greatest catabolic diversity occurred in soil under native grassland and lowest diversity under exotic plantations. However, while Graham and Haynes (2005) also found greatest catabolic diversity under native grassland, the lowest values were recorded under arable crops such as sugarcane and maize.

* Parts of this chapter have been published in: Wallis *et al.* (2010), *Applied Soil Ecology* 46, 147-150.

Soil organic matter status and microbial biomass size and activity are also affected by soil management practice within a particular land use, as shown by studies on the long-term (65 years) sugarcane trash management trial at Mount Edgecombe, KwaZulu-Natal, South Africa. Green cane harvesting with retention of a trash blanket, resulted in an accumulation of organic matter and a concomitant increase in microbial biomass and basal respiration, when compared with pre-harvest burning (Graham *et al.*, 2002a, b). In addition, fertilized plots generally had a greater soil organic C content and microbial biomass than unfertilized plots due to higher crop yields and, therefore, greater returns of organic residues to the soil (Graham *et al.*, 2002b). In studies of catabolic diversity, Graham and Haynes (2005; 2006) showed that the catabolic capability of soil bacteria under trashed cane differed markedly from that of communities under burnt cane, with the addition of fertilizer having a slight effect. Their results demonstrated that catabolic diversity was substantially greater under trashing than burning (Graham and Haynes, 2005).

While the above studies investigated the effects of land use and management on bacterial functional (catabolic) diversity, no investigations into their effects on the genetic diversity (community composition) have, thus far, been undertaken at these sites. Functional diversity is not necessarily a reflection of genetic and taxonomic diversity since one species of microorganism may perform many functions or, conversely, a multiplicity of microbial species may perform a particular function (Giller *et al.*, 1997). However, studies in other parts of the world have shown that both land use and management can alter the taxonomic diversity and composition of soil microbial communities (Kowalchuk *et al.*, 2000; Marschner *et al.*, 2001; Webster *et al.*, 2002; Miethling *et al.*, 2003).

Molecular profiling using DGGE analysis of amplified 16S rDNA is a powerful tool in the study of bacterial diversity in complex environments (Smalla *et al.*, 2001; Smit *et al.*, 2001). Therefore, PCR-DGGE was used to determine the impact of different land uses and management practices on the genetic (structural) diversity of the soil bacterial communities at two sites in KwaZulu-Natal, where the organic matter status and the size, activity and catabolic diversity of the microbial communities had previously been studied (Dominy and Haynes, 2002; Graham *et al.*, 2002a, b).

3.2 MATERIALS AND METHODS

3.2.1 Sites and soils

Two long-term experimental sites located in KwaZulu-Natal, South Africa, were selected for this study. These sites were chosen as all the land uses and management practices have been in place for many years, and the ecosystem was deemed to have achieved a degree of equilibrium before sampling took place.

For comparison of the effects of different land uses on soil microbial populations, fields on Baynesfield Estate (site 1) in the KwaZulu-Natal midlands (27° 22'S and 30° 45'E) previously described by Dominy and Haynes (2002), were used. Cropping histories were: > 50 years permanent kikuyu grass pasture (*Pennisetum clandestinum*, Chiov) (KIK); > 30 years continuous, pre-harvest burnt sugarcane (*Saccharum officinarum*, Linn.) (SC); > 30 years continuous maize (*Zea mays*, Linn.) under conventional tillage (M); > 20 years pine plantation (*Pinus patula*, Schiede) (PF); 6 years wattle plantation (*Acacia mearnsii*, De Wild) (W) planted between the stumps of a harvested 20-year *Eucalyptus* plantation; and undisturbed native grassland (NAT). The native, high diversity grassland is situated in an area separated from agricultural land on one side by a roadway and on the other by old buildings. It has never been cultivated or fertilized and is typical of grasslands in the area. The vegetation is dominated by *Themeda* grassland, with grasses such as *Monocymbium ceresiiforme*, *Trachypogon spicatus*, *Tristachya leucothrix*, *Eragrostis racemosa* and *Diheteropogon amplexans* prominent (Low and Rebelo, 1996). All the agricultural fields had been fertilized with typical annual rates for arable and grassland sites of approximately 100–300 kg N ha⁻¹, 50–150 kg K ha⁻¹ and 25–50 kg P ha⁻¹. For forestry plantation soils, typical fertilizer rates are approximately 50–100 kg N ha⁻¹, 10–60 kg K ha⁻¹ and 10–25 kg P ha⁻¹, applied at the start of each rotation. The maize, sugarcane and pasture sites are irrigated during the dry winter period. Mean annual rainfall is 844 mm and mean monthly temperatures range from a maximum in January of 21.1°C (maximum 25.9°C, minimum 16.3°C) to a minimum of 13.3°C in June (maximum 16.3°C, minimum 5.6°C). The site is located on soils classified as Hutton form

(Farmingham series) (Soil Classification Working Group, 1991) or as Rhodic Ferralsols (IUSS Working Group WRB, 2006).

To compare trash management effects on the soil microbial communities, an experimental site situated at the South African Sugarcane Research Institute (SASRI) at Mount Edgecombe (31° 04'S and 29° 43'E) was used (site 2). All sugarcane varieties used at SASRI are hybrids, in this case *Saccharum officinarum* x *S. spontaneum* var. N27. The site BT/139, previously described by Graham *et al.* (2002a), was established in 1939 and is a long-term pre-harvest burning and crop residue (trash) retention trial. Experimental treatments studied were: (i) green cane harvested with retention of a trash mulch (100% cover), either fertilized [TF] or unfertilized [TFo]; and (ii) pre-harvest burnt cane with harvest residues (tops) raked off, either fertilized [BtoF], or unfertilized, [BtoFo]. Annual fertilizer applications are 160 kg N ha⁻¹, 32 kg P ha⁻¹ and 160 kg K ha⁻¹. The experiment is replicated in a randomized split plot design, the main plots being trash management treatments. Mean annual rainfall is approximately 950 mm. The soil is classified as Arcadia form, Lonehill family (Soil Classification Working Group, 1991) or as a Chromic Vertisol (IUSS Working Group WRB, 2006).

3.2.2 Site sampling

Soil samples from both sites were collected from a depth of 0–5 cm during the fallow period at the end of the dry winter season in 2004. For the different land uses at Baynesfield, each field or plantation was divided into three separate sampling areas (each approximately 60 m²). Composite (bulked) samples, consisting of ten random subsamples per sampling area were collected, resulting in three separate bulked samples per land use. For comparison of trash management at Mount Edgecombe, 10 soil samples (0–5 cm depth) were randomly taken from under the rows of three replicates of each treatment over the entire area. The subsamples from each replicate were bulked so that there were three independent samples for each land management. All bulked, field-moist samples were thoroughly mixed and sieved (2 mm) in the laboratory. Subsamples for DNA extraction were stored in plastic bags at 4°C and further subsamples for chemical analysis were again sieved (< 1 mm) and air-dried.

3.2.3 Chemical analysis

The air-dried, sieved soil samples were analysed by routine testing methods (Manson *et al.*, 1993) by the Soil Fertility and Analytical Services Division of KwaZulu-Natal Department of Agriculture and Environmental Affairs, Cedara. Exchangeable acidity and exchangeable Ca and Mg were extracted with 1 M KCl (1:10 soil:solution ratio) for 10 minutes. The pH of the extract was measured with a glass electrode and exchange acidity by titration. Exchangeable K and extractable P, Zn and Mn were extracted with AMBIC reagent (0.025 M NH_4HCO_3 ; 0.01 M NH_4F ; 0.01 M EDTA [ethylenediaminetetra-acetic acid] at pH 8.0) using a 1:10 soil:solution ratio for 10 minutes. Total exchangeable Ca, Mg, K, Mn and Zn were analysed by atomic absorption spectrophotometry and P by the molybdenum blue method. Total exchangeable Ca, Mg, K and acidity were summed to give 'total cations', an approximation of the effective cation exchange capacity (ECEC). While N (the C/N ratio) is important in determining the structure and function of the soil microbial communities, this factor was not considered here as none of the current extraction methods can predict soil N supply accurately (Ros, in press). Organic C content was estimated by the Walkley-Black dichromate oxidation procedure (Walkley, 1947) in the University of KwaZulu-Natal laboratories.

3.2.4 DNA extraction

The UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc. CA, USA), was used to extract and purify DNA directly from the soil samples according to the manufacturer's instructions (using the protocol designed to maximize yields). For the Baynesfield samples, 0.5 g field-moist soil from maize, sugarcane, native grassland, kikuyu pasture, pine and wattle respectively, were added to MO BIO bead-beating tubes. For the Mount Edgecombe samples, because the DNA concentration was low, 0.7 g field-moist soil from each replicate of the four treatments was added to the MO BIO tubes. All DNA samples were prepared in duplicate, one set being stored at -20°C for subsequent analyses and the other at -80°C as a long-term back up.

3.2.5 PCR amplification of 16S rDNA fragments

Initially, three primer pairs were used to amplify prokaryotic 16S rDNA from the different soil communities, namely, P63fGC/534r, 341fGC/534r, and 968fGC/1410r (Watanabe *et al.*, 2001). While all primer pairs amplified efficiently, only 341fGC/534r consistently gave good results in subsequent DGGE analysis. Therefore, this universal bacterial primer pair, which amplifies the variable V3 region of bacterial 16S rDNA gene sequences, from nucleotides 341–534 (*E. coli* numbering) (Dilly *et al.*, 2004), was chosen for PCR amplification of soil bacterial DNA. To ensure efficient separation of fragments in subsequent DGGE, a GC clamp was added to the 5' end of the forward primer, 341f (Muyzer *et al.*, 1993, 1996) (Table 3.1).

TABLE 3.1 Primers used for PCR amplification of soil bacterial 16S rDNA

| Primer ^a | Position ^b | Sequences (5'- 3') | References |
|---------------------|-----------------------|---|--------------------------------|
| 341 f GC | 341-357 | CCTACGGGAGGCAGCAG (GC clamp attached to the 5' end of 341f) | Muyzer <i>et al.</i> 1993,1996 |
| 534 r | 518-534 | ATTACCGCGGCTGCTGG | " |
| GC clamp | | CGCCCGCCGCGCGGGCGGGC GGGGCGGGGGCACGGGGGG | " |

^a f: forward primer; r: reverse primer.

^b corresponding to the numbering in the *Escherichia coli* sequence.

Soil DNA was amplified in a Perkin Elmer Applied Biosystems Gene Amp 2400 thermal cycler (PE Corporation. Applied Biosystems, Foster City, CA, USA). Reaction mixtures were prepared using the PCR Core Kit (Roche Diagnostics) according to the manufacturer's instructions, and were optimized for the experimental soils. Each reaction contained 1.0 µl template DNA (Baynesfield soil samples) or 2.0 µl template DNA (Mount Edgecombe soil samples); 0.5 µM of each primer; 200 µM of each dNTP; 2.5 mM MgCl₂; 1.25 U Taq DNA Polymerase (Roche Diagnostics); sterile MilliQ H₂O; and 1.0 µl (20 mg ml⁻¹) Bovine Serum Albumen (BSA), added to

prevent inhibition of amplification, by organic compounds co-extracted from the soil (Pecku, 2003) in a buffered final volume of 50 μ l.

PCR conditions were as described in a method, which had been adapted and optimized for environmental samples, in the laboratories of the University of KwaZulu-Natal (Pecku, 2003). These were: an initial denaturation step (94°C/5 min), followed by 35 cycles comprising: a denaturing (92°C/1 min), annealing (55°C/1 min), and elongation (72°C/1 min) step, and finally, a single elongation step (72°C/10 min). Amplification products (~193 bp) were analyzed by electrophoresis of 5 μ l aliquots in 2% agarose gels stained with ethidium bromide, and visualized on a UV transilluminator.

3.2.6 Community fingerprinting by DGGE

A DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) was used for DGGE. Equal amounts of PCR amplicons (visual estimation from agarose gels) (Pennanen *et al.*, 2004), contained in 20 μ l aliquots, were loaded onto 8% (v/v) polyacrylamide gels (Sigma, acrylamide/bisacrylamide 40% solution, mix ratio 19:1) containing a linear chemical gradient ranging from 30–70% denaturant (100% denaturant corresponds to 7 M urea plus 40% (v/v) deionized formamide) in 1 \times TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM disodium EDTA, pH 8.3). The gels were allowed to polymerize for 1.5–2.5 hours, after which electrophoresis was run for 16 hours at 70 volts and 60°C in 1 \times TAE buffer. After the run, the gels were silver-stained (H. van Verseveld, *pers. comm.*),[†] scanned and photographed with the Bio-Rad VersaDoc gel documentation system.

3.2.7 Data analysis

Image analysis of the one-dimensional (1-D) DGGE gels, by Bio-Rad Quantity One™ software (version 4.5), was used for band detection and band intensity quantification. The Bio-Rad Versa Doc imaging device converts signals from biological samples into digital data. The signal intensity of the pixels in the bands is measured as OD units

[†] van Verseveld, H., 2001. Vrije Universiteit van Amsterdam.

(Bio-Rad Quantity One™ Instruction Manual, 2005). Bands were considered common if they migrated equidistantly in a gel (Nakatsu *et al.*, 2000). After background subtraction of individual lanes using the ‘rolling disk’ method, the position and total number of bands was determined for each sample, and manually checked and adjusted (Wakelin *et al.*, 2008a). Thereafter, banding patterns were scored as binary presence (1) or absence (0) matrices, and recorded in spreadsheets. Average relative intensity of individual bands was then calculated and this data also transferred into spreadsheets (Hoshino and Matsumoto, 2007). Band intensity was expressed as a percentage of the summed average band intensities across each gel. Each band was inferred to represent individual groups of species having similar melting domains, whereas the band intensity indicates the relative abundance of the group under the PCR conditions used (Eaton and Farrell, 2004; Wakelin *et al.*, 2008a). Comparisons of lanes were confined to those within single gels because of variation between gels (Pennanen *et al.*, 2004).

3.2.8 Statistical analysis

For practical reasons, as the sampling sites at the Baynesfield Estate are pre-existing land uses, the study was not established in a statistically rigorous experimental design (Lepš and Šmilauer, 2003). Accordingly, the data were analysed as a completely randomised design with three separate replications per land use, although the land use types were not truly replicated within a formal experimental design. Therefore, statistics were used only to describe the basic features of the study data and have limited application for extrapolation to other similar sites. By contrast, at Mount Edgecombe, the experiment is replicated in a statistically correct, randomized split plot design, so inferential statistics could be applied to this data.

Soil physicochemical properties were analysed using CANOCO 4.5 for Windows (Microcomputer Power, Ithaca, NY) (ter Braak and Šmilauer, 1997). Soil differences were tested by Multi-Response Permutation Procedures (MRPP) standardized to standard deviation = 1, using PC-ORD 4.25, and by Principal Component Analysis (PCA). Tests were done on the standardized data, where the original value for each soil variable from each site was divided by its standard deviation (across samples) to

allow comparisons of variables on a common scale (as was done in the PCAs). Dissimilarity between samples was measured by Euclidian distance.

Canonical correspondence analysis (CCA) using CANOCO 4.5 for Windows (ter Braak and Šmilauer, 1997) was used to assess the effects of measured soil physicochemical variables [organic C; pH (KCl); exchange acidity; total cations (ECEC); exchangeable K, Ca and Mg; and extractable P] on soil microbial community structure (described by DGGE profiles), under the different land uses at Baynesfield and trash managements at Mount Edgecombe. Because the soil variables at site 2 were highly collinear (variance inflation factors > 20 for most of the variables), a sub-set of variables with a relatively independent significant effect on soil microbial composition, was selected through permutation testing (Monte Carlo Permutation test, $n = 499$) in a stepwise selection process. CCA is specifically designed to compare data in the form of contingency tables where variables (in this case, bands) are enumerated.

Microbial community composition (presence/absence of DGGE bands) in soils from fields with different land uses (Baynesfield) or trash management plots (Mount Edgecombe), was compared by non-metric multidimensional scaling (NMS), rotated by PCA to produce low-dimensional plots, with the horizontal axis aligned to the direction of maximum compositional variation, based on Sørensen's distance coefficient, using the program WinKyst 1.0 (Šmilauer, 2002) and tested by MRPP, standard deviation = 1. The principle behind NMS is to find a graphical representation of the data in a few dimensions, the distance in the ordination of the samples reflecting the (dis)similarities between the respective DGGE patterns as closely as possible (Hernesmaa *et al.*, 2005).

Genstat Release 9.1 (PC/Windows XP) 2006, Lawes Agricultural Trust (Rothamsted Experimental Station) was used for analyses of variance (ANOVA). One-way ANOVA of species richness (S) (number of bands present) of soil microbial communities was carried out. Relative band intensity (mass) and position were used to determine bacterial community evenness (J) and the Shannon Weaver Diversity Index (H') (Peet, 1974). The latter provides a composite measure of diversity, based on both species richness (S) (the number of bands present) and Pielou's species

evenness (J') (a measure of the equitability of the relative band intensities across the gels) (Pielou, 1977). Pairwise comparisons were made, using the Ryan/Einot-Gabriel/Welsch multiple range test. For Mount Edgecombe data, a two-way ANOVA of soil variables, and another of the main effects and interaction of the trashing and fertilizer treatments on soil bacterial richness, evenness and diversity were carried out. The results of ANOVA however, are not unequivocal, because neither the richness nor evenness parameter can be determined with certainty for prokaryotes in soil samples (Tebbe and Schloter, 2007). Least significant differences were calculated at the 5% level.

3.3 RESULTS

3.3.1 Analyses of soils at the Baynesfield experimental site

Each of the soils tested varied with respect to pH, nutrient concentration and organic matter content, all of which may act as potential selection factors affecting plant growth and soil microbial communities. The means (\pm standard deviation) of the different physicochemical properties of soils under the various agricultural land uses are summarised in Table 3.2.

Soil organic C increased in the order: SC < M < NAT = W < PF < KIK. Soil pH in increasing order was: PF < SC < M < NAT = KIK < W. Concentrations of extractable P in soils under SC and M were high at 235 mg kg⁻¹ and 163 mg kg⁻¹, respectively. Under KIK, P concentrations were 14.7 mg kg⁻¹ and under NAT, PF and W, 6.0, 5.8 and 5.4 mg kg⁻¹, respectively. Similarly, soils under SC and M were enriched with extractable K relative to those of the other land uses. Compared with the other land uses, PF soils were characterised by low exchangeable Ca and Mg and high exchange acidity. Total exchangeable cations (ECEC) were similar for all the land use soils except under PF (Table 3.2).

TABLE 3.2 Means (\pm sd) for selected physicochemical properties of the study soils collected at 0–5 cm depth from different land uses at Baynesfield Estate

| Land use | pH (KCL) | Organic Carbon | Extractable P | Exchangeable cations | | | Exch. acidity | Total cations (ECEC) |
|----------|------------|----------------|---------------------|--|--------------|--------------|---------------|----------------------|
| | | | | K | Ca | Mg | | |
| | | % | mg kg ⁻¹ | ----- cmol _c kg ⁻¹ ----- | | | | |
| SC | 4.3 (0.12) | 3.7 (0.26) | 235 (53.60) | 2.24 (0.555) | 6.78 (1.102) | 3.04 (0.513) | 0.16 (0.027) | 12.24 (1.517) |
| M | 4.5 (0.12) | 4.1 (0.19) | 163 (17.70) | 1.43 (0.150) | 6.91 (0.841) | 2.69 (0.435) | 0.07 (0.062) | 11.11 (1.140) |
| KIK | 4.6 (0.17) | 9.9 (2.11) | 15 (3.38) | 0.93 (0.375) | 7.22 (0.238) | 5.03 (0.531) | 0.10 (0.874) | 13.30 (1.915) |
| NAT | 4.6 (0.05) | 5.1 (0.58) | 6.0 (1.66) | 0.47 (0.119) | 6.63 (0.406) | 4.19 (0.337) | 0.08 (0.055) | 11.38 (0.853) |
| PF | 4.1 (0.05) | 6.5 (0.26) | 5.8 (0.49) | 0.48 (0.295) | 3.95 (0.533) | 2.62 (0.311) | 1.53 (0.381) | 8.59 (0.671) |
| W | 5.2 (0.49) | 5.1 (0.56) | 5.4 (1.55) | 0.54 (0.145) | 9.08 (1.227) | 3.57 (0.608) | 0.08 (0.027) | 13.28 (1.937) |

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = permanent kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

Overall dissimilarities among the soil samples from the different fields and plantations were calculated by MRPP. A greater difference between the soils under the various land uses than among the three replicates of each soil was shown. However, soils under M and SC could not be differentiated nor those under KIK and NAT. Results are summarised in Table 3.3.

TABLE 3.3 Multi-Response Permutation Procedures (MRPP) of soil physicochemical properties, showing pairwise comparisons of the Baynesfield soils

| Land use | Test statistic (T) | Probability (p) |
|---------------|--------------------|-----------------|
| KIK vs. M | -2.655 | 0.024 |
| KIK vs. NAT | -1.510 | 0.075 |
| KIK vs. PF | -2.984 | 0.021 |
| KIK vs. SC | -2.666 | 0.023 |
| KIK vs. W | -2.404 | 0.027 |
| M vs. NAT | -2.920 | 0.021 |
| M vs. PF | -2.905 | 0.022 |
| M vs. SC | -0.991 | 0.152 |
| M vs. W | -2.581 | 0.025 |
| NAT vs. PF | -2.981 | 0.021 |
| NAT vs. SC | -2.851 | 0.022 |
| NAT vs. W | -2.443 | 0.026 |
| PF vs. SC | -2.946 | 0.021 |
| PF vs. W | -2.876 | 0.022 |
| SC vs. W | -2.684 | 0.023 |
| All land uses | -8.035 | <0.001 |

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = permanent kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

Analysis by PCA clustered the replicate soil samples from the different land uses on the basis of the correlation between the samples and soil variables (Figure 3.1). The closer the vector for an individual variable aligns with a principal component axis, the more that particular chemical variable can be used to explain the variation in the data along that axis. ECEC, Ca, pH and exchange acidity were highly correlated with PC1 and P, K and organic C were highly correlated with PC2. PC1 accounted for 45.8%,

PC2 for 32.6% and cumulatively, for 78.4% of the total variance in the soil data. Soils under the two arable crops SC and M were closely associated with high concentrations of P and K whereas PF soils were correlated with high exchange acidity, low pH and low concentrations of ECEC. KIK soils were characterised by high concentrations of organic C and Mg, with those of NAT also associated with these two soil variables, but at lower concentrations than those in KIK soils. Soils under W were correlated with the highest pH of all the land uses at this site.

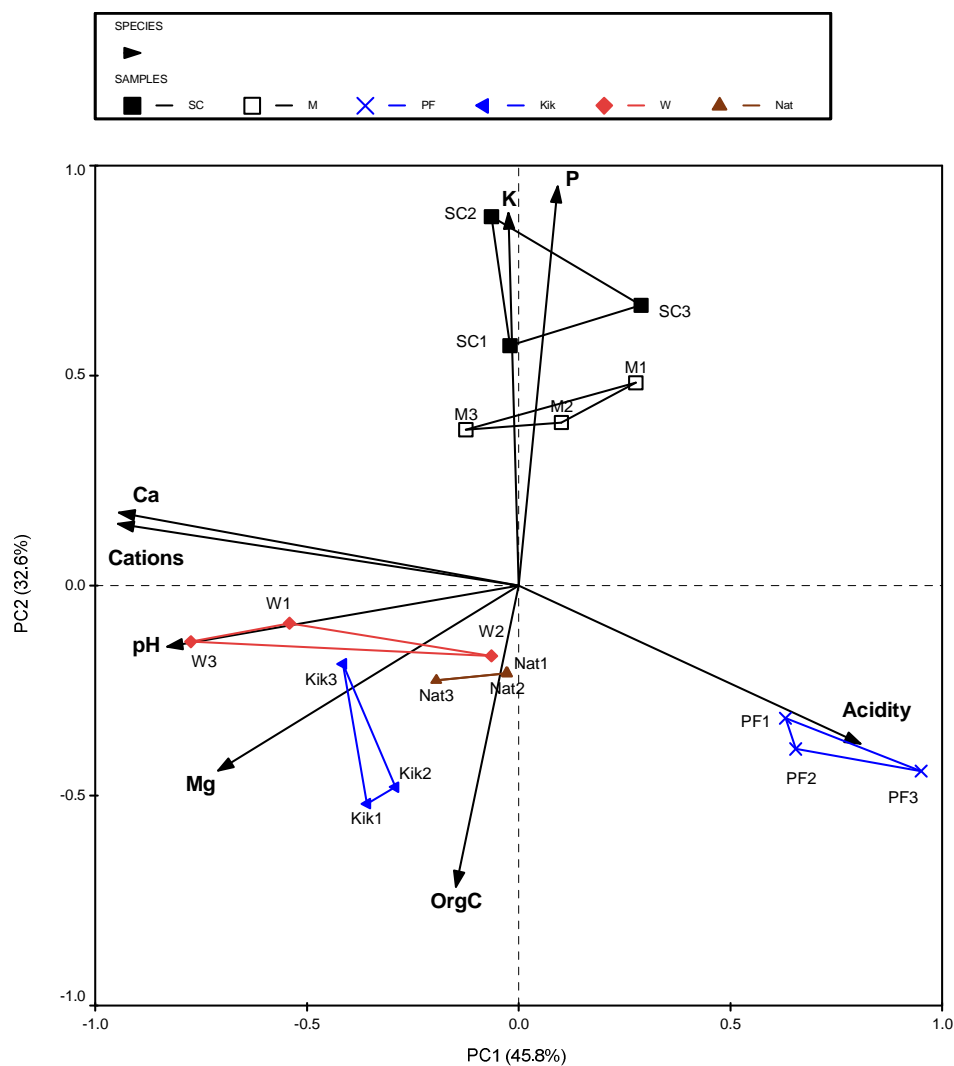


FIGURE 3.1 A PCA biplot (standardised and centred data) of sites and soil variables for subsamples of fields with various land uses at Baynesfield Estate. The PC1 (horizontal) and PC2 (vertical) components accounted for 45.8% and 32.6%, respectively, and, cumulatively for 78.4% of the total variance in the soil data.

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

CCA was used to show the effects of selected soil physicochemical variables on the bacterial community structure at site 1 (Figure 3.2). Eigenvalues for CCA 1 and CCA 2 were 0.234 and 0.161, accounting respectively, for 22.8% and 15.7% of the total variability (therefore these results should be interpreted with caution), and 33.3% and 22.9% (cumulatively 56.2%) of the variability in community composition related to soil. The soils were related to variation on axis 1 ($p = 0.002$) and along all canonical axes ($p = 0.002$). Bacterial community structure in soils under SC and M were closely associated with exchangeable K, extractable P and ECEC. Soil bacterial communities under KIK were associated with high concentrations of organic C (9.9%) and Mg, whereas lower concentrations of organic C (5.1%) and Mg were associated with bacteria in NAT soils. High acidity was correlated with the PF soil bacterial community structure.

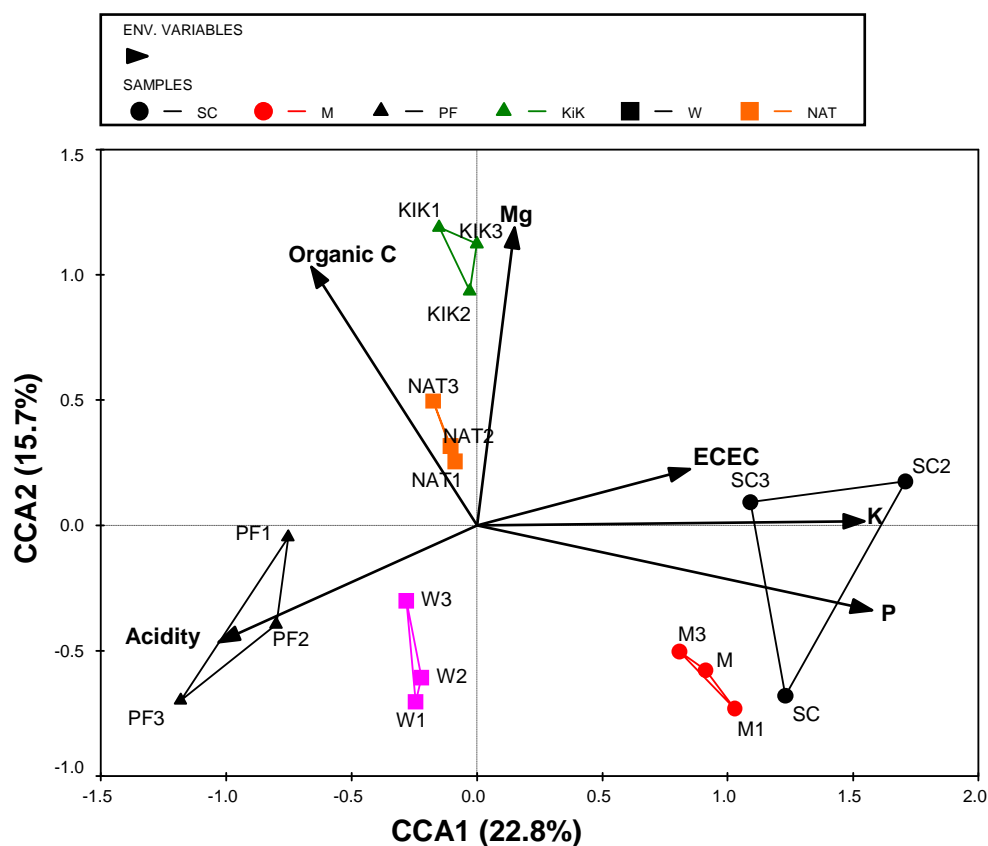


FIGURE 3.2 Plot of samples (classified by land use) and selected soil variables along the first two axes of a CCA of the effect of soils on bacterial composition (band presence) at Baynesfield.

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

The statistical significance of the relationship between environmental variables and variation in DGGE profiles was tested by the Monte Carlo Permutation test, which showed that P was the most significant variable affecting bacterial community composition, followed by acidity, Mg, ECEC, K, and organic C. (Table 3.4).

TABLE 3.4 Conditional effects of variables in a stepwise selection test in the CCA of soils on bacterial communities at Baynesfield

| Variable | Eigenvalue | P | F |
|------------------|-------------|--------------|-------------|
| P | 0.22 | 0.002 | 4.25 |
| Acidity | 0.14 | 0.006 | 3.36 |
| Mg | 0.11 | 0.008 | 2.70 |
| ECEC | 0.09 | 0.012 | 2.58 |
| K | 0.08 | 0.036 | 2.29 |
| Organic C | 0.06 | 0.042 | 2.24 |
| pH | 0.03 | 0.498 | 0.89 |
| Ca | 0.00 | 1.000 | 0.08 |

Note: All variables (in bold) were used in the CCA (significant effects on composition). In the CCA, the various inflation factors of the selected soil variables were acceptably low (< 10) indicating no severe problem of multicollinearity (i.e. the selected variables had relatively independent effects on composition).

The correlations between the selected soil variables used in the CCA and the ordination axes are shown in Table 3.5.

TABLE 3.5 Correlations between soil variables used in the CCA and compositional gradients (ordination axes)

| Variable | CCA1 | CCA2 |
|-----------|---------|---------|
| P | 0.8874 | -0.2020 |
| K | 0.8700 | 0.0099 |
| Mg | 0.0848 | 0.7079 |
| Acidity | -0.5805 | -0.2774 |
| ECEC | 0.4788 | 0.1332 |
| Organic C | -0.3724 | 0.6146 |

The results of the physicochemical analyses of Baynesfield soils showed that soil composition differed markedly under the various land uses, while CCA indicated which soil properties had affected bacterial community structure. These findings

concur with those of other workers at this site (Graham, 2003; Graham and Haynes, 2005).

3.3.2 Soil bacterial community structure at the Baynesfield experimental site

DGGE fingerprints of bacterial 16S rDNA amplicons from soils under the different land uses, contained from 15-44 bands, with some common to all lanes but varying with respect to relative band intensity. Some lanes showed several strong, dominant bands, while others had only a single dominant band. However there was a preponderance of low intensity, light bands in all the lanes, some unresolved and often closely spaced, which made subsequent analyses difficult (Plates 3.1 and 3.2).

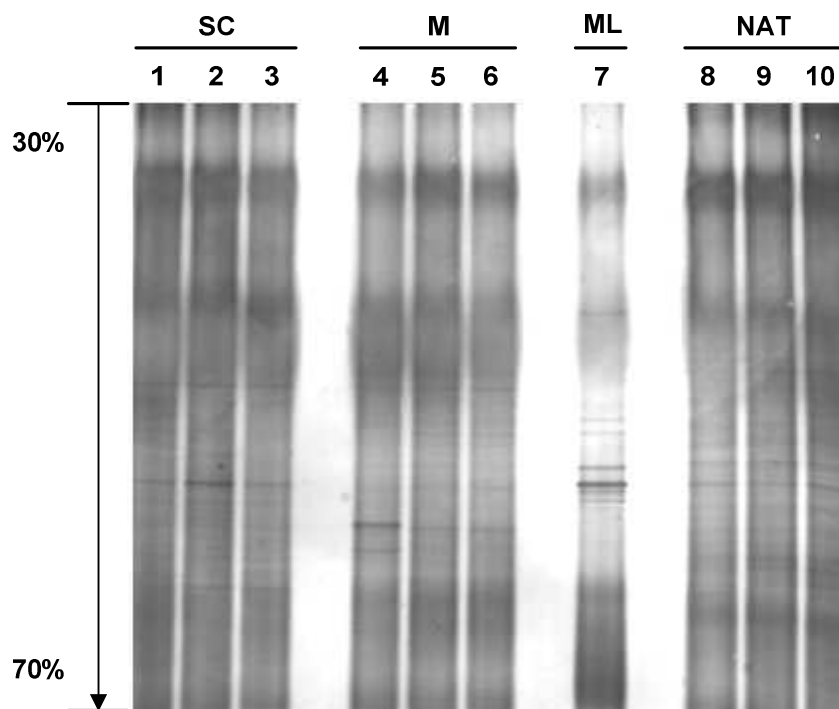


PLATE 3.1 A DGGE gel (denaturing gradient 30–70%) of 16S rDNA fragments from Baynesfield soils under different land uses.

Key: SC = sugarcane; M = maize and NAT = native grassland; ML = marker lane.

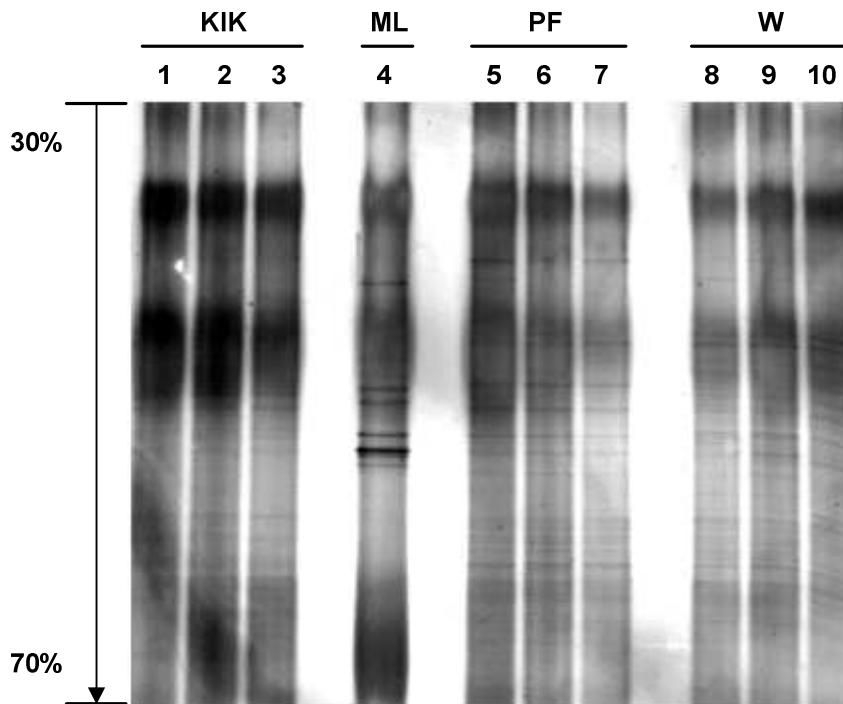


PLATE 3.2 A DGGE gel (denaturing gradient 30–70%) of 16S rDNA fragments from Baynesfield soils under different land uses.

Key: KIK = kikuyu pasture; PF = pine plantation; W = wattle plantation; ML = marker lane.

Banding patterns among the replicate samples from the same land use were highly repetitive, consistently more similar to each other, yet distinct from those of other land uses (visual assessment), indicating a low intraspecies variation but high interspecies variation. MRPP of the data confirmed that overall differences in bacterial community profiles between the different communities were greater than within the same community ($T = -9.750$, $p < 0.001$). A NMS site plot rotated by PCA (Figure 3.3) clustered the three replicates from each land use, on the basis of the presence or absence of bands. Community compositional variability within and between land use types is shown in Figure 3.3, and a plot in the NMS ordination of DGGE bands from all the replicate samples from the different land uses is shown in Figure 3.4.

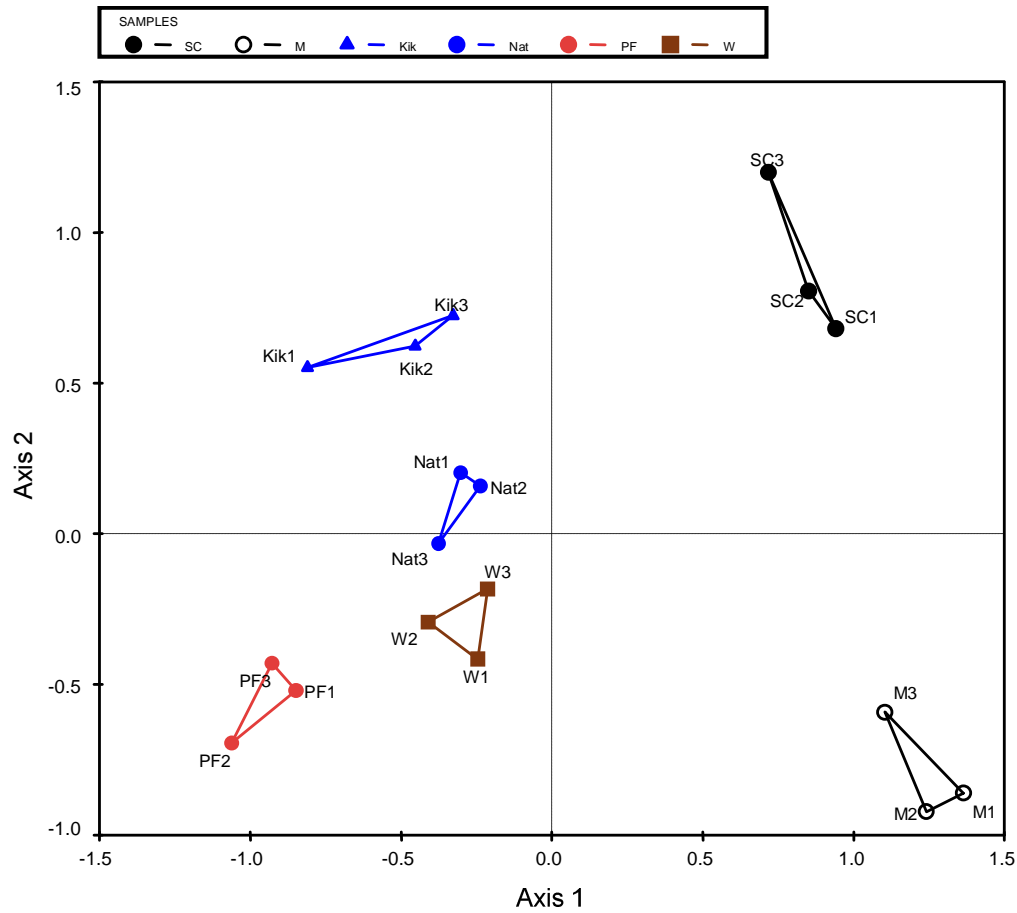


FIGURE 3.3 Non-metric multidimensional scaling (NMS) site plot (rotated by PCA) of bacterial populations (presence/absence of bands) at Baynesfield. NMS stress = 0.07963.

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

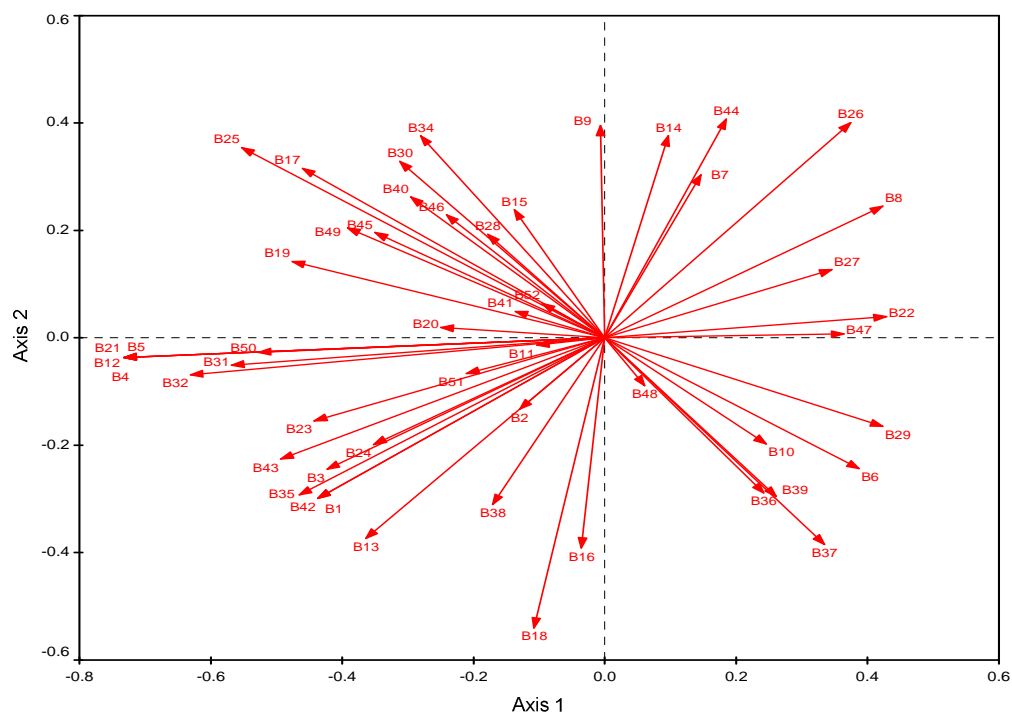


FIGURE 3.4 Plot of bands in the NMS ordination, generated by DGGE of soil bacterial communities (Figure 3.3) at Baynesfield.

Key: B = band.

One-way ANOVA indicated an overall difference in species richness between the soil bacterial populations at Baynesfield (Table 3.6). Trends for richness in increasing order were $M \leq SC < KIK \leq PF < NAT \leq W$, which did not follow the same order as those for organic C.

TABLE 3.6 ANOVA and land use means of bacterial species richness at Baynesfield

| Source of variation | d.f. | s.s | m.s. | f-ratio | p-value |
|---------------------|------|--------|--------|---------|---------|
| Land use | 5 | 713.16 | 142.63 | 98.75 | <0.001 |
| Residual | 12 | 17.33 | 1.44 | | |
| Total | 17 | 730.50 | | | |

| | | | | | | |
|------------|-----------------|----------------|------------------|------------------|-----------------|----------------|
| Grand mean | 24.83 | | | | | |
| Land use | SC ^a | M ^a | KIK ^b | NAT ^c | PF ^b | W ^c |
| Mean | 17.00 | 16.00 | 26.00 | 31.33 | 26.67 | 32.00 |

Note: Means with common superscript letters are not significantly different (l.s.d._{.5%} = 2.138).

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

Analysis of evenness (J) at this site showed that land use did not affect bacterial evenness (Appendix A, Table A1), whereas analysis of bacterial diversity by the Shannon Weaver Diversity Index (H') showed an overall difference in bacterial diversity. Communities under M were separated from those under PF and W but not from those under the other land uses (Table 3.7).

TABLE 3.7 ANOVA of bacterial community diversity and land use means at Baynesfield using the Shannon Weaver Diversity Index (H')

| Source of variation | d.f. | s.s. | m.s. | f-ratio | p-value |
|---------------------|------|--------|-------|---------|---------|
| Land use | 5 | 7.109 | 1.421 | 5.08 | 0.01 |
| Residual | 12 | 3.357 | 0.279 | | |
| Total | 17 | 10.467 | | | |

| Grand mean | 2.91 | | | | | |
|------------|-------------------|----------------|--------------------|--------------------|-----------------|----------------|
| Land use | SC ^{a b} | M ^a | KIK ^{a b} | NAT ^{a b} | PF ^b | W ^b |
| Mean | 2.61 | 1.72 | 2.97 | 3.03 | 3.54 | 3.58 |

Note: Means with common superscript letters are not significantly different ($p < 0.05$).

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

3.3.3 Analyses of soils at the Mount Edgecombe experimental site

The means (\pm standard deviation) of the different physicochemical properties of soils under the four sugarcane residue management practices are summarised in Table 3.8.

Soil organic C increased in the order: BtoFo < BtoF < TFo < TF. Soil pH in increasing order was: TF < BtoF < TFo < BtoFo. Under TF, concentrations of extractable P and exchangeable K were 33.2 mg kg⁻¹ and 0.61 cmol_c kg⁻¹, respectively, whereas in soils under the BtoF treatment, values for P and K were 27.0 mg kg⁻¹ and 0.60 cmol_ckg⁻¹ respectively. Concentrations for unfertilized mulched and no mulch treatments were 4.2 mg kg⁻¹ and 2.2 mg kg⁻¹ respectively, for P, and 0.26 cmol_ckg⁻¹ and 0.14 cmol_ckg⁻¹ respectively, for K. Exchangeable Ca and Mg concentrations were higher in unfertilized plots (Table 3.8). An overall significant difference in the various trash management soils was shown by MRPP ($p < 0.001$).

TABLE 3.8 Means (\pm sd) for selected physicochemical properties of soils under the four managements at Mount Edgecombe, collected at 0-5cm depth

| Trash Management | pH (KCL) | Organic Carbon % | Extractable P mg kg ⁻¹ | Exchangeable cations | | | Exch. acidity | Total cations (ECEC) |
|------------------|------------|---------------------|--------------------------------------|--|---------------|--------------|---------------|----------------------|
| | | | | K | Ca | Mg | | |
| | | | | ----- cmol _c kg ⁻¹ ----- | | | | |
| TF | 3.7 (0.11) | 5.9 (0.61) | 33.2 (10.61) | 0.61 (0.010) | 8.93 (0.942) | 3.27 (0.172) | 1.82 (0.898) | 14.63 (0.642) |
| TFo | 4.5 (0.15) | 5.1 (0.36) | 4.2 (1.24) | 0.26 (0.070) | 12.27 (1.960) | 5.48 (0.385) | 0.09 (0.038) | 18.11 (1.638) |
| BtoF | 3.8 (0.19) | 4.2 (0.26) | 27.0 (5.35) | 0.60 (0.111) | 6.34(1.503) | 3.06 (0.616) | 1.28 (0.854) | 11.28 (1.561) |
| BtoFo | 4.6 (0.15) | 3.9 (0.07) | 2.2 (0.06) | 0.14 (0.025) | 9.30(1.713) | 4.93 (0.230) | 0.08 (0.020) | 14.46 (1.915) |

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized

MRPP of Mount Edgecombe soils, which indicated dissimilarities between all possible soil pairs and also for all land treatments, are shown in Table 3.9. The data obtained for all treatments ($T = -4.585$, $p < 0.001$) showed a highly significant overall difference in the experimental soils except between BtoFo and TFo.

TABLE 3.9 Multi-Response Permutation Procedures (MRPP) of soil physicochemical properties, showing pairwise comparisons and a comparison of all soils under different trash managements at Mount Edgecombe

| Land treatments | Test statistic (T) | Probability (p) |
|-----------------|--------------------|-----------------|
| BtoF vs. BtoFo | -2.791 | 0.022 |
| BtoF vs. TF | -2.245 | 0.031 |
| BtoF vs. TFo | -2.824 | 0.022 |
| BtoFo vs. TF | -2.982 | 0.021 |
| BtoFo vs. TFo | -1.699* | 0.061* |
| TF vs. TFo | -2.990 | 0.021 |
| All treatments | -4.585 | <0.001 |

* no significant difference

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

A PCA biplot was used to cluster the three replicate samples of each trash management soil from Mount Edgecombe, on the basis of the compositional relationship among the replicate subsamples. Exchange acidity, P, K, pH and Mg were all highly correlated with PC1 whereas organic C, total cations ECEC and Ca were correlated with PC2. PC1 accounted for 69.6%, PC 2 for 23.2%, and cumulatively, 92.8% of the total variance in the soil data (Figure 3.5). A lower pH was associated with soils from fertilized treatments of both TF and BtoF than with those from the unfertilized treatments TFo and BtoFo. TFo soils were also correlated with high levels of Ca, ECEC and Mg. In contrast, fertilized soils TF and BtoF were associated with higher levels of P, K and acidity than the unfertilized soils. Both trashed treatments, TFo and TF, were associated with higher levels of organic C than the burnt cane harvested managements, BtoFo and BtoF.

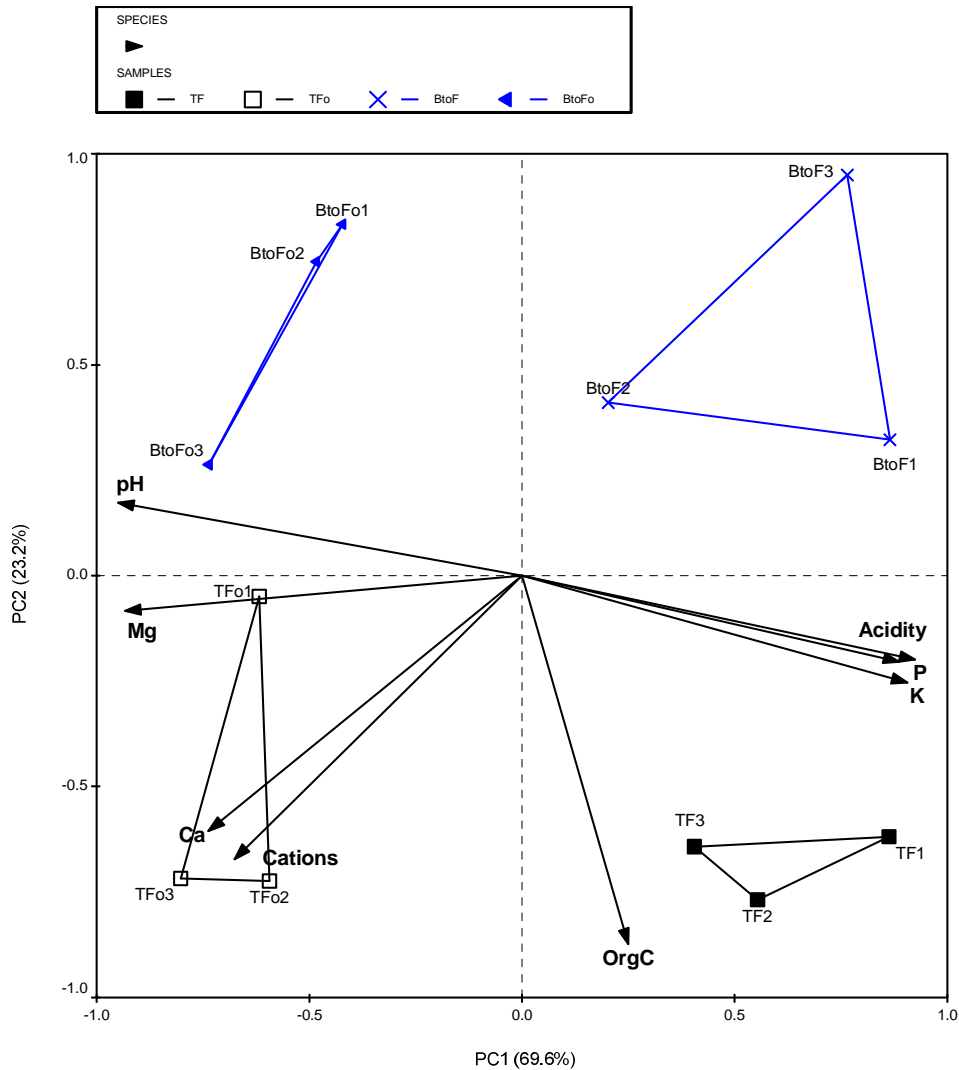


FIGURE 3.5 Plot of samples and soil variables along the first two axes of a standardised and centred PCA of different land treatments at Mount Edgecombe. The PC1 (horizontal) and PC2 (vertical) components accounted for 69.6% and 23.2% respectively, and cumulatively, for 92.8% of the total variance in the soil data.

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

A two-way ANOVA of the main effects of (i) trash mulch/no mulch, (ii) fertilizer/no fertilizer and (iii) the interaction of trash \times fertilizer on soil variables, showed that there were no significant interactive effects of trash \times fertilizer on any of the selected soil variables. However, fertilizer addition had a significant effect on pH, P, K, and Mg ($p < 0.001$), Ca ($p = 0.008$), ECEC ($p = 0.005$), exchange acidity ($p = 0.003$) and organic C ($p = 0.034$). The effects of trashing were seen only on organic C ($p < 0.001$), ECEC ($p = 0.004$) and Ca ($p = 0.016$).

CCA analysis showed the effects of selected soil physicochemical variables on bacterial community composition at site 2, (Figure 3.6). Eigen values for axes 1 and 2 were 0.228 and 0.160 respectively. CCA1 accounted for 30.1% and CCA2 for 21.1% of the total variability, and for 58.7% and 41.3% respectively, (cumulatively 100%) of the variability in bacterial community composition related to the selected soil variables. To reduce collinearity, variables were by forward selection. The soils were significantly correlated with variation on axis 1 ($p = 0.004$) and along all canonical axes ($p = 0.002$). Organic C was closely correlated with CCA1 and Mg with CCA2.

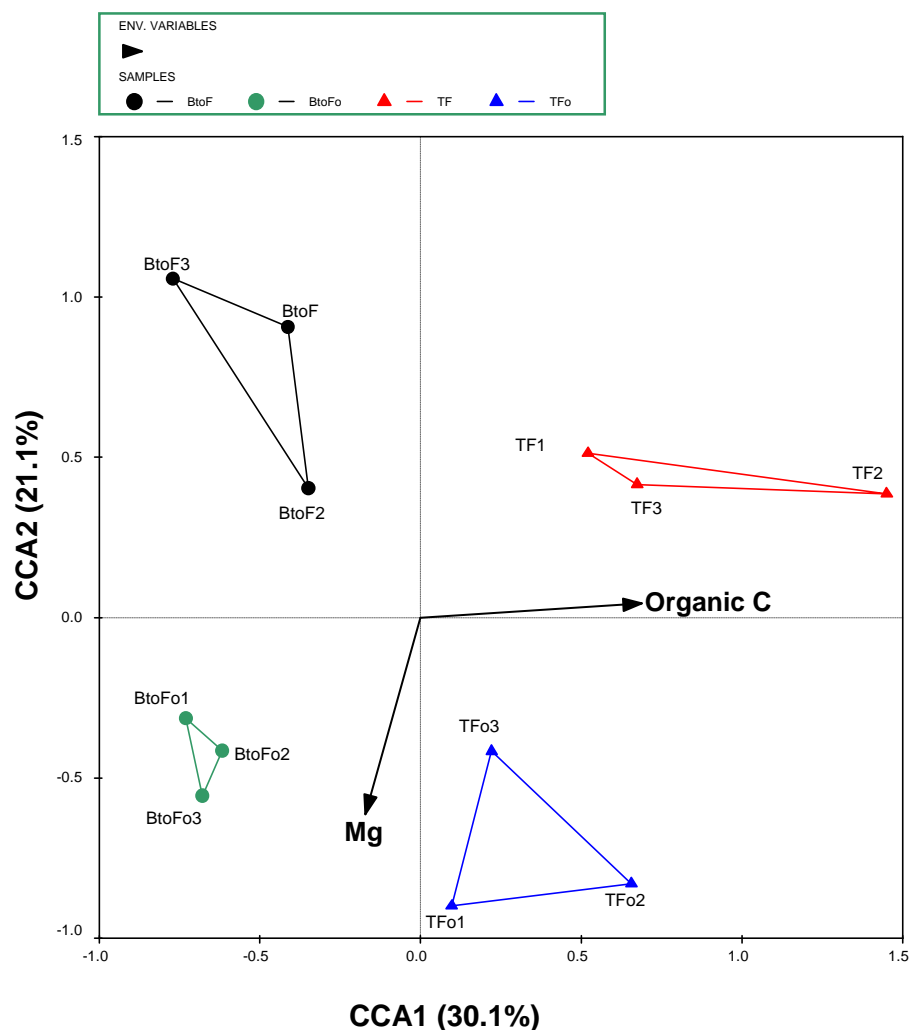


FIGURE 3.6 Plot of samples (classified by land treatment) and soil variables along the first two axes of a CCA of the effect of selected soil variables on bacterial composition (band presence) at Mount Edgecombe.

Soils were significantly related to variation on the first axis ($p = 0.004$) and along all the canonical axes ($p = 0.002$) (Monte Carlo Permutation test: $n = 499$).

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

The statistical significance of the relationship between environmental variables and variation in the DGGE profiles was tested by the Monte Carlo permutation test ($n = 499$). Organic C was shown to be the most significant variable affecting the bacterial communities at Mount Edgecombe, followed by Mg. These variables together accounted for 100% of the total variance explained by all the soil properties tested (Table 3.10). Correlations between the soil variables used in the CCA and the ordination axes are shown in Table 3.11.

TABLE 3.10 Conditional effects of variables in a stepwise selection test in the CCA of soils on bacterial community composition at Mount Edgecombe

| Variable | Eigenvalue | P | F |
|------------------|-------------|--------------|-------------|
| Organic C | 0.23 | 0.002 | 4.29 |
| Mg | 0.16 | 0.006 | 3.91 |
| K | 0.08 | 0.112 | 2.21 |
| P | 0.06 | 0.156 | 1.76 |
| Ca | 0.02 | 0.612 | 0.76 |
| Exchange acidity | 0.03 | 0.618 | 0.67 |
| pH | 0.01 | 0.756 | 0.46 |
| ECEC | 0.01 | 0.996 | 0.04 |

Note: All variables (in bold) were used in the CCA (significant effects on composition).

TABLE 3.11 Correlation between soil variables used in the CCA and compositional gradients (ordination axes)

| Variable | CCA1 | CCA2 |
|-----------|---------|---------|
| Mg | -0.2321 | -0.9079 |
| Organic C | 0.9252 | 0.0654 |

3.3.4 Soil bacterial community structure at the Mount Edgecombe experimental site

Bacterial fingerprints from soils under different trash managements at this site, contained from 20–30 bands, with only one or two bright, dominant bands in some lanes. The majority of bands in the different lanes were closely spaced, often very light, of low intensity and difficult to detect. Banding patterns of replicate samples of the same treatment were highly repetitive and, in general, profiles of the different

treatments across the gels were more similar to each other than those from site 1. Approximately 5–8 bands were common to all lanes across the gels, but differed from each other in relative intensity (Plate 3.3).

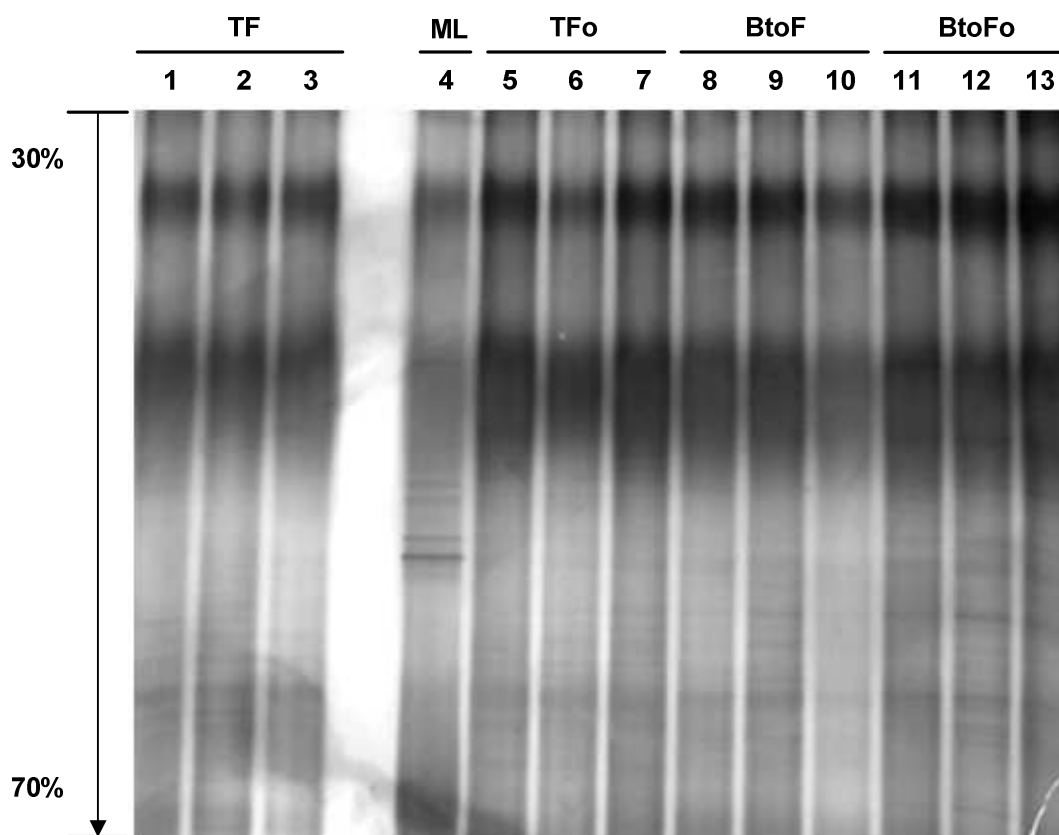


PLATE 3.3 A DGGE gel (denaturing gradient 30–70%) of 16S rDNA fragments from Mount Edgecombe soils under different treatments.

Key: TF = greencane harvested and fertilized; TFo = greencane harvested and unfertilized; BtoF = burnt cane harvested and fertilized; BtoFo = burnt cane harvested and unfertilized; ML = marker lane.

Bacterial community composition and a plot of the DGGE bands of soil DNA samples from the four different land treatments, are shown in an NMS two-dimensional plot rotated by PCA (Figure 3.7). Variability in prokaryote community composition within and between treatments based on the presence or absence of bands, is also shown in Figure 3.7. The presence/absence of bands was used for this analysis as it was deemed to be more reliable than band intensity. However, the analysis did not separate communities from the fertilized and unfertilized plots of the pre-harvest burnt (Bto) treatment (Figure 3.7). MRPP analysis of the data indicated a highly significant difference overall, in prokaryotic community profiles between managements ($T = -6.837$, $p < 0.001$).

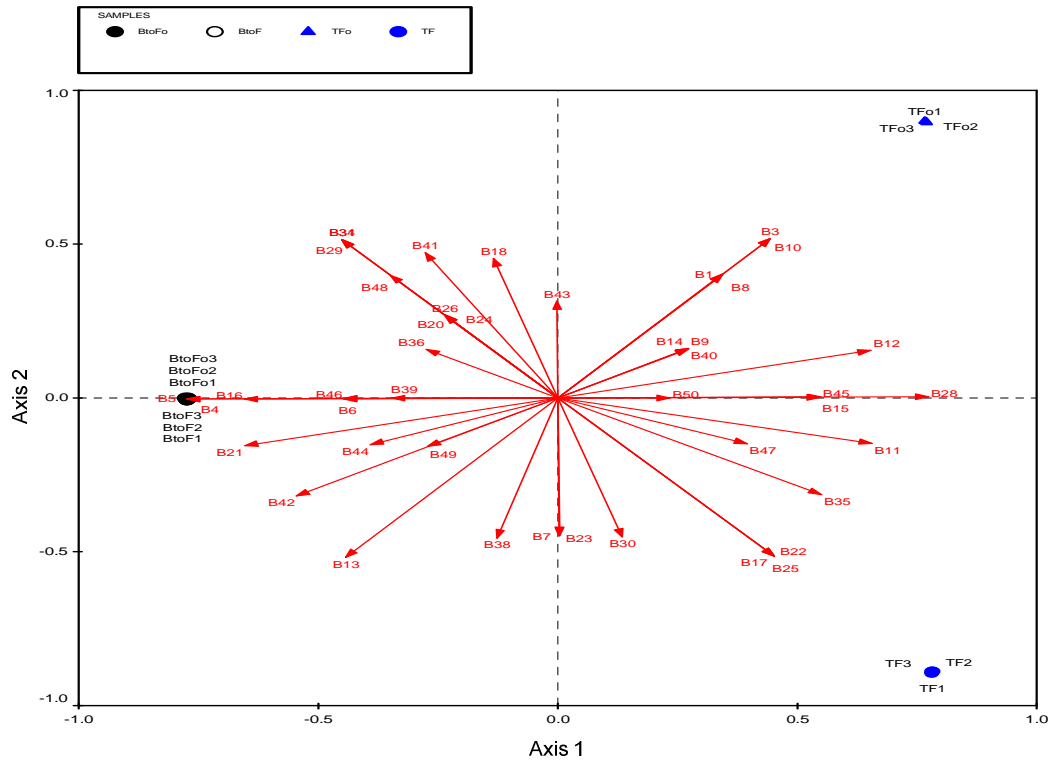


FIGURE 3.7 NMS two-dimensional plot (rotated by PCA) of bacterial communities (presence or absence of bands) under four land treatments at Mount Edgecombe. NMS stress = 0.00049.

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized. B = band.

One-way ANOVA showed no significant differences in bacterial richness (S), evenness (J) or the Shannon diversity index (H') (Appendix A, Table A2). However, two-way ANOVA of the main effects and interaction of trash mulch (T) / no mulch (Bto) and fertilizer (F) / no fertilizer (Fo), on bacterial community richness, evenness and diversity showed a significant difference in richness as an effect of fertilizer application (fertilizer / no fertilizer) ($p = 0.051$). No significant effect as a result of trash mulch / no mulch ($p = 0.519$), nor any significant interactions between trash / fertilizer treatments ($p = 0.373$) were found (Table 3.12). There were also no significant treatment effects on bacterial community evenness or diversity at Mount Edgecombe.

TABLE 3.12 Two-way ANOVA of the main effects and interaction of trash mulch (T) / no mulch (Bto) and fertilizer (F) / no fertilizer (Fo), on bacterial community richness at Mount Edgecombe

| Source of variation | d.f. | s.s. | m.s. | f-ratio | p-value |
|---------------------|------|--------|--------|---------|---------|
| Trash | 1 | 2.083 | 2.083 | 0.45 | 0.519 |
| Fertilizer | 1 | 24.083 | 24.083 | 5.25 | 0.051* |
| Trash/Fertilizer | 1 | 4.083 | 4.083 | 0.89 | 0.373 |
| Residual | 8 | 36.667 | 4.583 | | |
| Total | 11 | 66.917 | | | |

| | | | | | |
|------------------|-------|----------------|-------------------|-------|-------|
| Grand mean | 27.58 | | | | |
| Trash | | Burnt (Bto) | Trash (T) | | |
| Mean | | 28.00 | 27.17 | | |
| Fertilizer | | Fertilized (F) | Unfertilized (Fo) | | |
| Mean | | 26.17 | 29.00 | | |
| Trash/Fertilizer | | BtoF | BtoFo | TF | TFo |
| Mean | | 26.00 | 30.00 | 26.33 | 28.00 |

* significant

3.4 DISCUSSION

Agricultural biodiversity, particularly at agroecosystem levels, is determined by genetic resources, the physical environment and human management practices (Atlas and Bartha, 1987) with soil microbial community structure a sensitive indicator of sustainable land use (Gelsomino *et al.*, 2006; Yao *et al.*, 2006). Bacteria play a vital role in soil ecosystem processes by breaking down plant litter and exudates and are, therefore, important mediators of nutrient cycling in the soil-plant system (Alexander, 1977). Previous studies in KwaZulu-Natal (Graham, 2003; Graham and Haynes, 2005; 2006) of the effects of agricultural practices on the soil microbiota, focused on measurements of total biomass and gross microbial processes in crop soils. Therefore

in the present study, to assess genetic diversity in these soils, molecular techniques based on 16S rDNA analysis were used.

DGGE (section 2.4.2.2.6) of soil PCR amplicons (section 2.4.2.2) was used as a qualitative and semi-quantitative method to assess soil bacterial community diversity under various agricultural management practices and land uses. The DNA banding profiles obtained permitted the study of the complex communities in the soil samples, as most bacterial genotypes (species) generally produce a single band (Dilly *et al.*, 2004). In addition, the intensities of the DNA bands are strongly correlated with the relative level of abundance (mass) of the different bacterial strains (Dilly *et al.*, 2004; Hoshino and Matsumoto, 2007). Cognisance was taken of the fact that the relationship between bands in a profile and the number of bacterial species in the sample is not simply one-to-one, as both over- and under-estimation of the actual numbers present can occur. Also, as 16S rDNA was used as the template for PCR amplification, DNA from both living and dead microorganisms was amplified, so the DGGE profiles reflect this.

3.4.1 Bacterial community structure under different land uses at the Baynesfield experimental site

PCR-DGGE of 16S rDNA sequences from Baynesfield soils clearly differentiated bacterial community composition under all the agricultural land uses, on the basis of band number and the varying distances the PCR amplicons migrated in the gel. However, results of analyses based on average relative intensity of bands were less definitive. The banding patterns observed in the soil bacterial DNA profiles, with numerous, low intensity, closely-spaced bands interspersed with either a single or a few bright, dominant bands, were possibly due to the high heterogeneity of directly extracted soil DNA, indicating the complexity of the bacterial community composition. The similarity in the band profiles of the replicate samples from the same land use indicated greater differences in bacterial population structure between the different land uses than within the same one. In a study of soil microbial community responses to differing management practices, Crecchio *et al.* (2004) reported a similar type of banding pattern when using directly isolated soil DNA.

They suggested that the bright bands possibly indicated the presence of a limited number of dominant, ecologically well-adapted bacterial types in the soil. On the other hand, the numerous light bands possibly indicated that many, equally abundant populations characterised each soil.

The bacterial community structure was compared between widely different land uses with very different vegetation, fertilizer, and tillage histories. Consequently, large differences in nutrient status, pH, acidity and organic matter content between sites were found. NMS analysis of DGGE profiles clearly separated the bacterial populations in the six land use soils, indicating substantial differences in community composition between sites. This could be due to many interacting factors for example, the rhizosphere of different plant species is known to support bacterial communities of different species composition (Marschner *et al.*, 2001; Miethling *et al.*, 2003). It was shown by CCA that a range of physicochemical soil properties (P, acidity, Mg, ECEC, K and organic C) had influenced bacterial community composition, with P and acidity showing the strongest correlations. Nonetheless, CCA1 and CCA2 accounted for only 22.8% and 15.7% respectively, of the total variability, suggesting that other unmeasured factors were also influential.

Surface soils are physically, chemically and structurally heterogeneous and may provide numerous micro-environments for the survival and growth of microbes (Yao *et al.*, 2006). Analysis of selected soil variables by MRPP separated all the soils under the different land uses from each other, except those under sugarcane and maize and those under kikuyu pasture and native grassland. Despite this, visual assessment and NMS analysis of DGGE profiles of bacterial communities, clearly distinguished between both maize and sugarcane and also between kikuyu and native grassland. As was shown by CCA, soils under the sugarcane and maize monocultures had a high P and K content (from fertilizer additions) and the lowest organic C content of all the land use types. In an earlier study at this site, soils under sugarcane and maize were found to have lost organic matter due to repeated tillage, and had a smaller microbial biomass in comparison with soils under kikuyu, native grassland and exotic forests (Haynes *et al.*, 2003). In the DGGE gels, sugarcane and maize bacterial communities displayed fewer bands per lane than the other land uses. Therefore, as each band theoretically represents a different bacterial population (group of species), the soil

communities under sugarcane and maize had a lower richness and genetic diversity than those under the other land uses. This could largely be due to the effects of tillage, which cause intensive mixing of soil with organic matter and organic residues from the field (e.g. roots, straw and stubble). Tillage mechanically loosens the soil, resulting in increased mineralization of organic matter, causing a lowering of organic matter content, an increase in mineral-available nitrogen (nitrate), and increased erosion (Honermeier, 2007). More easily decomposable C fractions are lost preferentially when land use or management leads to the promotion of organic matter decomposition. These labile C fractions are thought to support a substantial proportion of the heterotrophic microbial community and their loss could, therefore, lead to a disproportionate decline in microbial diversity (Degens *et al.*, 2000; Graham, 2003).

Tillage may also cause a reduction in microbial diversity by, for example, mixing the soil, which results in a more homogeneous soil volume, thus reducing the number of microhabitats or “unit communities” (species assemblages of soil organisms which occupy each distinct organic resource) and thereby reducing the structural diversity of the community (Giller *et al.*, 1997). In contrast, in the relatively undisturbed surface soils of land uses such as permanent kikuyu pasture or exotic forests, the soil volume is likely to be extremely heterogeneous in both the size and continuity of pores, and in the distribution of “hot spots” of microbial activity. These occur around randomly distributed decomposing litter such as dead or dying roots and earthworm casts (Beare *et al.*, 1995). Despite the fact that at Baynesfield, the maize fields are tilled annually, whereas the sugarcane fields are tilled only at replanting (at approximately six yearly intervals) the bacterial richness and evenness were similar under the two crops, suggesting that at this site, the above-mentioned effect was of little importance.

In uncultivated soils in the study area a low P status (i.e. 1–10 mg kg⁻¹) is common. Conversely in the sugarcane and maize soils, exceptionally high levels of extractable P (i.e. > 150 mg kg⁻¹, almost ten times the recommended level of 15–20 mg kg⁻¹) were found. As suggested by CCA (Figure 3.2), P was a major factor in distinguishing bacterial communities under sugarcane and maize from those under other land uses at this site, indicating that P status had influenced bacterial community composition considerably. Thus exceptionally high P levels seemed to be associated with a

reduction in genetic diversity, which could possibly be as a result of the loss of bacterial species adapted to growth at low P levels. These results are indicative of the detrimental effects of excessive fertilizer applications on soil microbial diversity.

Soil organic C content was similar under native grassland, wattle and pine, but was greatest under permanent kikuyu pasture. Previously, Haynes *et al.* (2003) had reported similar findings at the same site. Under grassland, large amounts of soil organic matter accumulated characteristically, due to the turnover of the extensive root system and to above-ground inputs such as stem and leaf tissue and animal dung. Under improved kikuyu pasture, organic matter accumulation was greater than under native grassland due to greater dry matter production (and greater organic matter inputs) induced by improved, high-yielding cultivars, irrigation and regular fertilizer applications (Haynes *et al.*, 2003). In the present study, bacterial communities under kikuyu produced slightly fewer bands per lane in DGGE gels than did those from native grassland soils. This indicated a lower richness and genetic diversity in the soil bacterial community under kikuyu than under native grassland. Trends for richness, derived from ANOVA, confirmed this. On the basis of the number of bands present, the kikuyu soil bacterial community could be differentiated from all those studied at this site except the pine community.

The greater richness and genetic diversity of bacterial communities under unimproved native grassland, compared to that under pine, kikuyu, maize and sugarcane may reflect the physicochemical heterogeneity of undisturbed soil as well as a greater heterogeneity in the above-ground plant community. A more diverse plant community in native grassland would result in a greater variety of organic matter inputs to the soil than those of the agricultural or forestry monocultures. In grassland biomes, major plant constituents added to soils include cellulose and hemicellulose. Microbial degradation of these materials requires a diversity of microbes to break down complex substances into simple substrates for use by a variety of other organisms (Alexander, 1977). The larger number of potential substrates could account for the greater richness and genetic diversity of the bacterial community observed under native grassland than under the long-term pine, kikuyu, maize, and sugarcane monocultures. Pairwise comparisons of richness data separated native grassland from all land uses except wattle.

It is well known that the quality of soil organic matter differs between forests and grasslands, with carbohydrates tending to be higher in pasture than in forest soils, while phenolic compounds are usually significantly higher under forests (Yao *et al.*, 2006). Carbon in forest soil enters as litter and roots, and by rhizodeposition (Hernesmaa *et al.*, 2005). The organic C content of pine soils at site 1 was similar to that of native grassland and wattle, although the nature of the communities differed substantially between these soils as indicated by their clear separation by CCA (Figure 3.2). The separation of the communities under pine from those under the other land uses was due primarily to the effects of acidity, as pine soil had the lowest pH and the highest exchangeable acidity of all the soils at the study site. The replicate soil bacterial communities under pine produced numerous bands per lane in the DGGE gels, indicating a high richness. ANOVA analysis of this data separated pine soil bacteria from those of all the other land uses except kikuyu.

In the pine forest, a thick layer of needles covered the soil surface. Because pine needles are rich in lignocellulosic and phenolic compounds, which are recalcitrant and difficult to metabolise, a microbial succession, initiated by the fungi, is required to effect their breakdown into simple aromatic substances and, finally, into low molecular weight organic acids (Alexander, 1977; Ratering *et al.*, 2007). The production of the latter, together with humic acids, could help account for the high acidity observed in pine soil. The pathways involved in this degradation are complex, resulting in the formation of a large number of metabolic intermediates, which could provide substrates for a wide variety of microorganisms (Alexander, 1977; Ratering *et al.*, 2007). This may account for the observed richness in the pine prokaryotic community. The low pH, exchangeable Ca and Mg, and higher exchange acidity under pine than other land uses were probably important in differentiating the pine bacterial community from those of the other land uses. In an earlier study by Haynes *et al.* (2003), the microbial biomass was found to be higher under pine than under native grassland although that study did not take community richness or genetic diversity into account.

In wattle soil, organic C content was higher than under maize and sugarcane; similar to native grassland soil; slightly lower than under pine; and much lower than in kikuyu soil. Wattle soil had the highest pH and the highest Ca and Mg content of all

the land use types at this site. Replicate samples of DNA amplicons from wattle soil bacteria consistently produced most bands per lane in DGGE gels when compared to those of all the other land uses. MRPP tests of community profiles clearly differentiated between the wattle and pine communities at this site. A difference in richness between wattle and pine and all the other land uses except native grassland, was also shown by ANOVA. This was unexpected because of the greater variety of plant organic matter inputs to native grassland soil than to those of the wattle monoculture. However, the wattle plantation at Baynesfield had been established for < 10 years and was situated on the site of an old Eucalyptus (gum tree) plantation. Litter on the forest floor included wattle leaves and twigs, with rotting gum tree stumps still visible. These could all provide substrates for microbial metabolism. In addition, as wattle is a legume, nitrogen fixing bacteria associated with their root nodules would be released into soil in rotting root material. Wattle is an acacia species and, while it is an exotic, many other acacias are indigenous to the area. Therefore soil microbes associated with these acacias could possibly adapt to growth in the rhizosphere of wattles. All these factors could account for the high richness found under this land use. The observed lower organic C content under wattle than under pine is probably associated with loss of organic matter following the harvest of the Eucalypt plantation previously on this site.

Based on the Shannon Weaver Diversity index, calculations from the DGGE data showed an overall difference in diversity but not in evenness in the various soil bacterial communities. The Shannon Weaver index is a general diversity index, which increases with the number of species and is higher if the mass is distributed evenly over the species. Evenness is independent of the number of species and is lower if a small number of bands are dominant, and highest if the relative abundance of all bands is essentially similar. The equitability correspondingly indicates the presence of dominant bands (Dilly *et al.*, 2004). As the banding profiles of all the different soil communities contained a large number of light, low intensity bands, together with one or a few, bright dominant bands, a low evenness resulted. Nonetheless, an overall difference in diversity was shown. Pairwise comparisons separated maize bacterial communities from those under pine and wattle, although no differences were found in pairwise comparisons of the communities under the other land uses. Tebbe and Schloter (2007) reported a case study, where soil microbial diversity of

conventionally-managed fields was compared to fields under short-term and long-term organic farming, respectively. The microbial biomass of the investigated field plots from the different treatments did not differ from each other, whereas significant changes in the microbial community were detected at all taxonomic levels that were analysed. However, the overall diversity as calculated by the Shannon index was not significantly different between treatments. In addition, strain (ecotype) diversity showed that although the overall diversity (Shannon index) was unaffected, a clear shift in the type of strains could be detected. As the Shannon index is dependent on both 'richness' (for which clear, reproducible assignments of individual organisms to species level are required) and 'evenness' (for which a reliable quantification method is needed) and as neither of these parameters can be unequivocally determined for soil prokaryotes (Tebbe and Schloter, 2007), this method of determining bacterial diversity has some shortcomings. In the present study, analyses based on the presence or absence of bands or on band numbers clearly separated the various communities under the different land uses, whereas the Shannon Weaver diversity index based on the average relative intensity of the bands (mass), showed only slight differences, or none at all, because of the low evenness.

3.4.2 Bacterial community structure under different trash management practices at the Mount Edgecombe experimental site

Miethling *et al.* (2000) concluded that crop species was the main factor influencing the soil microbiota, whereas Marschner *et al.* (2001) found that a complex interaction of soil, plant species and root zone influenced the microbial communities. Other studies have reported that management practices were secondary to factors such as soil type and cultivation when determining soil microbial community structure (Girvan *et al.*, 2003; Yao *et al.*, 2006). On the other hand, Kirk *et al.* (2004) found that many anthropogenic activities could potentially affect soil microbial diversity.

DGGE of 16S rDNA amplicons from Mount Edgecombe soil successfully separated the different bacterial communities under the four experimental treatments, on the basis of band numbers and the distance migrated by the bands in the gels. Visual

assessment of the DGGE gels indicated that band numbers and banding patterns in the profiles of all the treatments were much more similar to each other than were those of the different land uses at the Baynesfield experimental site. However, as each band represents a different group of species and as only 5–8 bands were common to all four treatments at site 2, this indicates that the different communities were genetically diverse.

Two-way ANOVA analysis of the main effects and interaction of trash and fertilizer managements on soil bacterial community richness, evenness and diversity, showed that richness had been affected significantly by additions of fertilizer but not by trash retention, nor by any interaction between trash \times fertilizer. Interestingly, fertilizer additions had significantly reduced bacterial community richness compared to unfertilized treatments, from a mean of 29.00 in unfertilized plots to 26.17 in fertilized plots. It has been suggested that fertilizer applications tend to result in a less diverse, more specialized soil microbial community (Tiquia *et al.*, 2002). Applications of inorganic N to a silage corn field were shown by Peacock *et al.* (2001) to enhance the relative abundance of Gram positive bacteria. Mendum and Hirsch (2002) reported that plots fertilized with NH_4NO_3 were dominated by Group 3 AAO bacteria, whereas those receiving no fertilizer were dominated by Group 4. Demoleng *et al.* (2008) showed that soil bacteria were limited by a lack of carbon, which was exacerbated by fertilization.

Species diversity of a community is similar to genetic diversity of a population in that it allows for a varied response within a dynamic ecosystem. If an environment is dominated by a strong selective force such as fertilizer additions, less flexibility is needed to maintain stability. In such cases it is adaptive for a community to become stenotolerant (highly specialised) and to be dominated by a few populations (Atlas and Bartha, 1987). In the present study, this effect was observed on bacterial community richness, but no significant differences were evident between the treatments with respect to evenness or diversity. This indicates that although the communities from the different managements are genetically different, they are nonetheless similar in terms of the evenness of species present. The difference in bacterial community composition observed here could presumably account for the differences in catabolic diversity found by Graham and Haynes (2005).

Analyses of soil variables at this site by two-way ANOVA, suggested that two major factors, induced by fertilizer additions, interact to affect the size and activity of the microbial community, namely: (a) an increase in organic matter content in the surface soil, mediated by fertilizer-induced higher yields (and thus greater organic matter returns as roots and trash) and (b) fertilizer-induced soil acidification (Table 3.8). This agrees with the findings of previous studies at this site (Graham *et al.*, 2002 a, b). These two effects as shown by CCA, were the main factors influencing (either directly or indirectly) the nature of the soil bacterial communities at Mount Edgecombe. Under the four treatments, MRPP analysis of selected soil variables showed an overall significant difference in the various soils, as well as in all pairwise comparisons, except between BtoFo and TFo. Analysis by NMS (Figure 3.7) showed that fertilizer additions caused bacterial communities to differ substantially under green cane harvesting but not under burning. Bacterial communities under TF and TFo were clearly separated from each other and also from those of BtoF and BtoFo, whereas those under BtoF and BtoFo were very closely correlated and could not be separated. A significant difference in the bacterial communities between all the land treatment types was shown by the MRPP analysis.

CCA clearly separated the bacterial communities under the four treatments, with soil organic C being the most important variable (Table 3.10) accounting for the differences in bacterial community structure under the trashed (T), and burnt (Bto) plots, on CCA1 (Figure 3.6). Wakelin *et al.* (2007) reported similar findings in their study on maize stubble management. Communities associated with the trashed treatments are likely to have been involved principally in decomposition of the sizable inputs of crop residues at the soil surface, whereas those from burnt plots were probably either associated with the sugarcane rhizosphere or involved in the slow turnover of soil humic material. The major limiting factor to microbial growth under burning is likely to be a paucity of available C, leading to a community dominated by species able to use relatively recalcitrant humic substances.

Graham (2003) indicated that long-term retention of cane crop residues on the soil surface at this site had resulted in a 26.5% increase in organic C, and greater aggregate stability in comparison with soils under burning. Both C and N are volatilized during burning and this is a major cause of soil degradation and loss of soil

organic matter under long-term sugarcane production (Biederbeck *et al.*, 1980; Rasmussen and Collins, 1991). It has already been shown in the present study that trash retention results in an accumulation of soil organic matter and, as reported by Graham *et al.* (2002b), this is associated with an increase in the size and activity of the soil microbial community.

Soil pH was lower under fertilized managements (F) than under unfertilized (Fo), with high exchangeable acidity correlated with both the TF and BtoF treatments. The lowest pH was measured in soils under TF, and the highest under BtoFo. Soil acidification of fertilized plots is primarily attributable to nitrification of annually applied fertilizer NH_4^+ (Graham, 2003). As acidification proceeds and a decrease in pH occurs, Al-containing amorphous clay minerals begin to dissolve, resulting in an increase in soluble and exchangeable Al (and exchangeable acidity, i.e. exchangeable $\text{Al}^{3+} + \text{H}^+$). The exchangeable Al^{3+} displaces exchangeable bases (e.g. Mg^{2+} , Ca^{2+} and K^+) from exchange sites, with subsequent leaching of the bases down the profile (Graham *et al.*, 2002a). Therefore, fertilized soils had a lower pH and exchangeable Ca and Mg than unfertilized soils, but had higher exchangeable acidity. Fertilizer also increased extractable P and K. Thus, the considerable influence of exchangeable Mg in separating the different bacterial communities from the fertilized and unfertilized plots (as shown by CCA, Figure 3.6), is associated with the large losses of Mg from the acidified fertilized plots. This was confirmed by two-way ANOVA of soil variables. The substantial difference in the soil chemical environment between fertilized and unfertilized treatments resulted in large differences in bacterial community composition, particularly under trash retention (Figure 3.7).

3.5 CONCLUSIONS

A combination of soil physicochemical factors (P, acidity, Mg, ECEC, K and organic C, crop plants and long-term land use, affected the genetic diversity of soil bacterial communities at Baynesfield (site 1). Land uses such as pastures and exotic plantations increased bacterial community genetic diversity in comparison with arable agriculture, where organic matter was lost. At the long-term trash management trial at Mount Edgecombe (site 2), the main influences on soil bacterial community diversity

were the retention of organic residues, application of inorganic fertilizers and soil pH. However, the effects of the different treatments on the soil bacterial communities at site 2 were less marked than the effects of different land uses at site 1.

The PCR-DGGE fingerprinting method used successfully separated the complex bacterial communities in the different experimental soils. It proved to be a relatively simple and cost-effective method for screening multiple soil samples to determine genetic (community) diversity. However it is only semi-quantitative because of inherent shortcomings in PCR amplification and in the resolving power of DGGE. For future studies, PCR-DGGE combined with microarrays will provide a better quantitative assessment of the genetic diversity of the soil bacterial communities.

Chapter 4

EFFECTS OF LAND USE AND MANAGEMENT ON SOIL FUNGAL DIVERSITY AS MEASURED BY PCR-DGGE

4.1 INTRODUCTION

Most of the ecological data on fungal species diversity and richness is derived from studies in phytopathology, and is pathogen-orientated. In contrast, studies on the biodiversity of microfungi (hyphomycetes) in agricultural ecosystems are comparatively rare (Fischer, 2007). Worldwide, little is known about fungal population structure and dynamics in agricultural soils, or about how soil management and land use affect fungal community composition (Hagn *et al.*, 2003).

Fungi are a very diverse group of organisms containing an estimated 1.5 million species, of which only 72 000–100 000 have thus far been identified (Hawksworth and Rossman, 1997; Bridge and Spooner, 2001). Of these, the great majority are likely, at some stage in their life cycle, to occur in soil (Bridge and Spooner, 2001), where they are important in nutrient cycling, plant health and development, as well as in soil aggregate stability (Gomes *et al.*, 2003; Hagn *et al.*, 2003). Soil fungi include taxa from the Ascomycotina (mainly anamorphic forms) Zygomycotina (e.g. *Aspergillus*, *Fusarium*, *Mortierella* and *Penicillium*) and taxa that occur mainly as mycelium in soil (e.g. *Rhizoctonia* spp.). In addition, both arbuscular mycorrhiza (AM) and ectomycorrhiza (EM) are common soil inhabitants. Saprophytic basidiomycetes (e.g. cap and decomposer fungi) may also be included as their mycelia colonise the plant debris in the top soil layer (Fischer, 2007).

Hitherto, no critical assessment of the number of species isolated from soil appears to have been made, although their role is extremely complex and fundamental to the ecosystem (Bridge and Spooner, 2001). This is in part due to the fact that detecting which fungi are present in soil is a difficult task, as only an estimated 17% are culturable due to the fastidious nature of the vast majority of species (Hawksworth

and Rossman, 1997). More specifically, the influence of saprotrophic fungal species richness and evenness on organic matter decomposition is not known (Deacon *et al.*, 2006).

Molecular methods are increasingly being used to investigate both culturable fungi and total fungal communities in soil (Hagn *et al.*, 2003; Mitchell and Zuccaro, 2006). With the development of universal fungus-specific primers designed to amplify partial SSU rDNA (18S rDNA) fragments, PCR analysis (section 2.4.2.2) can be used to reveal the species composition of a mixed DNA sample (Vainio and Hantula, 2000; Hagn *et al.*, 2003). Comparisons of 18S rDNA provide insight into the diversity of unrelated or distantly related fungal taxa whereas an alternative target for fungal PCR, namely, the ITS region, is mainly used to compare closely-related species and strains (Hagn *et al.*, 2003).

As mentioned in Chapter 3, studies in KwaZulu-Natal have shown that soil organic matter status and the size, activity and catabolic diversity of the soil microbial (mainly bacterial) community are affected by both agricultural land use (Dominy and Haynes, 2002; Haynes *et al.*, 2003; Nsabimana *et al.*, 2004) and by soil management practices (Graham *et al.*, 2002a, b). Using PLFA analysis (section 2.4.1.3) to determine microbial community size and structure, Graham (2003) found clear differences in the PLFA composition of microbial communities under different land uses and management practices, with the fungal:bacterial ratio being lowest under kikuyu and ryegrass pasture, and highest under natural grassland at Baynesfield (site 1, Chapter 3). At Mount Edgecombe (site 2, Chapter 3), on the other hand, the ratio of 18:206 fungal fatty acid: bacterial fatty acid was higher under green cane harvested with trash retention than under unfertilized burnt plots with the tops raked off.

As information on fungal communities in South African soils is sparse, particularly with regard to the genetic diversity (community composition) of fungi in agricultural soils, another aim of this study is to use DGGE analysis of 18S rDNA PCR amplicons, to profile soil fungal communities at Baynesfield and Mount Edgecombe, and thereby to determine how land use and management practices have affected these communities at the two long-term sites.

4.2 MATERIALS AND METHODS

Methods were optimised in a long series of preliminary experiments. Only the most efficient protocol is described and discussed.

4.2.1 Study sites and soil sampling

The study sites and soil sampling procedures (0–5 cm depth) are described in section 3.2.1 and 3.2.2.

4.2.2 Chemical analysis

Chemical analysis followed the methods specified in section 3.2.3.

4.2.3 DNA extraction and purification

Total soil genomic DNA was extracted according to the procedure described in section 3.2.4.

4.2.4 PCR amplification of fungal 18S rDNA fragments

Fungal 18S rDNA from soil samples was PCR-amplified according to the procedure of Vainio and Hantula (2000), using the universal fungus-specific primer pair FR1/NS1. These primers amplify almost the entire 18S rDNA region, resulting in the formation of phylogenetically informative gene fragments for fungal analysis (Oros-Sichler *et al.*, 2006). The reverse primer FR1, targets the invariant region near the 3' end of fungal SSU rDNA, so has highly enhanced selectivity for fungi, but may also anneal to the DNA of a limited set of other organisms, including animals and plants (Vainio and Hantula, 2000). The specificity of primer NS1 was not tested in this aspect of the work (see Chapter 5). The relative locations of the primers in the SSU rDNA region are shown in Figure 4.1 and the primer sequences in Table 4.1.

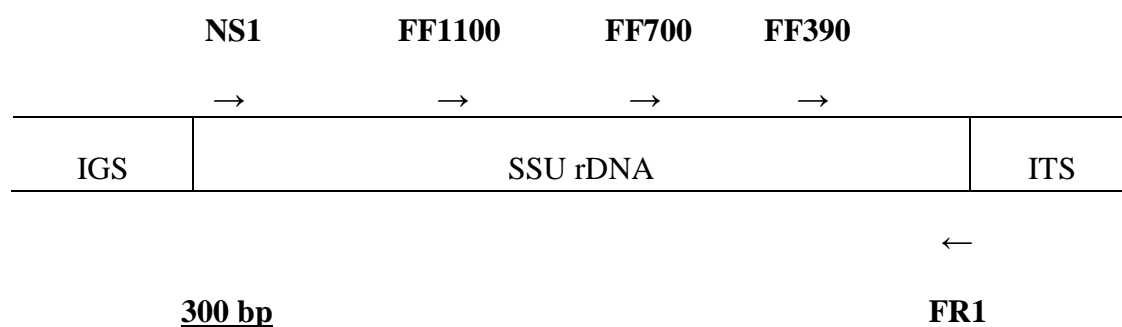


FIGURE 4.1 Schematic representation of annealing sites of PCR primers in the region coding for fungal SSU rDNA. The relative positions of the primers and their direction of extension are indicated by arrows (after Vainio and Hantula, 2000).

TABLE 4.1 PCR primers used in this study for amplification of partial SSU fungal rDNA

| Primer | Sequence (5'→3') | Product size (bp)† | Reference |
|--------|------------------------------|--------------------|--------------------------|
| FR1* | AIC CAT TCA ATC GGT AIT | — | Vainio and Hantula, 2000 |
| FF390 | CGA TAA CGA ACG AGA CCT | 390 | " |
| NS1 | GTA GTC ATA TGC TTG TCT C | 1650 | " |

† The approximate product length observed in the majority of reference species.

* Fungal-specific reverse primer used in combination with forward primers FF390 and NS1.

To separate PCR amplicons efficiently in subsequent DGGE, a GC clamp sequence (underlined) was added to reverse primer FR1, resulting in a 58 meric primer: 5' CCC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GCC GAI CCA TTC AAT CGG TAI T 3' (where I is inosine). The Inosine nucleotides accommodate variations in the fungal sequences covered by oligonucleotide FR1 (Vainio and Hantula, 2000).

Soil DNA was amplified in a Perkin Elmer Applied Biosystems Gene Amp 2400 thermal cycler. Reaction mixtures were prepared using the PCR Core Kit (Roche Diagnostics) according to the manufacturer's instructions, and were optimized for the experimental soils. Each 50 µl reaction contained 5 µl of 10× reaction buffer containing 15 mM MgCl₂ (1.5 mM MgCl₂ final concentration), 0.5 µM of FR1GC

reverse primer and 0.5 μM of NS1 forward primer, 200 μM of each dNTP, 1.25 U Taq DNA Polymerase (Roche Diagnostics), sterile MilliQ H_2O , and 1 μl (20 mg ml^{-1}) Bovine Serum Albumen (BSA) added to prevent amplification inhibition by organic compounds co-extracted from soil (Pecku, 2003). Template DNA, 1 μl for all Baynesfield soil samples and 2 μl for all Mount Edgecombe soil samples, were used for optimal PCR amplification.

PCR conditions were as described in a method by Vainio and Hantula (2000), namely: an initial cycle of 95°C/8 min; followed by 35 cycles consisting of 95°C/30 s, 47°C/45 s, and 72°C/3 min; and a final single 10 min extension at 72°C. This sufficed for the Baynesfield DNA samples but as the yield of amplicons from Mount Edgecombe soil DNA was low, the above method was modified by increasing the number of denaturation/annealing/ elongation cycles to 38. Amplification products (~1650 bp) were analyzed by electrophoresis of 5 μl aliquots in 0.8% agarose gels stained with ethidium bromide, visualised under UV and photographed.

4.2.5 Community fingerprinting by DGGE

The partial SSU rDNA fragments were analyzed by the DCode™ Universal Mutation Detection System (Bio-Rad). Equal amounts of PCR products, estimated visually from the agarose gels (Pennanen *et al.*, 2004), were loaded in 20 μl aliquots, onto 7.5% (v/v) acrylamide gels (Sigma acrylamide/bisacrylamide 40% solution, mix ratio 19:1). The denaturant gradients were produced by diluting 100% denaturing solution containing 40% (v/v) deionized formamide and 7 M urea. The gels were allowed to polymerize for 1.5–2.5 hours. Electrophoresis was then run in 1× TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM disodium EDTA, pH 8.3) at a constant temperature of 58°C according to the conditions shown in Table 4.2.

TABLE 4.2 DGGE conditions for analysis of fungal SSU rDNA fragments (after Vainio and Hantula, 2000)

| Primer Pairs | % Denaturing Gradient | Electrophoresis |
|--------------|-----------------------|----------------------|
| FR1GC + NS1 | 18–43 | 17h at 180V and 58°C |

After the run, gels were either silver-stained (H. van Verseveld, *pers. comm.*)³ or stained for 30 min with SYBR[®] Green I (Roche Diagnostics) (10,000-fold diluted in 1× TAE) according to the manufacturer's instructions. The SYBR[®] Green I stained gels were visualized under UV and all gels were photographed and documented with a Bio-Rad VersaDoc imaging system.

Of the two different staining procedures used, SYBR[®] Green was far simpler and more rapid and gave reasonably good results. However, the much more laborious and time-consuming silver-staining method proved to be more sensitive, enabling bands to be detected that were not visible with SYBR[®] Green, so was the stain of choice.

4.2.6 Data analysis

The banding patterns of the DGGE profiles were analyzed using Bio-Rad Quantity One™ (version 4.5) image analysis software, with band detection and quantification of banding patterns as described in section 3.2.7.

4.2.7 Statistical analysis

Statistical analyses were as previously described in section 3.2.8.

4.3 RESULTS

4.3.1 Analyses of soil physicochemical properties at both sites

As variations such as pH, and nutrient and organic matter concentration may act as potential selection factors affecting plant growth and soil microbial communities, the relationship between selected soil variables and the fungal communities was investigated in each soil. The results of the soil analyses from both experimental sites by MRPP and PCA, are described in detail in sections 3.3.1 and 3.3.3.

³ van Verseveld, H., 2001. Vrije Universiteit van Amsterdam.

4.3.2 PCR-DGGE analysis of fungal communities

Amplification of soil fungal DNA by PCR, using the FR1GC/NS1 primer pair, yielded products of the expected size (~1650 bp) for all the samples from both sites. All soil DNA samples produced bright, clear bands in the agarose gels, indicating a good yield of amplicons for DGGE analysis (Plate 4.1).

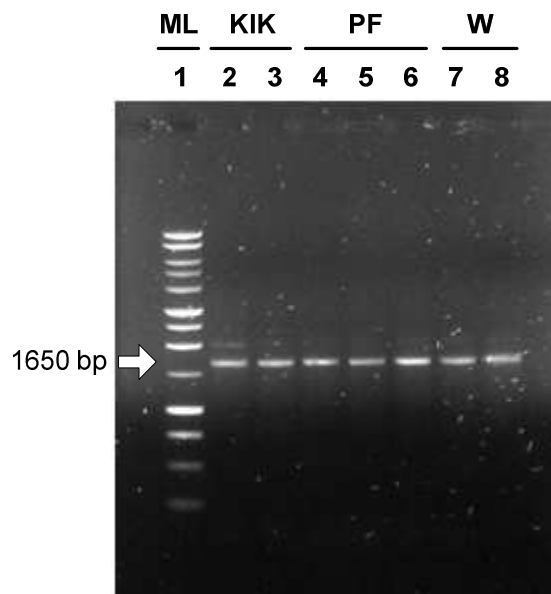


PLATE 4.1 Agarose gel showing some of the fungal DNA amplicons (~1650 bp) from Baynesfield soils amplified using primer pair FR1GC/NS1.

Key: ML = 1Kb ladder; KIK = kikuyu pasture; PF = pine plantation; W = wattle plantation.

The DGGE banding patterns of fungal 18S rDNA sequences (produced using primer pair FR1GC/NS1) from soils under the different land uses at Baynesfield were similar, not only between the replicate samples but also across the gels, unlike those of the bacterial communities (visual assessment). This suggested that intraspecies variation within the 18S rDNA fragments in this study were minimal. Kowalchuk *et al.* (2002) reported similar findings in their PCR-DGGE study of AM fungi, where interspecies variation was also very slight. This implied that the various fungal communities were more homogeneous than the bacterial populations from these soils (Plate 4.2).

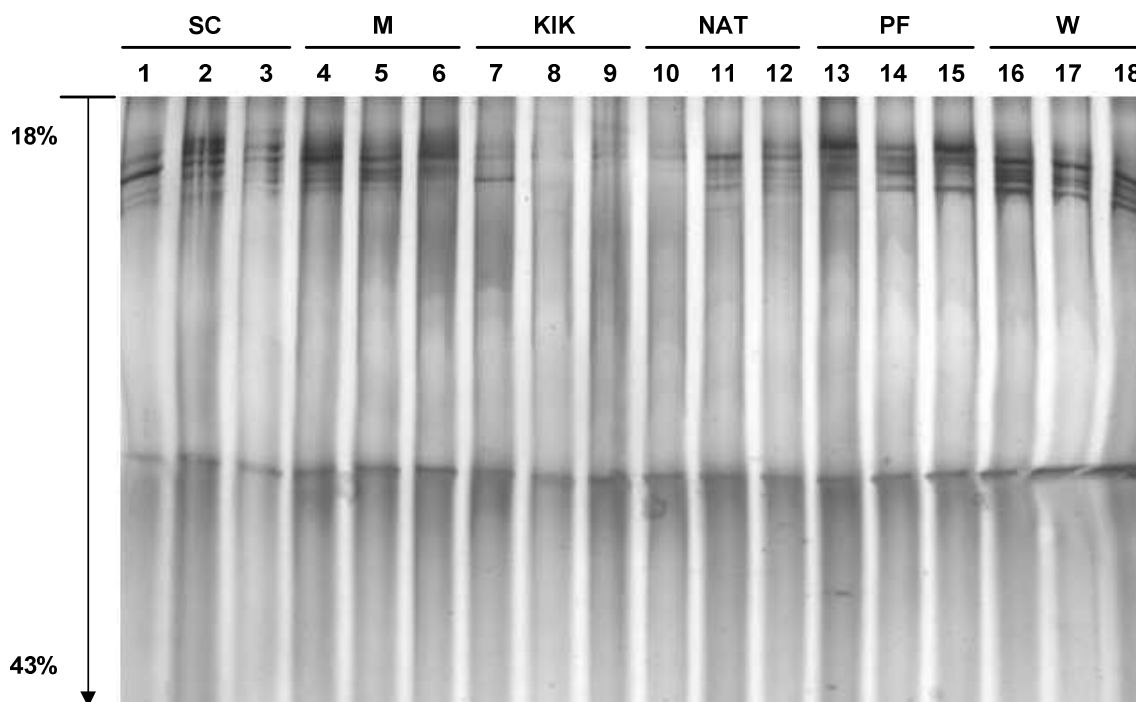


PLATE 4.2 A DGGE gel (denaturing gradient 18–43%), showing 18S rDNA banding patterns of soil fungal communities under different land uses at Baynesfield.

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

Quantity One analysis of the DGGE gel showed that the total number of bands detected per lane varied from only 5–10, with most bands clearly visible and more easily detectable than had been the case with the bacteria. Across the gels, three bands were common to all land uses, namely, bands 3, 4, and 11. The band intensity varied, however, with single bands in some lanes being of a higher relative intensity than the other bands in the same lane, indicating a low evenness in the various fungal communities (Figure 4.2).

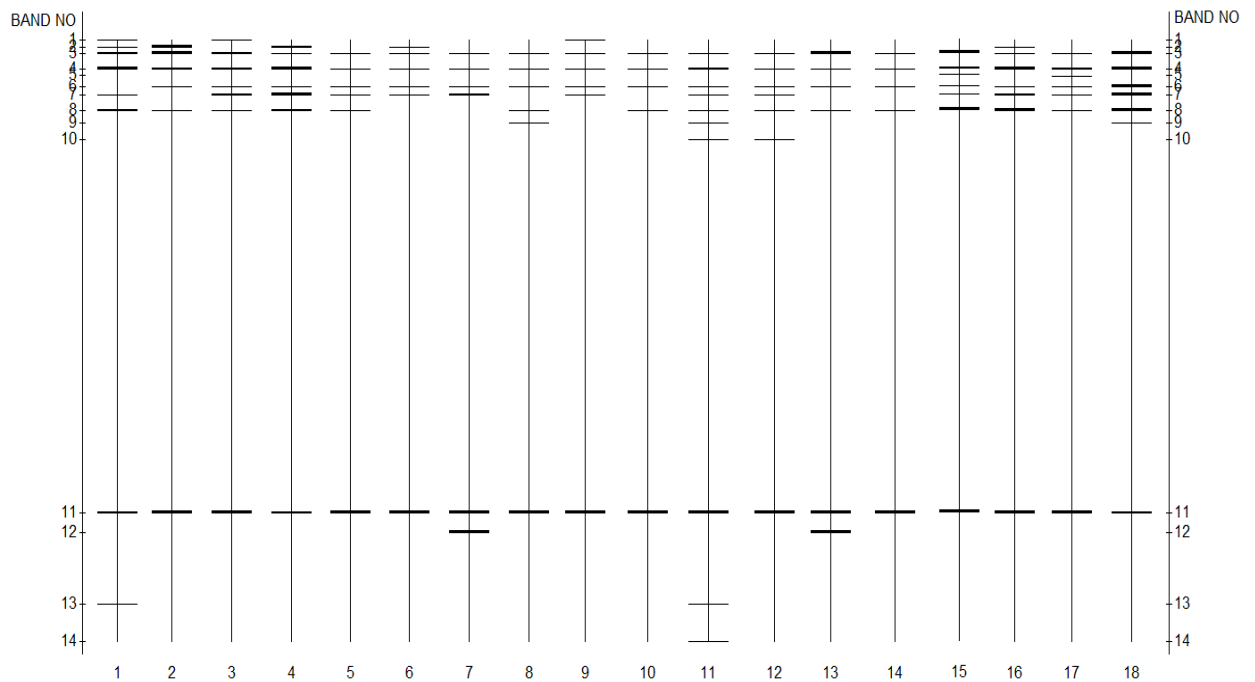


FIGURE 4.2 Quantity One diagram of DGGE gel (Plate 4.2) of Baynesfield soil fungal 18S rDNA banding patterns produced with primer pair FR1GC/NS1.

Key: Lanes 1–3 = sugarcane; lanes 4–6 = maize; lanes 7–9 = kikuyu pasture; lanes 10–12 = native grassland; lanes 13–15 = pine plantation; lanes 16–18 = wattle plantation.

Like those of Baynesfield, the fungal DGGE band profiles of 18S rDNA amplicons (produced with primer pair FR1GC/NS1) from Mount Edgecombe soils under the four different trash managements, were similar to each other across all the gels, again indicating that intraspecies variation in the 18S rDNA fragments was probably minimal. Again this suggested that these fungal populations were more homogeneous than the bacterial communities from the same site (Plate 4.3).

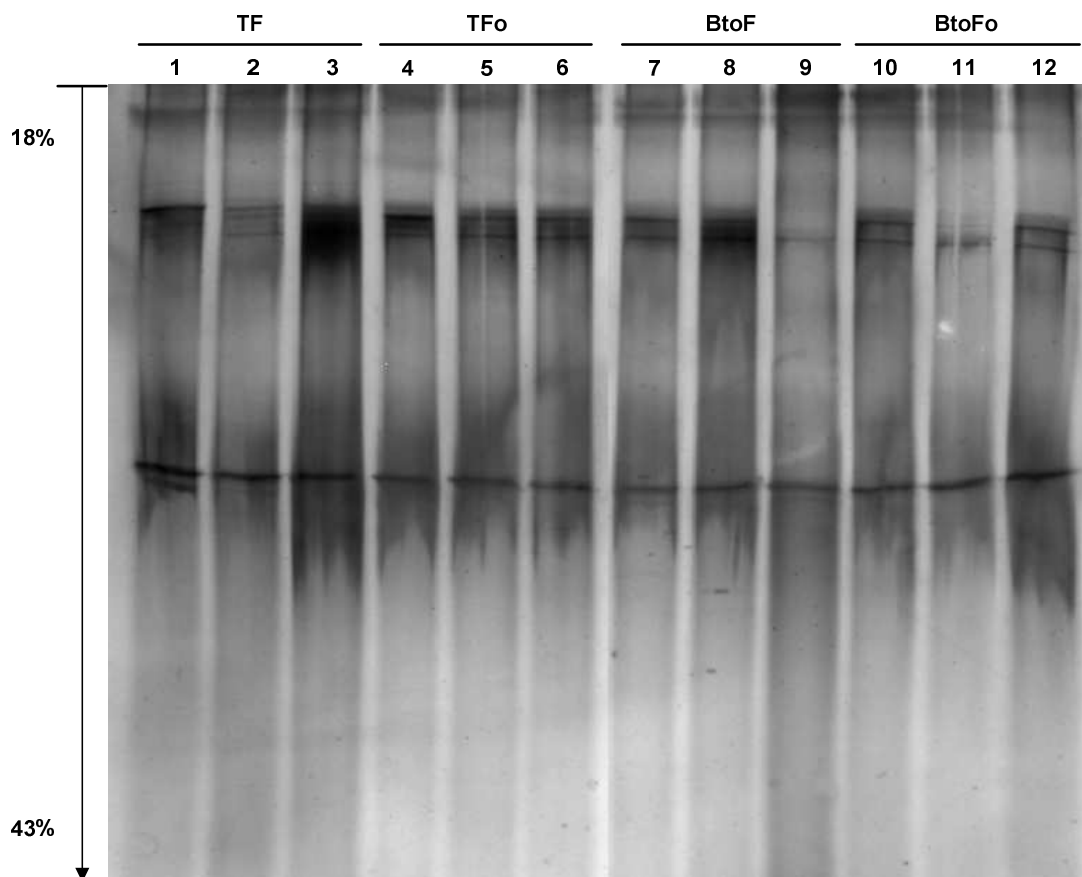


PLATE 4.3 A DGGE gel (denaturing gradient 18–43%), showing 18S rDNA banding patterns of soil fungal communities under different treatments at Mount Edgecombe.

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

Quantity One analysis (Figure 4.3) of the DGGE gel (Plate 4.3) showed that total band numbers varied from only 4–9, with two bands (numbers 6 and 8) common to all the replicates of the four treatments. Here again, band intensity varied, with 1–5 bands per lane being dominant and of a higher intensity than the remaining bands in the lanes (Figure 4.3). This proved problematic for subsequent statistical analysis, as a low evenness within the fungal communities under all the different treatments resulted.

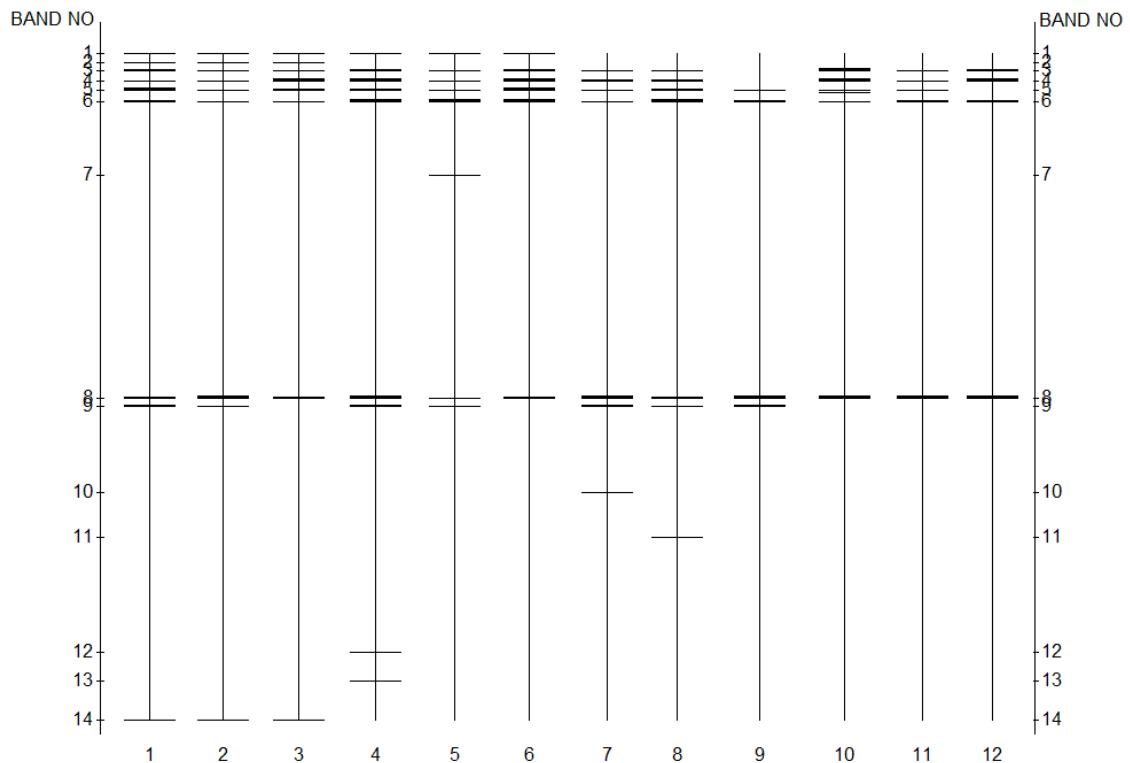


FIGURE 4.3 Quantity One diagram of DGGE gel (Plate 4.3) showing bands not visible in the photograph.

Key: Lanes 1–3: TF = green cane harvested, fertilized; lanes 4–6: TFo = green cane harvested, unfertilized; lanes 7–9: BtoF = burnt cane harvested, fertilized; lanes 10–12: BtoFo = burnt cane harvested, unfertilized.

For the fungal DNA samples from both sites, the majority of bands (from 2–7) in each lane were located near the top in all the DGGE gels. This indicated a relatively high A + T content (Hagn *et al.*, 2003). Only one or two bands ran midway across the gels, with 1-2 bands detected in some lanes towards the bottom of the gels. This banding pattern was consistent throughout all the fungal gels for both sites. Thus the profiles of fungi at both sites were more similar to each other than the respective bacterial profiles had been.

4.3.3 Statistical analysis of fungal communities at Baynesfield Estate (site 1)

Analysis of soil fungal community composition at this site, by means of a NMS two-dimensional plot rotated by PCA, clustered the replicates from each land use on the

basis of the presence or absence of bands (sequence types) (Figure 4.4). An MRPP test of the data showed a greater overall difference ($T = -7.244$, $p < 0.001$) in the fungal community profiles between the different fields and plantations than within them. However, the communities from soils under M and W were closely correlated with each other, and could not be separated.

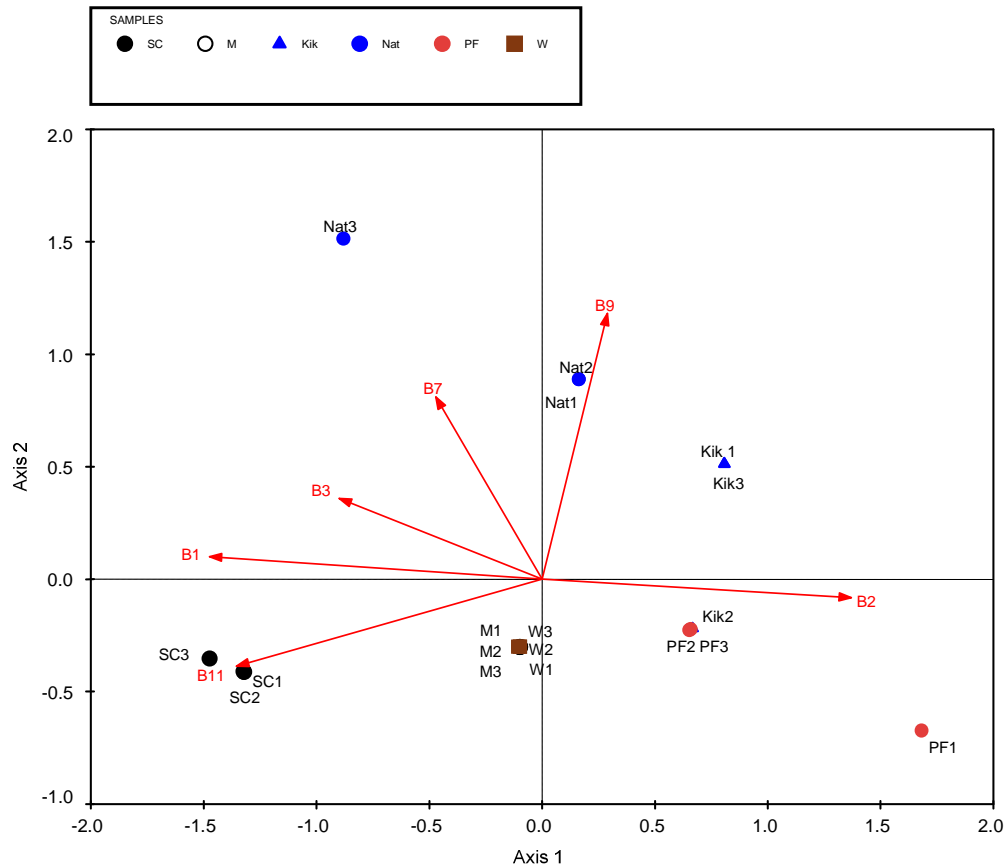


FIGURE 4.4 A NMS two-dimensional plot (rotated by PCA) of fungal communities (presence/absence of bands) at Baynesfield. NMS stress = 0.00049.

Key: M = maize; SC = sugarcane; NAT = native grassland; KIK = kikuyu pasture; PF = pine plantation; W = wattle plantation; B = band.

Analysis of species richness (S) (number of bands present = number of different groups of species) by one-way ANOVA of the soil fungal populations at this site, showed an overall difference between the various fungal communities (Table 4.3). However, the low number of bands present in the gels meant that richness could not reliably be determined. Trends for richness followed the order: $M = W \leq PF \leq KIK = NAT \leq SC$.

TABLE 4.3 ANOVA of species richness (number of bands) and land use means in the soil fungal communities at Baynesfield

| Source of variation | d.f. | s.s. | m.s. | f-ratio | p-value |
|---------------------|------|--------|-------|---------|---------|
| Land use | 5 | 12.000 | 2.400 | 7.20 | 0.002 |
| Residual | 12 | 4.000 | 0.333 | | |
| Total | 17 | 16.000 | | | |

| | | | | | | |
|------------|-----------------|----------------|--------------------|--------------------|-------------------|----------------|
| Grand mean | 7.00 | | | | | |
| Land use | SC ^c | M ^a | KIK ^{b c} | NAT ^{b c} | PF ^{a b} | W ^a |
| Mean | 8.00 | 6.00 | 7.67 | 7.67 | 6.67 | 6.00 |

Means with common superscript letters are not significantly different. ($p < 0.05$; l.s.d. = 1.03)

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

The Shannon Weaver Diversity index (H'), showed a low diversity in the fungal populations, due to the dominance of a single band in one of the samples (W2), which lowered the evenness (J) (Appendix B, Tables B1 and B2).

4.3.4 Statistical analysis of fungal communities at Mount Edgecombe (site 2)

Analysis of fungal community structure at site 2 by means of NMS two-dimensional plots rotated by PCA, clustered the replicate soil DNA samples from the four trash managements on the basis of the presence or absence of bands. Replicate samples of the TF treatments were highly correlated, as were those of TFo treatments. Similarly, replicates of BtoF were closely clustered, as were BtoFo, although the four treatments were clearly separated from each other (Figure 4.5). MRPP analysis of this data showed a significant difference in fungal population profiles between all land treatments ($T = -6.327$, $p < 0.001$).

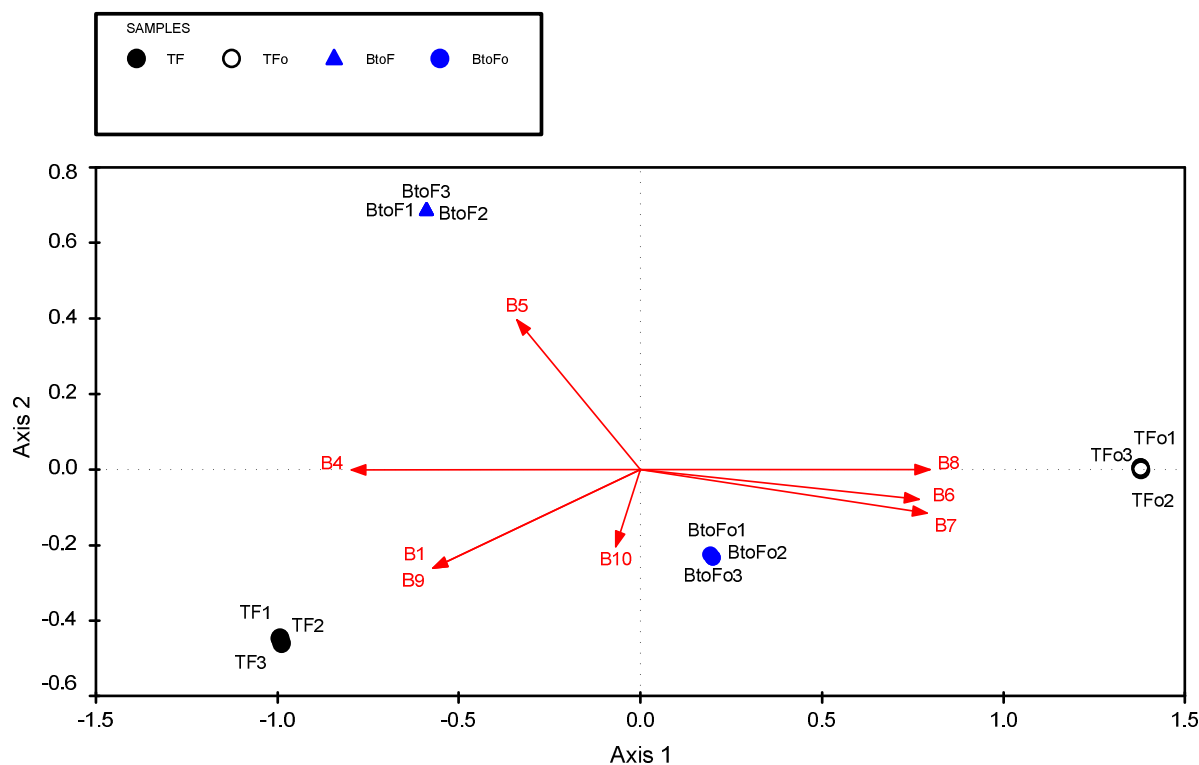


FIGURE 4.5 A NMS two-dimensional plot (rotated by PCA) of fungal communities (presence/absence of bands) under four land treatments at Mount Edgecombe. NMS stress = 0.00044.

Key: TF = green cane harvested with retention of crop residues, fertilized with N, P and K; TFo = green cane harvested with retention of crop residues but unfertilized; BtoF = pre-harvest burnt sugarcane with tops raked off, fertilized with N, P and K; BtoFo = pre-harvest burnt sugarcane with tops raked off but unfertilized; B = band.

An overall significant difference ($p < 0.001$) in species richness (S) of the fungal communities at this site was shown by one-way ANOVA (Table 4.4). Pairwise comparisons of the four treatments were carried out using Fisher's Protected Least Significant Difference test, which indicated a significant difference in richness between TF communities and those under TFo, BtoF and BtoFo. There was also a significant difference in richness between BtoFo and TFo communities, but not between BtoFo and BtoF nor between BtoF and TFo (Table 4.4). However, the low number of bands present in the gels meant that richness could not reliably be determined. Trends for fungal species richness followed the order: $BtoFo \leq BtoF < TFo < TF$. The soil organic C content in increasing order was: $BtoFo < BtoF < TFo < TF$.

TABLE 4.4 ANOVA of soil fungal species richness (number of bands) and land treatment means at Mount Edgecombe

| Source of variation | d.f. | s.s. | m.s. | f-ratio | p-value |
|---------------------|------|-------|------|---------|---------|
| Land treatment | 3 | 11.66 | 3.88 | 23.33 | < 0.001 |
| Residual | 8 | 1.33 | 0.16 | | |
| Total | 11 | 13.00 | | | |

| | | | | | |
|------------|------|--|--|--|--|
| Grand mean | 6.50 | | | | |
|------------|------|--|--|--|--|

| Land treatment | TF ^c | TFo ^b | BtoF ^{a b} | BtoFo ^a |
|----------------|-----------------|------------------|---------------------|--------------------|
| Mean | 8.00 | 6.67 | 6.00 | 5.33 |

Means with common superscript letters are not significantly different ($p < 0.05$; l.s.d. = 0.76)

Key: TF = green cane harvested, fertilized; TFo = green cane harvested, unfertilized; BtoF = burnt cane harvested, fertilized; BtoFo = burnt cane harvested, unfertilized.

Analysis of species evenness (J) of the soil fungal communities showed that the different trash managements at this site, had not influenced this parameter ($F_{3,8} = 0.32$, $p = 0.812$, grand mean = 0.876) nor the Shannon Weaver diversity index (H') ($F_{3,8} = 1.62$, $p = 0.26$, grand mean = 1.618).

4.4 DISCUSSION

Fungi are an important component of the microbiota and are responsible for many of the key steps in a wide range of ecosystem processes (Anderson and Parkin, 2007). One of these processes is the biological cycling of carbon, in which decomposition of cellulose, which constitutes a large part of the vegetation added to soil, is significant. At moderate moisture levels, conditions are conducive to growth of the cellulolytic fungi, which are a very diverse group. Hemicellulose, a major plant constituent added to soils, is utilized by many soil microorganisms including fungi, for growth and cell synthesis. Lignin, the third most abundant plant tissue and crop residue constituent, is degraded almost exclusively by the higher fungi, such as the basidiomycetes (Alexander, 1977; Deacon, *et al.*, 2006).

Decomposer (saprotrophic) fungi play a major role in carbon and nutrient cycling in ecosystems but are sensitive to disturbance, pollution and environmental change. Fungal successions on dead plant material generally consist of initial associations of weak pathogens, followed by primary saprotrophs and decomposers of more recalcitrant substrates, such as cellulose and lignin (Deacon *et al.*, 2006). In addition, the majority of plant species are mycorrhizal with approximately two thirds forming AM and one third, EM associations with fungi. In agricultural production systems however, AM fungal diversity seems to be much lower than in natural environments (Fischer, 2007).

Investigation of fungal communities in complex environmental samples such as soil, using culture-independent molecular techniques, has aroused major interest of late. In fungi, the tandemly repeated ribosomal gene cluster is an appropriate target for PCR amplification, as one to several hundred copies per cell may be present (Hagn *et al.*, 2003). Each major repeat contains the coding regions for the primary rRNAs and non-coding spacer regions. Within the rRNA gene cluster, the genes encoding the precursors for 18S rRNA namely, 18S rDNA, are among the most frequently used targets for ecological studies (Stanier *et al.*, 1987; Anderson and Parkin, 2007).

In this study, universal fungal primers were used, since the objective was to show the response of soil fungal communities to different land uses and management practices, without any deliberate selection by specific PCR primers. As the highest resolution in DGGE gels was obtained by Vainio and Hantula (2000) when using ~1650 bp amplicons produced by the primer pair FR1GC/NS1, these primers were selected for this study. Moreover, as these analyses were based on 18S rDNA and not on 18S rRNA, the abundance of individual fungal species in the DGGE profiles, was not necessarily indicative of fungal growth or activity, as fungi in latent or dormant forms, might be included (Bridge and Spooner, 2001; Hagn *et al.*, 2003).

Direct PCR amplification of fungal DNA, from directly isolated total soil DNA samples, using the above-mentioned primers, successfully amplified the 18S rDNA fragments from all the experimental soils. The advantage of direct, single-step PCR is the elimination of some of the possible bias caused by preferential amplification of specific sequences, which may be exacerbated by successive PCR steps (Vainio and

Hantula, 2000). The approach used in the present study, generated reproducible amplicons from each of the 30 soil samples used, which were subsequently separated in DGGE gels in which the band profiles described in section 4.3.2 were consistently produced. In contrast, Oros-Sichler *et al.* (2006), in a PCR-DGGE analysis of soil fungal communities, using the same soil DNA isolation kit, the same primer pair and the direct PCR method, were unable to amplify the sequences of interest from most of their test soils. They had to devise a new, semi-nested, two-step PCR protocol before distinct, reproducible 18S rRNA gene fragments could be amplified for subsequent DGGE. Thus the method used in the present study was more efficient than that of Oros-Sichler *et al.* (2006).

The highly repetitive banding patterns in all the gels from both sites seem to indicate that not only were the soil fungal populations homogeneous, they were also very stable and were not as easily influenced by different land uses and managements as the bacterial communities from the same sites. However, the relatively few bands obtained per DNA sample (5–10 at site 1, and 4–9 at site 2), meant that species richness could not be determined reliably. This tended to indicate that fungal richness was low when compared with that of the equivalent bacterial communities. This may possibly have been due to the use of a single PCR primer pair, which could have limited the number of bands subsequently detected in DGGE. By using other primer pairs such as FR1GC/FF390, as well as FR1GC/NS1, PCR amplification of additional, partial SSU rDNA fragments of different lengths would have resulted (Vainio and Hantula, 2000). A more comprehensive assessment of the soil fungal communities would thus have been possible (see Chapter 5). Pennanen *et al.* (2004), when using FR1GC/FF390 primers in a soil fungal analysis, reported a large number of bands (sequence types) in their DGGE gels although there were fewer than in their bacterial analysis of the same arable soil.

The DGGE profiles observed in this study, with several bands located in the upper part of the gels and one or two bands midway down, are similar to those reported by other workers on soil fungal DNA, regardless of the primers used. Hagn *et al.* (2003) reported an overall pattern of five or six major bands in the DGGE gels, three in the upper part, others in the middle and a few minor additional bands. Kowalchuk *et al.* (2002), analysing AM fungi from soil, reported DGGE profiles yielding a total of

three to five bands per sample, occurring in an upper and a lower grouping in the gels. Some bands comprised more than one DNA fragment of slightly different sequence but the same mobility, so some minority populations could have been masked by more dominant ones, resulting in an underestimation of the number of fungal species present. It was reported by van Elsas *et al.* (2000) that a limited number of bands in DGGE gels were produced from soil fungal DNA, with the fungal profiles showing a much lower diversity than that commonly observed for soil bacteria. This was attributed to the restricted sequence divergence in fungal 18S rDNA together with the presence of large G + C stretches in the separated sequences, which together led to an aberrant and possibly converging melting and migration behaviour.

Since the separation of bands in DGGE gels is based on differences in base sequence, which determine the melting properties (Muyzer *et al.*, 1993; 1996), this could explain some of the observed grouping of bands in the eukaryotic (fungal) profiles compared to the better spread of bands in the bacterial profiles from the same soils. Prokaryotic DNA varies in G + C content from ~24%–76% and particular taxonomic groups only include organisms varying in G + C content by no more than 3–5% (Tiedje *et al.*, 1999). By contrast, in eukaryotes (such as fungi) the range of G + C content is relatively narrow (~35–40%) and is quite similar in plants and animals. This results in two organisms with identical mean DNA base composition differing greatly in genetic constitution, which is evident from the very similar DNA base ratio values from all plants and animals (Stanier *et al.*, 1987). This could possibly account for the clustering behaviour of the majority of bands observed in the fungal gels in this study as well as in those of other workers. The relatively high A + T content (Hagn *et al.*, 2003) of most of the sequences in the fungal DNA bands, caused them to melt at low concentrations of denaturant and therefore to be located near the top of the gel. Also, as observed by Kowalchuk *et al.* (2002), the tendency for more than one fungal species of similar sequence to separate out in the same band, could possibly be due to the highly similar G + C content of all eukaryotes. This could have led to the observed apparent reduction in the richness and genetic diversity in the soil fungal communities under the various land uses and treatments compared to the soil bacterial communities from the same sites. An improved spread of bands might have been achieved in this investigation had other denaturing gradients been tested, besides the

18–43% suggested by Vainio and Hantula (2000). This is discussed further in Chapter 5.

It is thus apparent that many factors, including PCR bias, could have influenced the nature of the DGGE fungal banding profiles obtained. The use of a single primer pair; the denaturing gradient; similarities in eukaryote G + C content; and limited sequence divergence in fungal 18S rDNA compared to bacterial 16S rDNA, could all have contributed to the observed sparse banding patterns.

4.4.1 Effects of different land uses and management practices on soil fungal community structure at the experimental sites

4.4.1.1 Baynesfield Estate (site 1)

Soil fungal communities under the different land uses at this site were found to differ more from each other than from the replicate samples within the same land use. The fungal community under pre-harvest burnt sugarcane, showed the highest richness, unlike the bacterial community from the same land use. As the amount, type and availability of organic matter determine the size and composition of the soil heterotrophic population (Alexander, 1977) and as the organic C level was lowest in soil under sugarcane, a lower richness in the fungal community under sugarcane than under the other land uses was expected. The higher richness observed may, in part, be explained by the fact that soils under this land use had a low pH (~4.3) which would tend to favour fungal growth over that of bacteria. In neutral or alkaline soils, many organisms are capable of hydrolysing cellulose, whereas at acid reaction, the degradation of cellulose is mediated largely by filamentous fungi. The process is rapid below pH 5.0 and occasionally below 4.0 (Alexander, 1977). Another contributing factor might be the loss over time, of readily metabolizable C from soils under continuous sugarcane production (Graham *et al.*, 2002b). This would result in a soil with a low overall organic C content but a high proportion of relatively recalcitrant humic C. Fungi are known to be particularly effective at degrading such organic compounds, so the low organic matter content may have acted as a selection pressure favouring fungal proliferation (Alexander, 1977). However, a similar effect would

also be expected in the maize soil, which had a slightly higher pH (4.5) but a slightly lower organic C content than the sugarcane soil. In addition, soil physicochemical analyses also indicated, that exchangeable K and, to a lesser extent, extractable P, had influenced microbial communities under these two land uses (Figure 3.1). As previously noted in section 3.4.1, the sugarcane fields are tilled only every 6–7 years (at replanting), whereas maize fields are tilled annually. Repeated primary and particularly secondary tillage is likely to result in mechanical disruption and destruction of filamentous fungal hyphae (Pennanen *et al.*, 2004), thus affecting the fungal biomass.

Under pine at this site, the soil pH was low (~4.1), and the dense layer of pine needles covering the soil surface, together with decaying root tissues and small branches, provided large inputs of organic matter to the soil. In the destruction of forest litter, wood and woody tissues, fungi such as cellulolytic and ligninolytic basidiomycetes are especially prominent (Alexander, 1977). Thus a large, diverse fungal community would have been expected to occur under this land use. However, community richness was lower than that under sugarcane, kikuyu pasture and native grassland, suggesting that the pine soil fungal community possibly consisted of a limited number of highly-specialised fungal species, adapted to the prevailing conditions. During a study of fungal succession on leaf litter, Atlas and Bartha (1987) found that a simultaneous, parallel succession of bacterial populations probably occurred, which may have affected the observed fungal succession. In the present study, the rich, widely diverse bacterial community found at this site, may possibly have had a dampening influence on the fungal population, as a consequence of substrate competition, resulting in a less rich, less genetically diverse fungal community than expected.

At this site, permanent kikuyu pasture and native grassland soil fungal communities were of similar richness, although the soil organic C content was higher under the former than under the latter land use. Exchangeable Mg was also higher under kikuyu than under native grasslands and PCA analysis (Figure 3.1) showed that organic C, Mg and pH were the main factors influencing microbial communities at this site. Grasslands throughout the world owe their continued existence to fungal activity, because saprotrophic fungi decompose dead organic matter, thereby recycling carbon and other nutrients. In grasslands as in forests, basidiomycetes are the major

decomposers of dead organic matter (Deacon *et al.*, 2006). NMS analysis of fungal community composition, based on the presence or absence of bands (groups of species) in the DGGE gels, clearly separated the kikuyu and native grassland communities. However, ANOVA indicated that differences in fungal community evenness were low; accordingly, the Shannon Weaver index showed a low diversity. In an earlier study at this site, Graham (2003), using PLFA analysis, found that the fungal/bacterial ratio was lowest under kikuyu pasture and highest under native grassland. The results also indicated that, in comparison with native grassland, improved kikuyu pasture soil had a higher organic matter content and a smaller fungal community of similar richness, but with lower evenness and a lower diversity index. Therefore, although the number of fungal species in the soil under kikuyu and native grassland was essentially the same, the low evenness and diversity was regarded as indicative of the presence of a small number of dominant species in the kikuyu fungal community. This was interpreted as a reflection of the complexity of the interactions between land use and the size and composition of soil microbial communities. These results support the findings in the current study.

The close relationship between fungal communities under maize (conventional tillage) and wattle (Figure 4.4) at this site, was unexpected and in contrast to the findings in the equivalent bacterial study. The banding patterns, both in terms of band number and bands common to both maize and wattle fungal communities, were very similar in duplicate DGGE gels. This indicated that fungal populations at both sites contained essentially the same groups of species. However, soil physicochemical analyses (Figure 3.1) showed that soils under these two land uses differed considerably. Maize soil had a higher P and K content, whereas wattle soil had a higher pH, organic C and exchangeable Ca and Mg content. Other research has shown that fungal/bacterial ratios are about five times lower in arable than in forest soils, due to the frequent disturbances associated with tillage and the presence of a distinct, thick litter layer under forests (Pennanen *et al.*, 2004). One factor which may possibly have contributed significantly to the similarity in the two communities is that wattle trees drop only small amounts of litter, so the soil surface is essentially bare (Dlamini and Haynes, 2004). Other important factors to be considered, are that wattle soil pH (~5.5), was the highest of all the land uses at this site, and the bacterial community was the most diverse. These conditions would tend to favour bacterial growth over

that of fungi, causing the former to proliferate more rapidly than the latter, and to become the dominant microbial population.

4.4.1.2 Mount Edgecombe (site 2)

At this site there was an overall significant difference in fungal community structure under the various sugarcane trash managements. Analysis of the DGGE profiles clearly separated the different fungal communities from the four crop residue management soils. Those treatments retaining a full trash blanket (T) supported communities with a greater richness than those under pre-harvest burnt sugarcane (Bto). TF had the highest richness and BtoFo the lowest. CCA showed that soil organic C content was one of the main factors involved in differentiating fungal communities under trashed treatments from those under pre-harvest burning. Loss of exchangeable Mg (associated with soil acidification) was the main factor differentiating communities under fertilized conditions, from those under unfertilized conditions. However, addition of fertilizer increased fungal community richness relative to unfertilized treatments. As previously noted, fungi have a selective advantage over bacteria in acid soils, so applications of fertilizer that increase acidity due to nitrification of ammonium sulphate (Graham, 2003) would tend to favour fungal dominance over bacteria. This author had previously reported that for unfertilized treatments (Fo), at this site, trash retention had greatly increased the fungal to bacteria ratio, which supports the findings in the present study. In contrast however, this did not apply to fertilized plots (F).

At this site, both the greater soil fungal richness and the larger diversity index under trash retention compared to burning, were as expected. Surface retention of crop residues stimulates fungal growth more than that of bacteria, causing the fungi to predominate (Holland and Coleman, 1987). In the present study, fungal mycelium was clearly visible, both ramifying through the decaying trash layer and on the soil surface below. This suggests that the decaying crop residues provide a medium which supports a more diverse fungal community than that under the bare soil surface of pre-harvest burnt sugarcane. Normally a few dominant species occur in a trophic level, although the less abundant species largely determine the species diversity of that level

and of the whole community. Diversity generally decreases when one or a few populations attain high densities, with high numbers tending to signify successful competition but also the dominance of a single population (Atlas and Bartha, 1987).

4.4.2 Overall assessment of the DGGE analysis of the soil fungal communities from both sites

In this study, the total number of bands detected per lane in all the DGGE gels, for soil fungi from both sites was low (4–10). In addition, the bands varied in relative intensity (fungal mass), indicating that individual groups of species in the brighter bands were more abundant than those in the lighter bands. However, several different species with nearly identical sequences and melting domains may have co-migrated within a single band (Kowalchuk *et al.*, 2002). This could have resulted in the low evenness and, consequently, the low diversity observed at the experimental sites. These findings support the observation that the resolving power of DGGE gels is limited for the separation of fragments in excess of 500 bp (Kennedy and Gewin, 1997). The DGGE technique was originally designed to analyse point mutations in relatively short DNA fragments (Vainio and Hantula, 2000) and is based on sequence differences (Muyzer *et al.*, 1993; Kowalchuk *et al.*, 2002). Therefore, when using very long molecules, some of the base-pair differences may remain undetected (Vainio and Hantula, 2000). Notwithstanding this, the last named authors, obtained the highest resolution in DGGE when using the ~1650 bp amplicons, despite resolution in DGGE gels usually decreasing for DNA molecules with large melting domains. However, these authors were working with wood-inhabiting fungi so relative to soil-based studies, there would have been only a limited number of different species to separate on the gels, in contrast to the large numbers from soil.

4.5 CONCLUSIONS

This study demonstrated that the direct PCR-DGGE system used was successful in showing that different land uses and sugarcane residue managements had affected the community structure (genetic diversity) of the soil fungal communities at the sites under investigation. In comparison with cloning techniques, it is also a relatively rapid

method of screening multiple soil samples. Moreover, consistent banding profiles were obtained in duplicate DGGE gels, indicating good reproducibility.

The fungal communities at Baynesfield Estate were found to be much more stable and homogeneous than the bacterial communities at the same site so were less influenced by differences in land use. Fertilizer applications, organic C and pH were all shown to have had a positive influence on the fungal community richness and genetic diversity under the various land use types. At Mount Edgecombe, the fungal communities were also very homogeneous and stable, showing very little variation both within and between treatments. Here too, pH affected the fungal communities, with applications of fertilizer and organic C increasing community richness. This effect was particularly noticeable in fertilized soils where organic residues were retained. Therefore, treatments causing an accumulation of organic matter (together with applications of fertilizer) increased fungal genetic diversity relative to those unfertilized treatments where organic matter was depleted.

Chapter 5

DNA-DERIVED ASSESSMENTS OF FUNGAL COMMUNITY COMPOSITION IN SOILS UNDER DIFFERENT LAND USES AND MANAGEMENT PRACTICES

5.1 INTRODUCTION

To manage soil better and to minimise negative environmental impacts, a more detailed understanding of the activities of microbial communities in soil, and of their response to environmental stress and climate change is needed (Hirsch *et al.*, 2010). This applies particularly to the fungal component. Various methods have been developed to assess soil fungal communities, which could potentially contribute to a better understanding of their ecological role in soil habitats (Schwarzenbach *et al.*, 2007).

Few studies have investigated how specific changes in edaphic characteristics may influence soil fungal community composition across land use types (Lauber *et al.*, 2008). In addition, the critical role of plant root exudates in structuring soil fungal communities is poorly understood (Broeckling *et al.*, 2008).

The use of molecular techniques in ecology has provided a framework linking organisms to the processes they control, with PCR-based analyses dominating molecular diversity assessments (Mitchell and Zuccaro, 2006). Genetic fingerprinting of PCR amplicons using DGGE has been used increasingly in studies of microbial ecology (Lynch and Thorn, 2006). However, this approach has seldom been used to characterize eukaryotic diversity (Aguilera *et al.*, 2006). Even with the introduction of molecular techniques, the diversity of soil fungi, particularly of the basidiomycetes, has not been fully explored (Lynch and Thorn, 2006).

Broad-based diversity studies can be performed with the small (18S rRNA) subunit ribosomal RNA gene, which can be used in phylogenetic analyses to determine evolutionary relationships between taxa. However, as the nuclear SSU rRNA gene is highly conserved, it resolves little phylogenetically beyond the family level (Mitchell and Zuccaro, 2006). A large, constantly expanding electronic database of SSU gene sequences (both of 16S in prokaryotes and 18S in eukaryotes) enables many soil bacteria, archaea and fungi to be identified to the genus, species and, occasionally, sub-species level. Nonetheless, many remain unassigned to known phyla and are classified as ‘uncultured’ (Hirsch *et al.*, 2010).

The aim of this component of the current study was to expand the scope of the initial work, conducted over the 2004–2006 period, by: (i) using an additional universal fungal primer pair to that used in section 4.2.5, to provide a wider spectrum of sequences; (ii) modifying the DGGE protocols and testing different denaturing gradients to improve band separation; and (iii) sequencing some of the prominent, common bands to show whether individual bands contained more than one fungal species, to reveal the presence of chimeras and to identify whether apparently ‘common’ bands in different lanes contained the same fungal groups. This approach, allowed a more accurate assessment to be made of the effects of the different land uses and management practices on the soil fungal community structural diversity at the two sites.

5.2 MATERIALS AND METHODS

5.2.1 Study sites and soil sampling

The study sites are the same as those described in section 3.2.1. Re-sampling of soils from both sites (0–5 cm depth) took place in August/September 2008, at the end of the dry winter season, and followed the same procedure described in section 3.2.2. Immediately after collection in the field, replicate soil subsamples were stored on ice. On return to the laboratory, subsamples from each replicate were bulked so that there were three independent samples for each land use and also for each land management. All bulked, field-moist samples were thoroughly mixed and sieved (2 mm).

Subsamples for DNA extraction and CLPP (sections 6.2.4 and 7.2.4) were stored in plastic bags at 4°C, and further subsamples for chemical analysis were again sieved (< 1 mm) and air-dried.

5.2.2 Chemical analysis

Chemical analysis of fresh soil samples followed the methods described in section 3.2.3.

5.2.3 DNA extraction, purification and quantification

Total genomic DNA was directly extracted and purified, from soils at both sites, using an UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc. CA, USA). For the Baynesfield site, 0.5 g of each replicate soil sample was added to separate bead solution tubes, and DNA was extracted and purified according to the manufacturer's instructions, using the protocol designed to maximize yields. Duplicate samples for each soil were prepared, and the DNA concentrations were determined using a WPA Biowave II spectrophotometer. Electrophoresis of the DNA samples (5 µl) in 0.8% agarose gels was carried out, and the gels were then stained with ethidium bromide, visualised with UV and photographed.

Difficulty was experienced repeatedly when isolating DNA from the various Mount Edgecombe soil samples. Accordingly, both direct and indirect DNA extraction procedures, and two different DNA extraction kits, were tested. The most successful method was by direct DNA extraction with the MO BIO UltraClean™ Soil DNA isolation kit, using a special protocol recommended by the manufacturers for recalcitrant soils/samples. In this method, 0.4 g of each soil subsample was added to separate bead solution tubes, after which the protocol to maximise yields was followed but with the following modifications. Prior to the bead beating step: (i) two freeze/thaw steps were added (samples were frozen for ± 30 min. at -20°C until solid and then allowed to thaw completely [± 10 min] at 65°C in a waterbath) to assist in the lysis of recalcitrant microbes and fungal spores; and (ii) the inhibitor removal solution (IRS) step was eliminated to reduce DNA loss. Before centrifugation in the

spin columns, the pH of each sample was measured and was found to be within the ideal range (5.6–6.5). Despite the additional measures, no bands were visible in the subsequent agarose gel, which was attributed to the DNA concentration being below the level of detection of the gel (~2–5 ng) and indeed this was found to be the case. Therefore, in order to conserve the samples, no spectrophotometric estimation of DNA concentration from these soils was carried out. All samples were then stored at -20°C.

5.2.4 PCR amplification of fungal 18S rDNA fragments

To amplify fungal 18S rDNA fragments of a different length (~390 bp) to those produced by the original primer pair, FR1GC/NS1 (~1650 bp) (section 4.2.5), the universal primer pair FR1/FF390 was used. Pennanen *et al.* (2004) reported that reproducible DGGE fingerprints, reflecting the sequence diversity of soil fungal communities, were consistently generated with the latter primer pair. A GC clamp sequence was again added to the reverse primer FR1, (Vainio and Hantula, 2000) (section 4.2.4). The relative locations in the SSU rDNA region, of both sets of primers and their sequences, are shown in Figure 4.1 and Table 4.1, respectively (section 4.2.4).

Partial SSU rDNA fragments were amplified in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Each 50 µl reaction mixture contained 5 µl of 10× reaction buffer, 3 µl of 25 µM MgCl₂ (1.5 mM MgCl₂ final concentration), 0.5 µM of FR1GC reverse primer and 0.5 µM of FF390 forward primer, 200 µM of each dNTP (ABgene, Rochester, NY, USA), 1.25 U SuperTherm Taq DNA Polymerase, sterile MilliQ H₂O and 1 µl Bovine Serum Albumen (BSA) (20 mg ml⁻¹), added to prevent amplification inhibition by organic compounds co-extracted from soil (Pecku, 2003). Template DNA, 1 µl for the Baynesfield samples and 10 µl for the Mount Edgecombe samples (as the DNA concentration in the latter was low), were added to each PCR reaction.

PCR conditions were as described by Vainio and Hantula (2000) for primer pair FR1GC/FF390, namely, an initial denaturation step (95°C/8 min), followed by 30

cycles comprising a denaturing (95°C/30s), annealing (50°C/45s), and elongation (72°C/2 min) step, and a final single elongation step (72°C/10 min). This protocol was suitable for the Baynesfield soil DNA samples, but not for those from Mount Edgecombe, as the yield of fungal amplicons using this procedure was low. Therefore, for the latter samples the number of denaturing/annealing/elongation cycles was increased to 40. In addition, the concentration of Mount Edgecombe fungal amplicons was further increased for subsequent DGGE, by adopting a nested PCR approach. This comprised a first round of PCR, using total community DNA as the template, followed by a second round, using first round amplicons from the nested PCR as template (Clegg, 2006). The same primer pair was used for both rounds of PCR. The amplicons (~390 bp) were analysed by electrophoresis of 5 µl aliquots (Baynesfield) and 10 µl aliquots (Mount Edgecombe) in 2% agarose gels stained with ethidium bromide, then visualised under UV and photographed.

5.2.5 Community fingerprinting by DGGE

PCR fungal amplicons were analysed by DGGE with the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using a modified protocol of Pennanen *et al.* (2004). PCR amplicons (20 µl) were loaded onto 8% (v/v) polyacrylamide gels (Sigma, acrylamide/bisacrylamide 40% solution, mix ratio 19:1) with a 30–50% denaturing gradient (100% denaturant contains 7 M urea and 40% deionized formamide in 1× TAE buffer, pH 8.3). The gels were allowed to polymerise for 1.5–2.5 h. Electrophoresis was run in 1× TAE buffer at a constant temperature of 60°C for 16h at 65V. The gels were then stained for 35 min with SYBR® Gold (Invitrogen™, Molecular Probes™) (10,000-fold dilution in 1× TAE), visualized under UV, and scanned and photographed with a Bio-Rad VersaDoc system. SYBR® Gold nucleic acid stain was chosen for this study, as it is currently the most sensitive fluorescent stain for detecting double-stranded DNA when using standard UV transilluminators (Molecular Probes™, Eugene, OR, USA).

5.2.6 Data analysis

The banding patterns of the DGGE profiles were analysed with Bio-Rad Quantity One™ (version 4.6) image analysis software, with respect to band number, position and relative band intensity (details in section 3.2.7).

5.2.7 Statistical analysis

Statistical analysis of the DGGE profiles was as previously described in section 3.2.8, except with respect to the following:

Genstat Release 11.1 (PC/Windows) 2008, VSN International Ltd, Hemel Hempstead, U.K., was used for one-way (ANOVA). Species richness (S) (number of bands present) was determined, and pairwise comparisons were tested by Tukey's test (95% confidence intervals). Relative band intensity (mass) was used to determine Pielou's species evenness (J) (a measure of the equitability of the relative band intensities across the gels) and the Shannon Weaver Diversity Index (H') (Peet, 1974) was used to give a composite measure of microbial community diversity, based on both species richness and evenness. Least significant differences were calculated at the 5% level.

5.2.8 DNA sequencing analysis of DGGE bands

To obtain samples for sequencing from both sites, prominent DGGE bands were excised (using separate, sterile scalpel blades for each band), from the UV-illuminated acrylamide gels, following a method adapted from Ma *et al.* (2005). A low intensity transilluminator was used (365 nm wavelength, UVB) to minimise damage to the DNA. Each excised band was placed in a separate sterile microfuge tube, to each of which, 30 μ l sterile MilliQ water was added. This was followed by a brief pulse-spin in a microfuge. The DNA was allowed to elute overnight in an incubator at 28°C, after which the tubes were again subjected to a pulse-spin in a microfuge, and the supernatant containing the eluted DNA, transferred to clean, sterile microfuge tubes.

The eluted DNA was used for PCR amplification as described in section 5.2.4, and the resultant amplicons were analysed by DGGE, but using a narrower denaturing gradient (35–45%). The position of these bands relative to the original bands was verified by running excised DNA amplicons alongside controls consisting of non-excised DNA amplicons (Sekiguchi *et al.*, 2008). The gel was then analysed with respect to band position and band number, using Quantity One software. The aim was to test the assumptions made previously (section 4.4), that single DGGE bands may comprise more than one DNA fragment of slightly different sequence but of the same mobility (Kowalchuk *et al.*, 2002), or that a band may contain fragments from different fungi, which may have run together due to the similarity of the G + C content among eukaryotic DNA (Stanier *et al.*, 1987). These amplicons were then gel-purified and sequenced with forward primer FF390 (no GC clamp) by Inqaba Biotechnical Industries, Pretoria, South Africa, using the Sanger sequencing method with AB1 3130 XL sequencers (Applied Biosystems, Foster City, CA, USA).

Comparisons of the partial soil fungal 18S rDNA sequences from both sites, obtained from the excised bands, and the closest matches in the National Centre for Biotechnology Information (NCBI) nucleotide database, were made using the online standard BLAST (Basic Local Alignment Search Tool) programme (<http://www.ncbi.nlm.nih.gov/>). In addition, the Ribosomal Database Project (RDP) (http://wcdm.nig.ac.jp/RDP/docs/chimera_doc.html) was used for online screening of possible chimeric sequences. Sequences which were: (i) of poor quality, (ii) of fewer than 200 base pairs in length, or (iii) suspected of being chimeric, were eliminated from the analysis (Jangid *et al.*, 2008; Lauber *et al.*, 2008). Each sequence was identified according to the first BLAST match, but as most indicated the sequences were from ‘uncultured’ soil fungi, the first 5 matches were considered. However, as only one of these matches was deemed reliable (sample PF3, Table 5.2), the other sequences remained classified as ‘uncultured’ (Allison *et al.*, 2007).

5.3 RESULTS

5.3.1 Analyses of soils at the Baynesfield experimental site (site 1)

The means (\pm standard deviation) of the selected physicochemical variables of the fresh soil samples collected at this site in 2008, are presented in Table 5.1.

At this site, soil organic carbon increased in the order: M < W < SC < NAT < PF < KIK, and soil pH in increasing order was: PF < SC < M = W < KIK < NAT. High concentrations of extractable P were recorded in the fertilized M and SC soils, and lowest concentrations in soil under undisturbed NAT. The highest exchangeable K concentration was in SC soil followed by M and KIK, and lowest under W, whereas exchangeable Ca was highest under KIK, followed by W and M and lowest under SC. The Mg concentrations were highest in KIK soil followed by that of NAT, and lowest under SC. Soil pH was lowest under PF and highest in NAT soils (Table 5.1). While some of the selected physicochemical variables from the soil sampling in the initial study (2004) were similar to those of the present study, others had changed (Table 3.2, section 3.3.1)

TABLE 5.1 Means (\pm sd) for selected physicochemical properties of soils collected at 0–5 cm depth from different land uses at Baynesfield Estate

| Land use | pH (KCL) | Organic Carbon | Extractable P | Exchangeable cations | | | Exch. acidity | Total cations (ECEC) |
|----------|------------|----------------|---------------------|--|------------|------------|---------------|----------------------|
| | | | | K | Ca | Mg | | |
| | | % | mg kg ⁻¹ | ----- cmol _c kg ⁻¹ ----- | | | | |
| SC | 4.4 (0.05) | 5.0 (0.17) | 72.4 (21.50) | 1.7 (0.19) | 3.5 (0.49) | 1.9 (0.29) | 0.6 (0.19) | 7.8 (0.90) |
| M | 4.6 (0.17) | 3.9 (0.16) | 156 (32.02) | 1.4 (0.07) | 6.8 (0.56) | 2.8 (0.14) | 0.1 (0.02) | 11.2 (0.56) |
| KIK | 4.7 (0.08) | 9.4 (0.22) | 25.0 (16.46) | 1.4 (0.20) | 7.8 (0.97) | 5.5 (0.82) | 0.1 (0.03) | 14.8 (0.67) |
| NAT | 4.8 (0.28) | 5.1 (0.12) | 3.5 (0.54) | 0.5 (0.11) | 5.4 (0.85) | 4.9 (1.47) | 0.1 (0.05) | 10.9 (2.19) |
| PF | 3.8 (0.03) | 6.3 (1.00) | 11.3 (2.21) | 0.4 (0.04) | 4.2 (0.43) | 2.5 (0.28) | 2.7 (0.21) | 9.8 (0.49) |
| W | 4.6 (0.04) | 4.8 (0.29) | 3.4 (0.46) | 0.4 (0.03) | 6.8 (0.64) | 2.7 (0.32) | 0.2 (0.02) | 10.0 (0.98) |

Key: SC = sugarcane (burnt cane harvested); M = maize (CT); KIK = kikuyu permanent pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

A PCA analysis of this soil data showed the compositional relationships among replicate subsamples from the different land uses (Figure 5.1). The closer the vector for an individual variable was aligned with a principal component axis, the more that particular variable accounted for the variation in the data along that axis. Calcium, ECEC, Mg and pH were correlated with PC1, and accounted for 45.2% of the total variance, whereas P, K, organic C and acidity were correlated with PC2, and accounted for 26.2% of the total variance. Cumulatively, PC1 and PC2 accounted for 71.4% of the total variance in the soil data. The PF soils were associated with high acidity and low pH, whereas the W soils were associated with lower acidity and organic C levels than PF soils. The SC and M soils were closely correlated with a high soil P content, with M soils also closely associated with a high soil K content. Soils under KIK were correlated with high organic C, Mg and ECEC. Considerable variability was shown among the samples with respect to soil physicochemical properties. (MRPP, $T = -8.965$; $p < 0.001$).

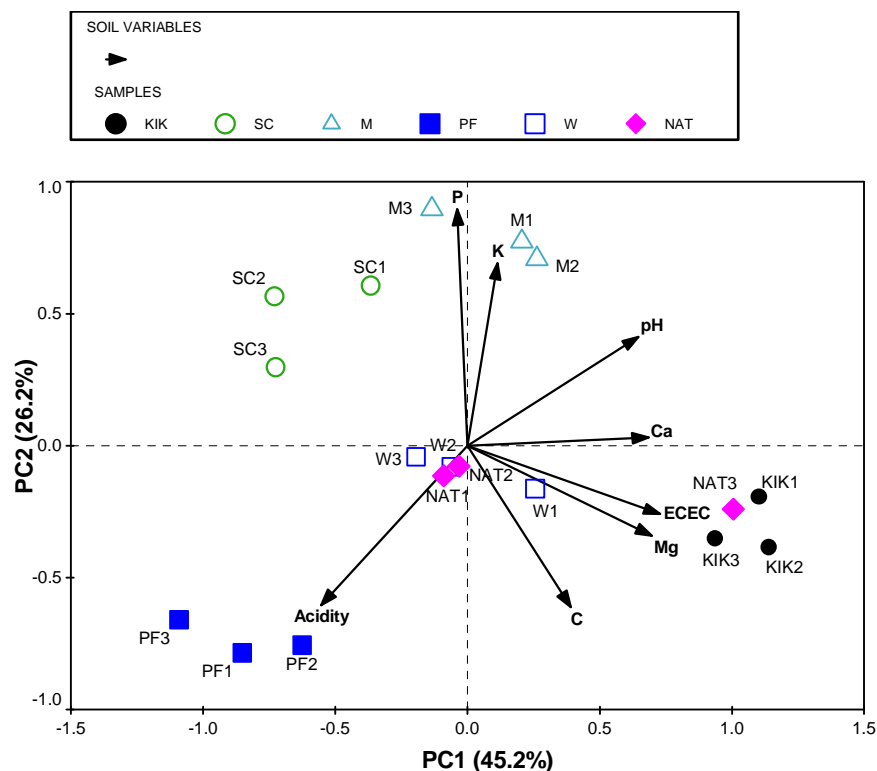


FIGURE 5.1 A PCA biplot (standardised and centred data) of sites and soil variables for subsamples of fields with various land uses at Baynesfield Estate. The PC1 (horizontal axis) and PC2 (vertical axis) accounted for 45.2% and 26.2%, respectively, of the total variance in the soil data.

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

5.3.2 PCR-DGGE analysis of fungal communities

The total genomic DNA isolated from the various land use soil subsamples at Baynesfield using a MO BIO Ultraclean™ kit is shown in Plate 5.1.

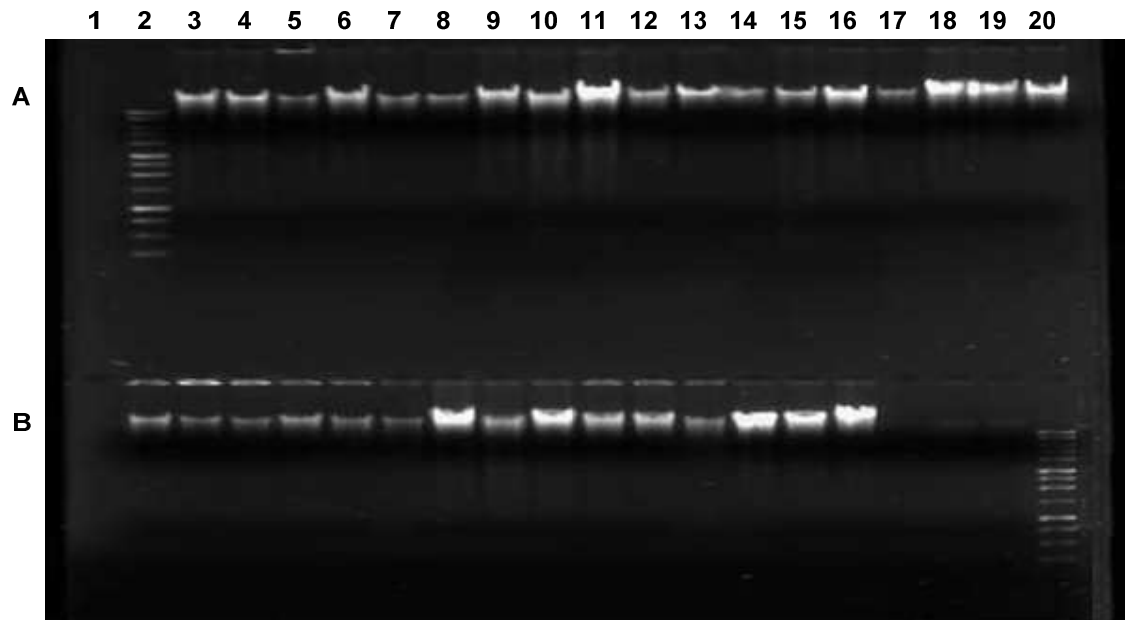


PLATE 5.1 Agarose gel showing total genomic DNA from soil subsamples from Baynesfield Estate.

Key: Row A, Lane 2, 1 Kb Molecular weight markers (MWM); lanes 3–5, sugarcane (SC); lanes 6–8, maize (M); lanes 9–11, kikuyu (KIK); lanes 12–14, native grassland (NAT); lanes 15–17, pine (PF); lanes 18–20, wattle (W). Row B: Lanes 2–16, duplicate samples of those in row A but excluding PF; lane 20, 1Kb MWM.

As previously noted, the concentration of total soil DNA at Mount Edgecombe was too low for the bands to be visible in the agarose gels. Fungal DNA was seen to be present only after all the samples had been PCR amplified, and the number of denaturing /annealing/elongation cycles increased to 40 (Plate 5.2).

Amplification of the fungal component of the soil microbial communities from both sites, by PCR of 18S rDNA with the universal fungal primer pair FR1GC/FF390, yielded products of the expected size (~390 bp) for all the replicate subsamples.

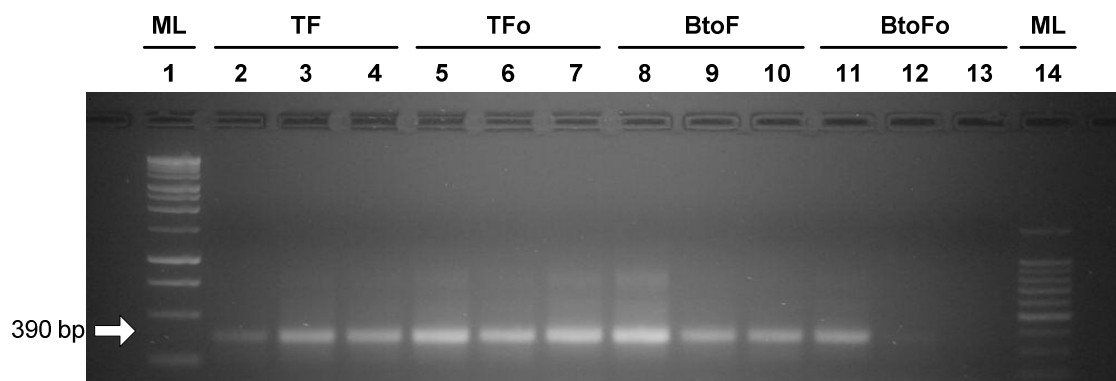


PLATE 5.2 Fungal PCR amplicons (~390 bp) from soils under different management practices at Mount Edgecombe.

Key: Lane 1, ML = 1 Kb MWM; TF = fertilized, green cane harvested; TFo = unfertilized, green cane harvested; BtoF = fertilized, burnt cane harvested; BtoFo = unfertilized, burnt cane harvested; lane 14, ML = 100 bp MWM.

5.3.3 Soil fungal community structure at Baynesfield Estate (site 1)

Fungal fingerprints from DGGE analysis of PCR amplicons (produced with FR1GC/FF390) of the various land uses at site 1, showed that the DNA banding profiles of the replicate subsamples of a single land use, were more similar to each other than they were to the subsample profiles of the other land uses (visual assessment) (Plate 5.3). This was confirmed by Quantity One analysis (Figure 5.2). In contrast, the banding patterns previously obtained when using primer pair FR1GC/NS1 were more similar across the gels regardless of land use (section 4.3.2). In the current study, Quantity One analysis showed the number of bands in each lane varied from 4–18 (Figure 5.2), which was greater than the 5–10 bands previously produced by FR1GC/NS1. Nonetheless, this was fewer than the 15–44 bands in the bacterial community DGGE gels (section 3.3.2). Since each band was inferred to represent a single OTU, soil fungal richness at Baynesfield, as revealed by FR1GC/FF390, was greater than that shown by FR1GC/NS1, but lower than the bacterial richness at the same site. Some bands were again common to all lanes although the band intensities varied, indicating a difference in mass (Plate 5.3; Figure 5.2).

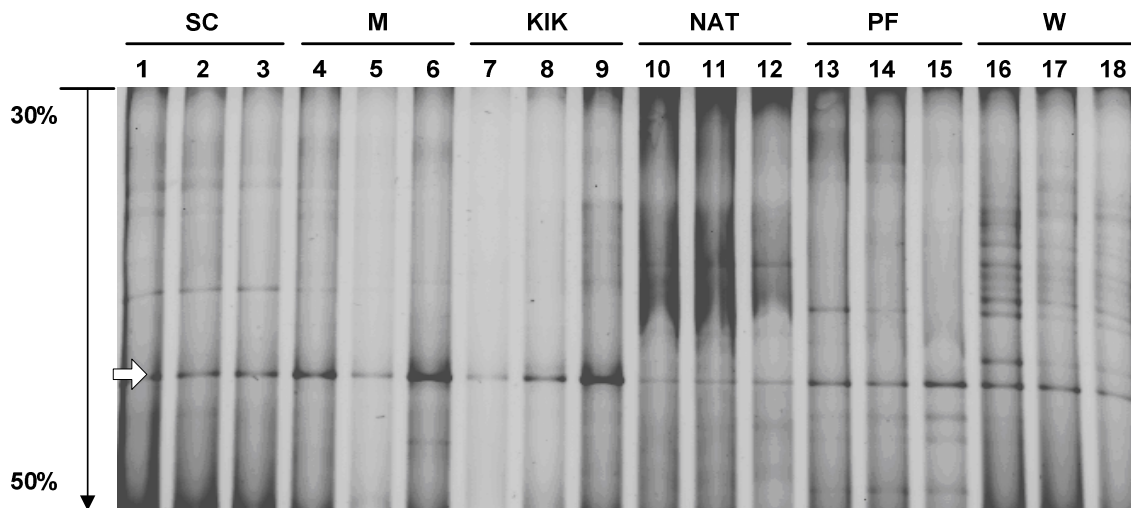


PLATE 5.3 DGGE gel (denaturing gradient 30–50%) of Baynesfield soil fungal amplicons from different land uses amplified with primer pair FR1GC/FF390.

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation. Arrow indicates bands excised for DNA elution.

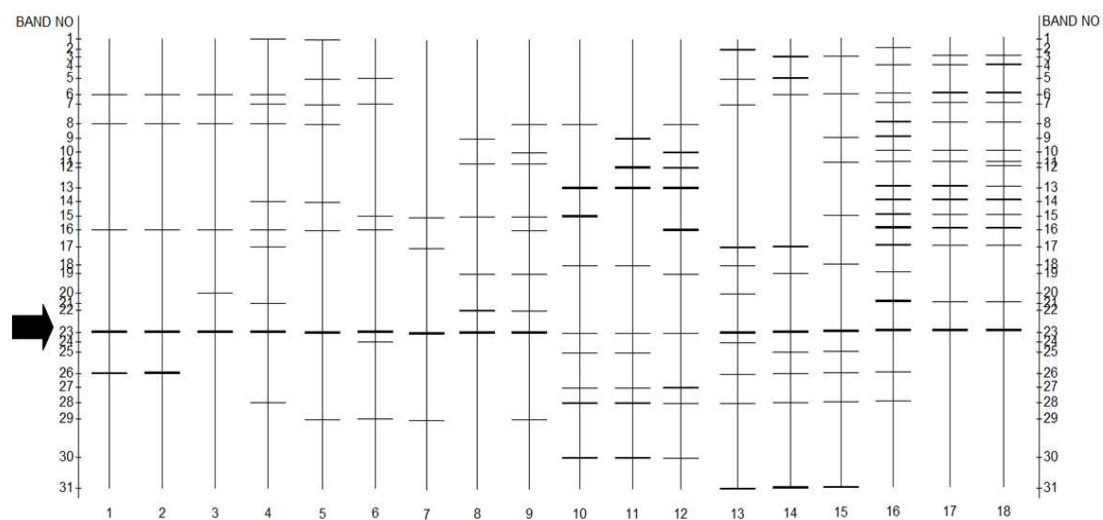


FIGURE 5.2 Quantity One diagram of DGGE gel (Plate 5.3) showing bands not visible in the photograph.

Key: Lanes 1–3: sugarcane; lanes 4–6: maize; lanes 7–9: kikuyu pasture; lanes 10–12: native grassland; lanes 13–15: pine plantation; lanes 16–18: wattle plantation. Arrow indicates band 23, excised from all lanes for DNA elution and sequencing.

A DGGE gel (denaturing gradient, 35–45%) of Baynesfield samples, containing the bands produced by eluted fungal DNA from single excised bands, together with non-excised control DNA (to verify band position), is shown in Plate 5.4.

Analysis of the gel (Plate 5.4) with Quantity One (Figure 5.3) showed that multiple bands, varying from 2–7, (excluding control samples) were contained within each, apparently ‘single’, excised, dominant band. The distance migrated in the gel lanes by the native grassland control DNA (NAT C), and the pine plantation control DNA (PF3 C), was slightly shorter than that by the excised DNA (Plate 5.4). This was possibly because these control bands contained several sequences. In contrast, the kikuyu pasture control DNA (KIK C), contained a single band only, and therefore ran a similar distance in the lane, to that of the excised DNA. While the narrower denaturing gradient (35–45%) served to separate the multiple bands within each single excised band, the control DNA amplicons, produced fewer bands per lane than they had in the DGGE gels with a 30–50% denaturing gradient (Plate 5.3, Figure 5.2). These results indicate that the narrower gradient would not have been suitable for the initial DGGE of mixed soil fungal communities in soil.

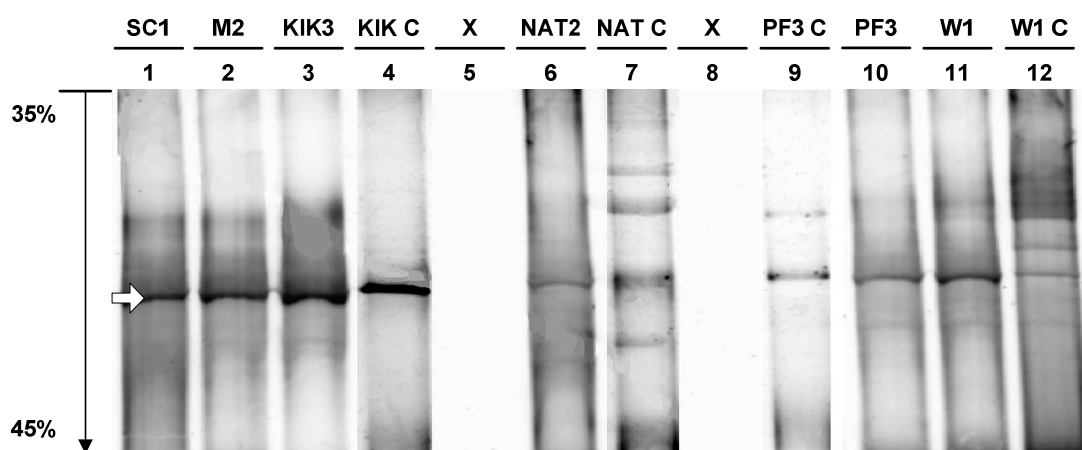


PLATE 5.4 DGGE gel (denaturing gradient 35–45%) to verify the position of some of the eluted fungal DNA samples, relative to non-excised controls from Baynesfield.

Key: SC1 = sugarcane, subsample 1; M2 = maize, subsample 2) KIK3 = kikuyu pasture, subsample 3; KIK C = control DNA from kikuyu subsample 3; lane 5, open; NAT2 = native grassland, subsample 2; NAT C = control DNA from native grassland subsample 2; lane 8, open; PF3 C = control DNA from pine plantation subsample 3; PF3 = pine plantation, subsample 3; W1 = wattle plantation, subsample 1; W1 C = control DNA from wattle plantation subsample 1. Arrow indicates bands excised for sequencing. Control DNA was not excised and sequenced.

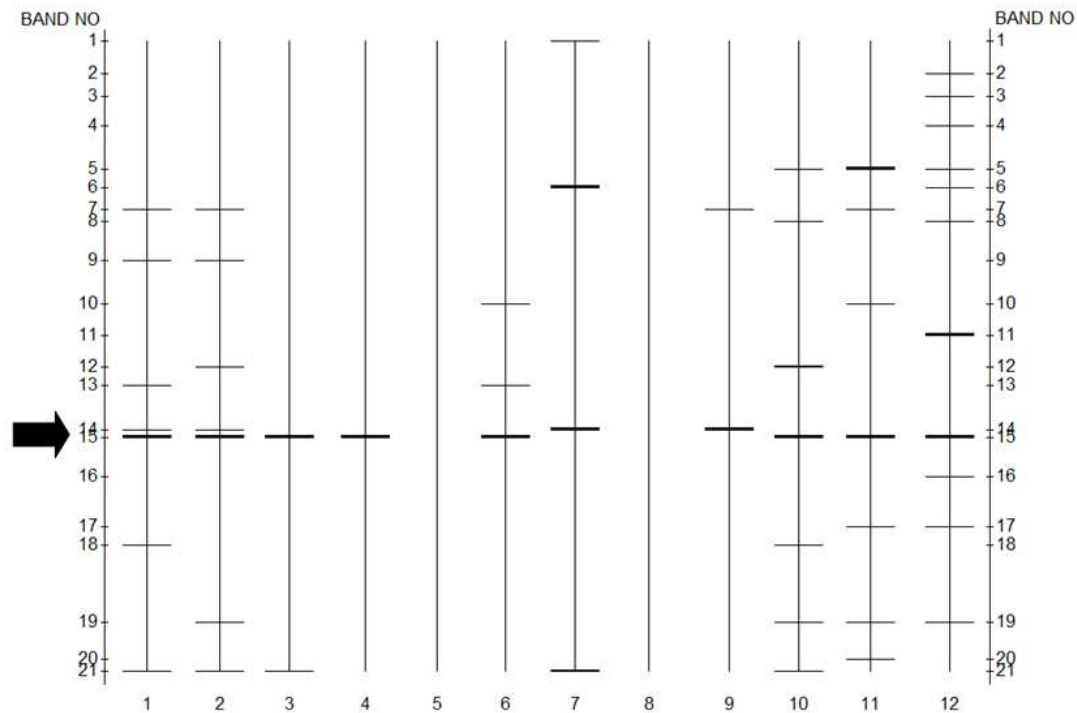


FIGURE 5.3 Quantity One diagram of DGGE gel (Plate 5.4) showing bands not visible in the photograph.

Key: Lane 1, SC1: sugarcane; lane 2, M2: maize; lane 3, KIK3: kikuyu pasture; Lane 4, KIK C: control DNA, kikuyu pasture; lane 5: open; lane 6, NAT2 : native grassland) lane 7, NAT C: control DNA, native grassland; lane 8: open; lane 9, PF3 C: control DNA, pine plantation; lane 10, PF3: pine plantation; lane 11, W1: wattle plantation; lane 12, W1 C: control DNA, wattle plantation. Arrow indicates excised bands (15).

The results obtained from sequencing the 18S rRNA gene fragments from the Baynesfield soil fungal communities under the different land uses (identified using the NCBI nucleotide database and the mega BLAST program), are presented in Table 5.2. When comparing sequences from environmental samples with fully identified reference sequences, it is common to rely on threshold values (e.g. 97%) for determining sequence similarity (Ovaskainen *et al.*, 2010). Of the 18 fungal sequences excised, 17 showed a similarity of 98–100% to Genbank sequences. Only band 2 from subsample W1 (W1/2) had a similarity of less than 90% and was, therefore, eliminated from the analysis (Green *et al.*, 2006).

TABLE 5.2 Identity of 18S rRNA gene sequences from excised DGGE bands of soil fungal communities from different land uses at Baynesfield

| Sequence designation | Closest match from Genbank | % sequence similarity (BLAST) | Genbank accession no. |
|----------------------|--|-------------------------------|-----------------------|
| SC1 | Uncultured fungus isolate 1 18S rRNA gene, partial sequence | 99% | AY769847.1 |
| SC2 | Uncultured fungus partial 18S rRNA gene, isolate 9 | 100% | FM202462.1 |
| SC3 | Uncultured fungus isolate 1 18S rRNA gene, partial sequence | 100% | AY769847.1 |
| M1 | Uncultured fungus clone Nikos_35 18S rRNA gene, partial sequence | 100% | HM104558.1 |
| M3 | Uncultured fungus clone Nikos_35 18S rRNA gene, partial sequence | 99% | HM104558.1 |
| KIK1 | Uncultured fungus isolate 6 18S rRNA gene, partial sequence | 99% | AY769852.1 |
| KIK2 | Uncultured fungus clone Nikos_35 18S rRNA gene, partial sequence | 100% | HM104558.1 |
| KIK3 | Uncultured fungus clone Nikos_35 18S rRNA gene, partial sequence | 99% | HM104558.1 |
| NAT1 | Uncultured soil fungus isolate DGGE gel band 12060835(SF01)FF390 | 100% | EU647857.1 |
| NAT2 | Uncultured fungus isolate 1 18S rRNA gene, partial sequence | 100% | AY769847.1 |
| NAT3 | Uncultured soil fungus isolate DGGE gel band 12060835(SF01)FF390 | 100% | EU647857.1 |
| PF1 | Uncultured soil fungus isolate DGGE gel band 12060835(SF01)FF390 | 100% | EU647857.1 |
| PF2 | Uncultured soil fungus isolate DGGE gel band 12060835(SF01)FF390 | 100% | EU647857.1 |
| PF3 | <i>Mortierella</i> sp.20006 18S rRNA gene, partial sequence | 100% | EU710842.1 |
| W1/1 | Uncultured fungus clone Nikos_35 18S rRNA gene, partial sequence | 98% | HM104558.1 |
| W1/2* | <i>Repetobasidium mirificum</i> 18S rRNA gene, partial sequence | 87%* | AY293155.1 |
| W2 | Uncultured fungus isolate 1 18S rRNA gene, partial sequence | 100% | AY769847.1 |
| W3 | Uncultured soil fungus isolate DGGE gel band 12060835(SF01)FF390 | 99% | EU647857.1 |

Sequence similarity values below 90% are not considered identical (Green *et al.*, 2006)

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

Soil fungal community composition under the different land uses at this site, is shown in an NMS two-dimensional plot rotated by PCA (Figure 5.4). The various land use soil replicates were clustered on the basis of the presence or absence (Sørensen coefficient) of bands (sequence types). The replicate PF soil fungal communities were separated from those of the other land uses, as were the W fungal communities. Soil fungi under the arable crops, SC and M, clustered together indicating the presence of similar fungal OTUs in soils under these land uses. Similarly, KIK replicates were closely clustered with those of NAT. MRPP confirmed that subsamples from W (av. distance 0.19, Sørensen) were the most closely correlated, indicating the structural similarity of their fungal communities. SC subsamples were also more closely clustered (0.27) than the NAT (0.33), PF (0.39), KIK (0.44) and M (0.66) subsamples, with those of KIK being more dissimilar than NAT and PF. However, the M subsamples were the most structurally dissimilar of all the land uses.

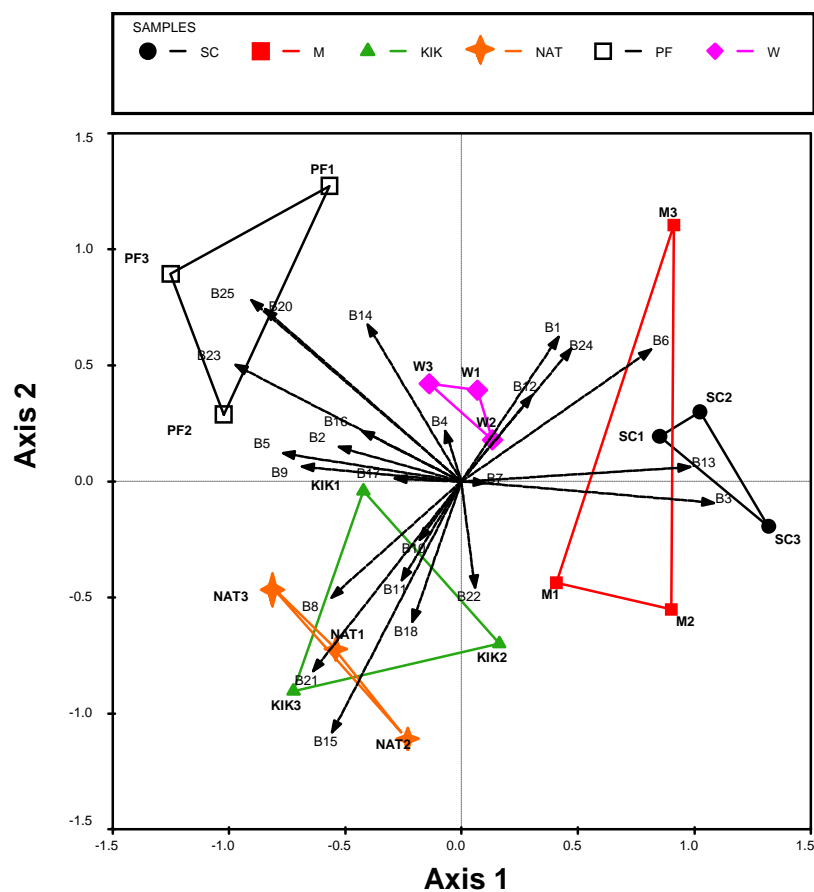


FIGURE 5.4 A NMS two-dimensional plot (rotated by PCA) of fungal communities (presence or absence of bands) at Baynesfield. NMS stress = 0.19955.

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation; B = band (OTU).

Analysis of species richness (S) (number of bands present = number of different groups of species) by one-way ANOVA of the soil fungal communities at this site, showed large differences between the various fungal communities (Table 5.3). Pairwise comparisons made using Tukey's test, indicated that soil fungal community richness under W differed from that of the soil fungi under M, SC and NAT, but not from that of the communities under PF or KIK (Table 5.3). Trends for richness followed the order: $M < SC < NAT < PF < KIK < W$.

TABLE 5.3 ANOVA of species richness (number of bands produced using primer pair FR1GC/FF390) and land use means in the fungal communities at Baynesfield

| Source of variation | d.f | s.s. | m.s. | f-ratio | p-value |
|---------------------|-----|--------|-------|---------|---------|
| Land use | 5 | 96.44 | 19.29 | 6.09 | 0.005 |
| Residual | 12 | 38.00 | 3.17 | | |
| Total | 17 | 134.44 | | | |

| | | | | | | |
|------------|-----------------|----------------|-------------------|------------------|------------------|----------------|
| Grand mean | 7.56 | | | | | |
| Land use | SC ^a | M ^a | KIK ^{ab} | NAT ^a | PF ^{ab} | W ^b |
| Mean | 5.33 | 5.00 | 8.33 | 7.00 | 7.67 | 12.00 |

Means with common superscript letters are not significantly different. ($LSD_{5\%} = 3.166$).

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

ANOVA of soil fungal community evenness (J) under the different land uses at Baynesfield, showed a marked overall difference in the evenness of the fungal species present (Table 5.4). Wattle soil fungal evenness differed from that of the communities under SC, M and KIK, but not from that of NAT and PF soil fungal communities. Trends for evenness were: $M < KIK \leq SC < NAT \leq PF < W$.

TABLE 5.4 ANOVA and land use means of soil fungal species evenness (J) at Baynesfield Estate

| Source of variation | d.f | s.s. | m.s. | f-ratio | p-value |
|---------------------|-----|------|------|---------|---------|
| Land use | 5 | 0.11 | 0.02 | 13.14 | < 001 |
| Residual | 12 | 0.02 | 0.00 | | |
| Total | 17 | 0.13 | | | |

| | | | | | | |
|------------|-------------------|----------------|-------------------|--------------------|------------------|----------------|
| Grand mean | 0.83 | | | | | |
| Land use | SC ^{abc} | M ^a | KIK ^{ab} | NAT ^{bcd} | PF ^{cd} | W ^d |
| Mean | 0.79 | 0.72 | 0.78 | 0.88 | 0.89 | 0.94 |

Means with common superscript letters are not significantly different. (LSD_{5%} = 0.072).

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

Analysis of soil fungal diversity by the Shannon Weaver diversity index (H') showed an overall difference in community structural diversity, with communities under W being separated from those of SC and M, but not from those of KIK, NAT or PF. Communities under M and SC could not be separated from each other nor from those under KIK, NAT and PF (Table 5.5). Trends for fungal diversity followed the order: M < SC < KIK < NAT < PF < W.

TABLE 5.5 ANOVA and land use means of soil fungal species diversity (H') at Baynesfield Estate

| Source of variation | d.f | s.s. | m.s. | f-ratio | p-value |
|---------------------|-----|------|------|---------|---------|
| Land use | 5 | 2.64 | 0.53 | 7.14 | 0.003 |
| Residual | 12 | 0.89 | 0.07 | | |
| Total | 17 | 3.53 | | | |

| | | | | | | |
|------------|-----------------|----------------|-------------------|-------------------|------------------|----------------|
| Grand mean | 1.65 | | | | | |
| Land use | SC ^a | M ^a | KIK ^{ab} | NAT ^{ab} | PF ^{ab} | W ^b |
| Mean | 1.29 | 1.15 | 1.64 | 1.70 | 1.77 | 2.34 |

Means with common superscript letters are not significantly different. ($LSD_{5\%} = 0.484$).

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

The results of a CCA of the relationship between the effects of selected (non-collinear) soil physicochemical variables, and the soil fungal community (genetic) structure under the different land uses at Baynesfield Estate are presented in Figure 5.5. CCA 1 accounted for 43.8% of the variance due to environmental effects, and 17.5% of the total variance. CCA 2 accounted for 30.4% of the variance due to environmental effects, and 12.1% of the total variance. CCA 1 was correlated with pH, whereas organic C, P and ECEC were correlated with CCA 2. Subsamples of M and SC were clustered together on the basis of a strong correlation with high soil P values as a result of fertilizer applications. PF replicate subsamples were correlated with a low soil pH. Soils with a higher pH and level of ECEC were associated with W, and organic C and ECEC with NAT and KIK soils. A plot showing the presence of species centroids (bands) in the CCA (Figure 5.5) is shown in Figure 5.6.

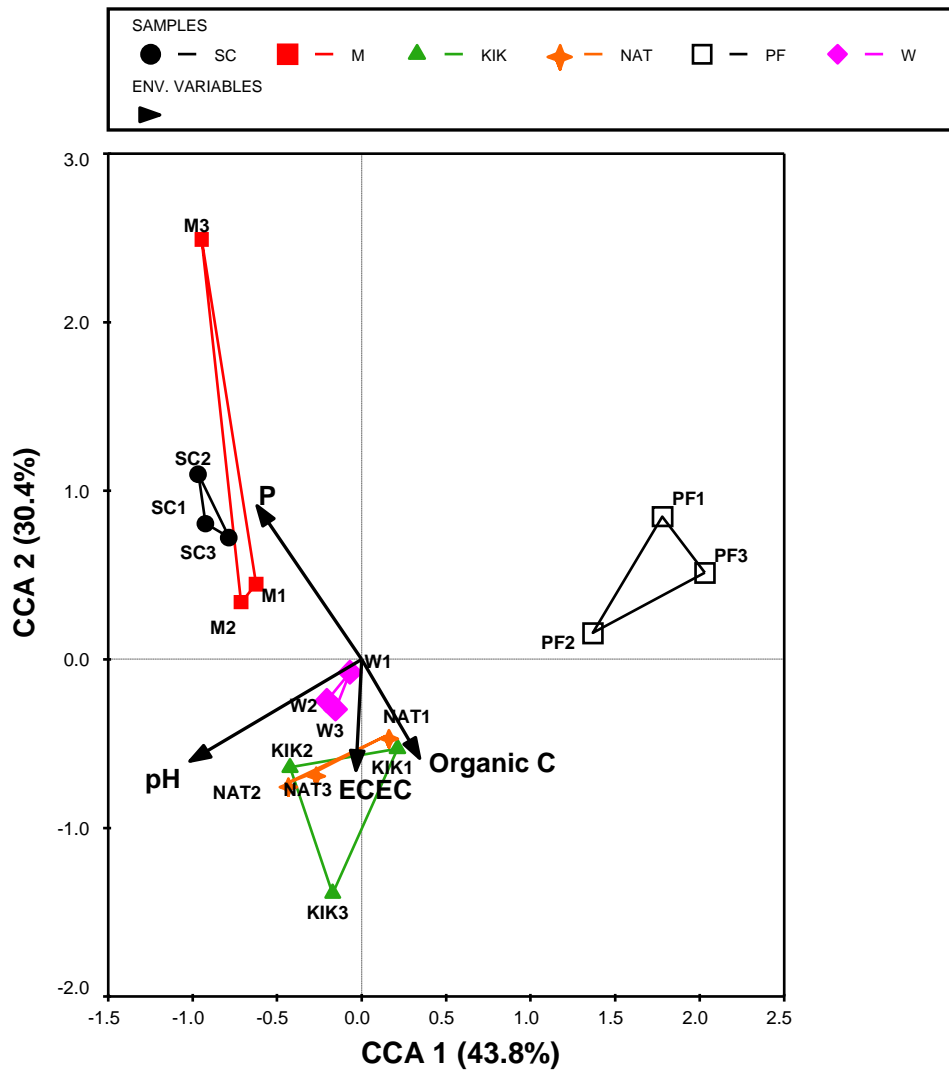


FIGURE 5.5 Plot of samples (classified by land use) and soil variables along the first two axes of a CCA of the effects of selected soil variables on fungal community composition (band presence) at Baynesfield Estate. CCA1 accounted for 43.8% and CCA2 for 30.4% respectively, of the variance due to environmental effects.

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

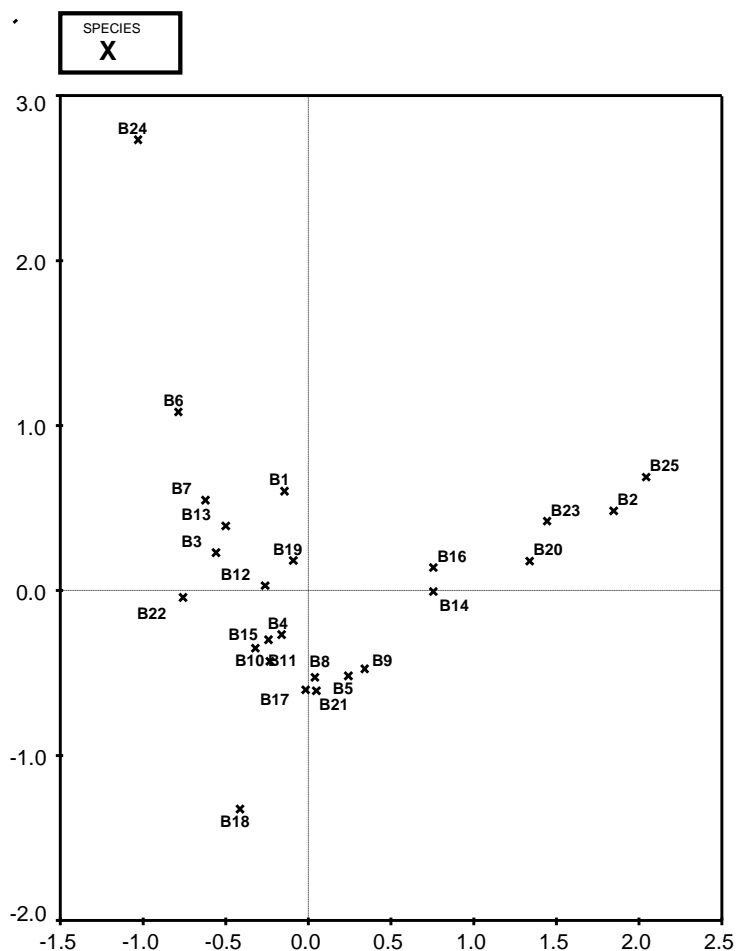


FIGURE 5.6 Plot of bands (centroids) in the CCA (Figure 5.5) showing the relationship of the different fungal OTUs to the land use soils at Baynesfield.

Key: B = band.

The Monte Carlo Permutation test for significant relationships within the CCA data showed that soil variables had affected fungal community structural diversity at this site. (Appendix C, Table C1).

5.3.4 Analyses of soils at the Mount Edgecombe site (site 2)

The means (\pm standard deviation) of the soil physicochemical properties selected for analysis at this site are summarised in Table 5.6.

TABLE 5.6 Means (\pm sd) for selected chemical properties of soils collected at 0–5 cm depth from different land management practices at Mount Edgecombe

| Trash Management | pH (KCL) | Organic Carbon | Extractable P | Exchangeable cations | | | Exch. acidity | Total cations (ECEC) |
|------------------|------------|----------------|---------------------|--|-------------|------------|---------------|----------------------|
| | | | | K | Ca | Mg | | |
| | | % | mg kg ⁻¹ | ----- cmol _c kg ⁻¹ ----- | | | | |
| TF | 3.5 (0.07) | 6.6 (0.27) | 65.3 (11.79) | 0.7 (0.08) | 6.0 (0.39) | 2.9 (0.37) | 3.6 (0.82) | 13.1 (0.42) |
| TFo | 4.5 (0.12) | 6.0 (0.22) | 4.3 (0.50) | 0.2 (0.03) | 10.7 (2.21) | 5.9 (0.83) | 0.1 (0.03) | 16.8 (1.43) |
| BtoF | 3.7 (0.04) | 5.1 (0.18) | 46.2 (10.38) | 0.9 (0.09) | 5.6 (1.30) | 2.6 (0.52) | 2.4 (0.47) | 11.5 (1.38) |
| BtoFo | 4.6 (0.14) | 4.9 (0.27) | 2.6 (0.43) | 0.2 (0.05) | 8.5 (0.14) | 6.4 (0.57) | 0.1 (0.02) | 15.1 (0.50) |

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

At this site, soil organic C increased in the order: BtoFo < BtoF < TFo < TF, indicating that retention of plant residues and applications of fertilizer resulted in the highest amounts of accumulated organic C in the soil. Soil pH increased in the order: TF < BtoF < TFo < BtoFo, showing that fertilizer application (F) had lowered the soil pH. Concentrations of extractable P and exchangeable K were higher under fertilized (F) than unfertilized (Fo) treatments, but exchangeable Ca and Mg were higher in unfertilized plots (Table 5.6). Results obtained in the initial study (2004–2008) showed that soil organic C content and soil pH followed the same order as in the present study, but that the values obtained for the selected soil variables differed overall (Table 3.9, section 3.3.3).

PCA analysis of this soil data showed the compositional relationships among replicate subsamples from under the different land management practices (Figure 5.7). PCA was used to project the maximum variance in soil data in multiple dimensions (e.g. axis 1 and axis 2), in an unconstrained ordination. Potassium, P, acidity, pH, Mg and Ca were all correlated with the PC1 axis, whereas organic C and ECEC were correlated with the PC2 axis. The PC1 axis accounted for 75%, the PC2 axis for 16%, and, cumulatively, for 91% of the total variance in the soil data. Treatments retaining harvest residues (T) were correlated with high organic C in contrast to burnt cane harvesting (Bto), whereas those receiving fertilizer (F) were associated with a low pH, and higher acidity, P and K levels. Unfertilized managements were correlated with higher pH, Mg, Ca and ECEC.

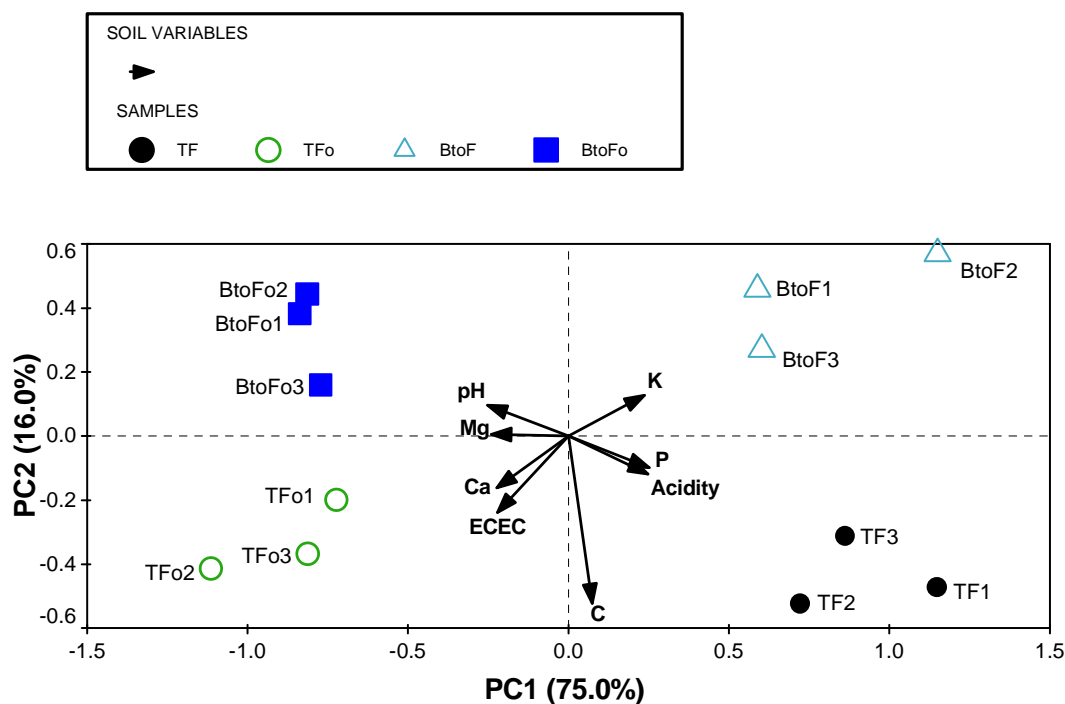


FIGURE 5.7 Plot of samples and soil variables along the first two axes of a standardised and centred PCA of different land treatments at Mount Edgecombe. The PC1 (horizontal) and PC2 (vertical) components accounted for 75.0% and 16.0%, respectively, and cumulatively for 91.0% of the total variance in the soil data.

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

At Mount Edgecombe, MRPP tests of the soil PCA data showed that differences among the soil subsamples from the four land managements were highly significant ($T = -4.976$; $p < 0.001$).

5.3.5 Soil fungal community structure at Mount Edgecombe (site 2)

Profiling by DGGE of fungal 18S rDNA amplicons (generated with FR1GC/FF390) from soils under the four different trash managements at this site, showed that the banding patterns of all the replicate subsamples appeared similar across the gels, regardless of management practice (visual assessment) (Plate 5.5). This was confirmed by Quantity One analysis (Figure 5.8) which revealed that total band numbers per lane, varied from 1–17, in comparison with the 4–9 bands produced previously with primer pair FR1GC/NS1 (section 4.3.2), and the 20–30 bands in the

lanes of the bacterial DGGE gels. This indicates that while primer pair FR1GC/FF390 revealed greater soil fungal community diversity than FR1GC/NS1, the communities were not as structurally diverse as those of the soil bacteria from the same site. Bands common to all replicates of the four treatments were observed (Plate 5.5). Band intensity varied, with some bands dominant and of higher intensity than others in the same lane (Figure 5.8).

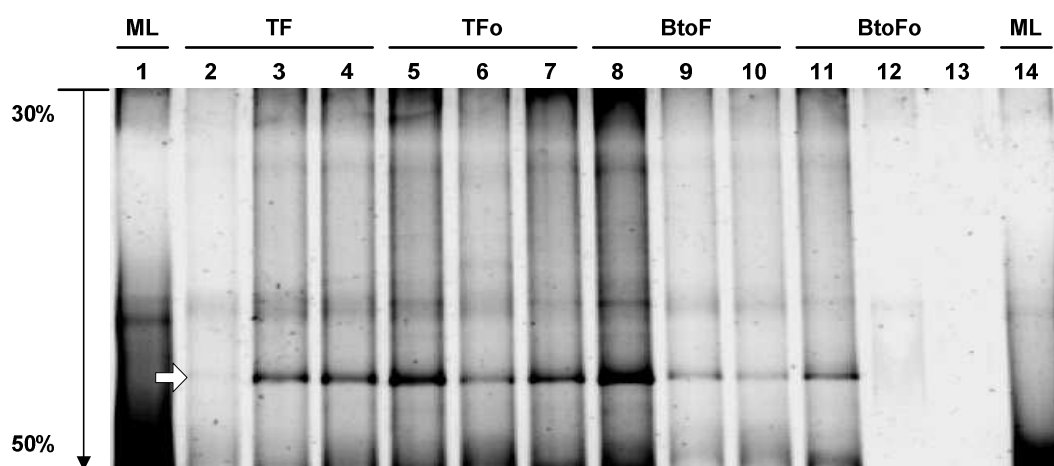


PLATE 5.5 DGGE gel (30–50% denaturing gradient) of Mount Edgecombe fungal amplicons from soils under different management practices.

Key: T = green cane harvested with retention of a trash mulch; Bto = burnt cane harvested, either fertilized (F) or unfertilized (Fo); ML (marker lanes). Arrow indicates the bands excised for fungal DNA elution.

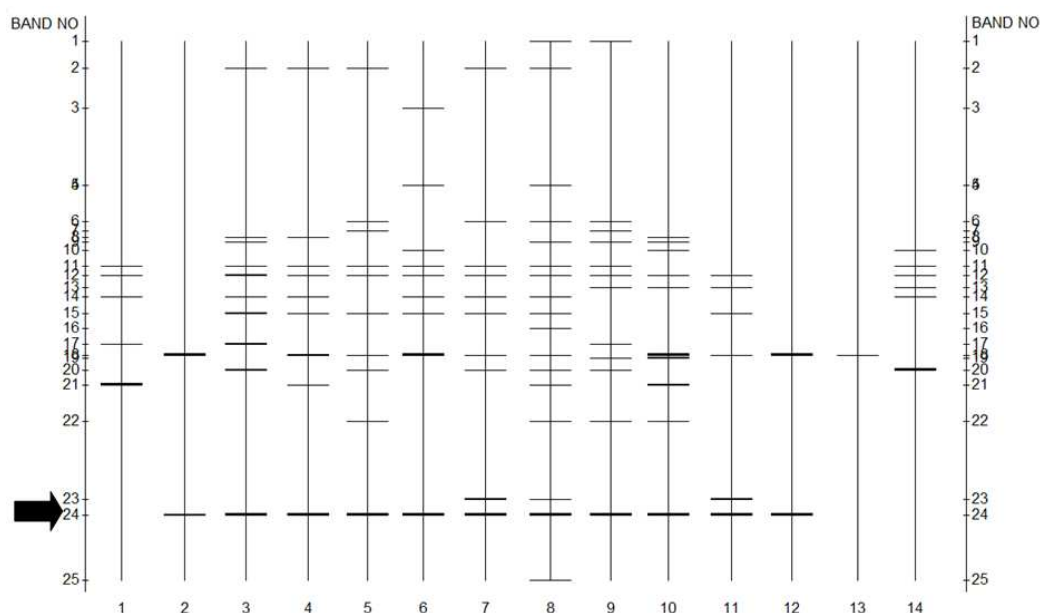


FIGURE 5.8 Quantity One diagram of gel (Plate 5.5) showing bands not visible in the photograph.

Key: Lanes 1 and 14: markers; lanes 2–4, TF: greencane harvested, fertilized; lanes 5–7, TFo: greencane harvested, unfertilized; lanes 8–10, BtoF: burnt cane harvested, fertilized; lanes 11–13, BtoFo: burnt cane harvested, unfertilized. Arrow = excised bands.

A DGGE gel (denaturing gradient 35–45%) of Mount Edgecombe samples, containing the bands produced by eluted fungal DNA from single excised bands, together with non-excised control DNA (to verify band position), is presented in Plate 5.6, and a Quantity One diagram of this gel, in Figure 5.9.

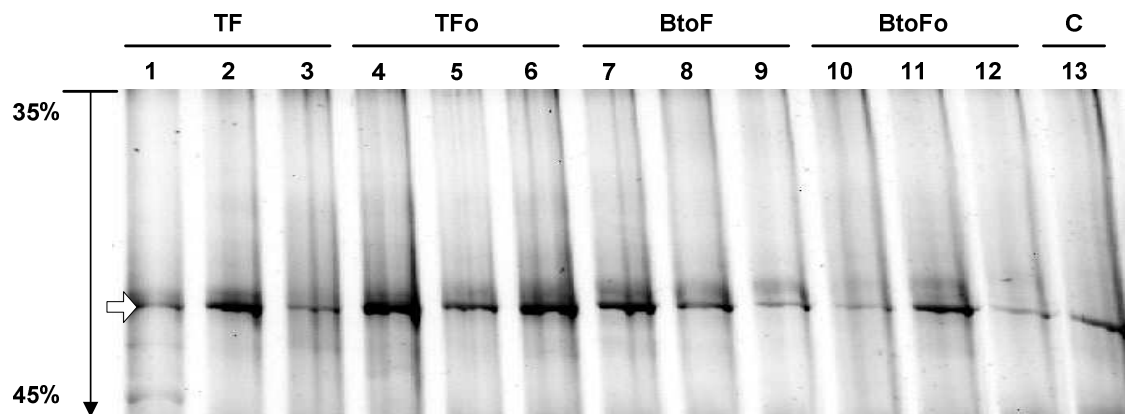


PLATE 5.6 DGGE gel (35–45% denaturing gradient) to verify position of eluted DNA from excised bands relative to control DNA at Mount Edgecombe.

Key: TF (trashed and fertilized); TFo (trashed and unfertilized); BtoF (burnt cane harvested and fertilized); BtoFo (burnt cane harvested but unfertilized); lane 13 (control DNA). Arrow indicates bands excised for sequencing.

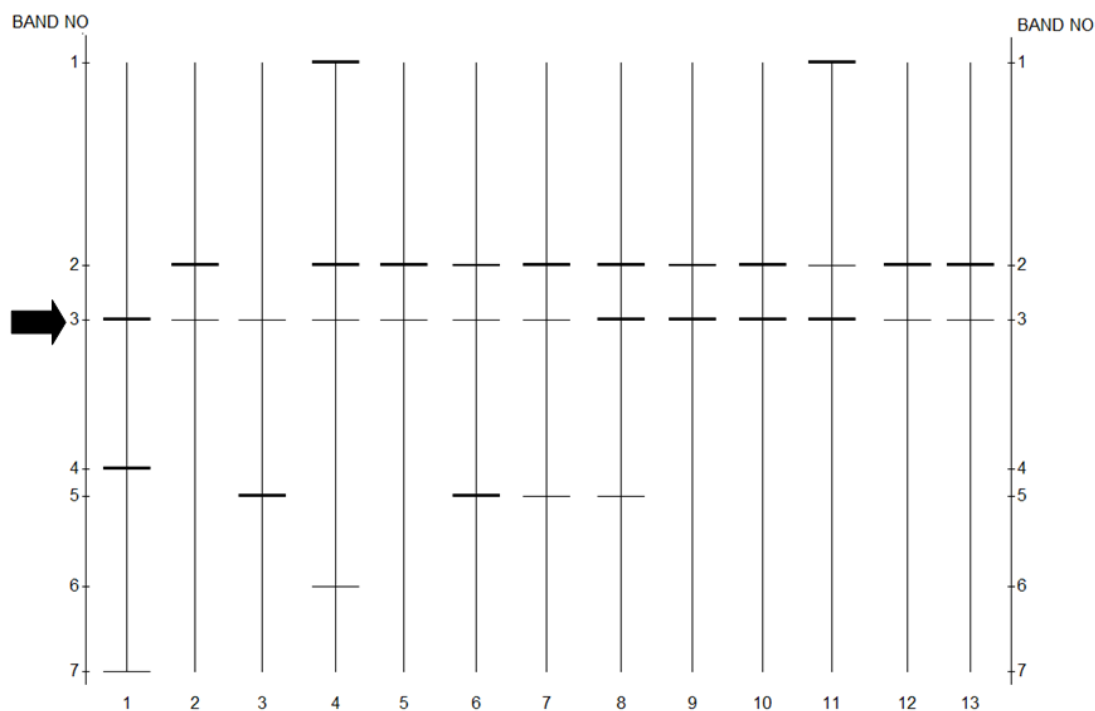


FIGURE 5.9 Quantity One diagram of DGGE gel (Plate 5.6) showing bands not visible in the photograph.

Key: Lanes 1–3, TF: greencane harvested, fertilized; lanes 4–6, TFo: greencane harvested, unfertilized; lanes 7–9, BtoF: burnt cane harvested, fertilized; lanes 10–12, BtoFo: burnt cane harvested, unfertilized; lane 13, control DNA. Arrow indicates bands excised for sequencing.

Analysis of the gel (Plate 5.6) by Quantity One (Figure 5.9) showed that 2–4 bands were contained within each apparently ‘single’ excised dominant band. As with the Baynesfield gels, the narrower 35–45% denaturing gradient separated the multiple bands within each excised band. The results of sequencing the 18S rRNA gene fragments from the excised bands of the soil fungal communities under the different treatments at Mount Edgecombe (identified using the NCBI nucleotide database and the mega BLAST program) are presented in Table 5.7. Of the 17 sequences excised, only five showed a 91–100% similarity to Genbank sequences. Of the remainder, three bands had a similarity of less than 90% and six showed no significant similarity, as their sequencing traces were either chimeric or mixed template traces. In addition, two sequences were identified as plant DNA, and one sequencing trace failed. Consequently, these poor quality/chimeric sequences were eliminated from the analysis (Green *et al.*, 2006).

TABLE 5.7 Identity of 18S rRNA gene sequences from excised DGGE bands of soil fungal communities from different land management practices at Mount Edgecombe

| Sequence designation | Closest match from Genbank | % sequence similarity (BLAST) | Genbank accession no. |
|----------------------|--|-------------------------------|-----------------------|
| TF2 | Uncultured soil fungus isolate DGGE gel band 12060835(SF01)FF390 | 93% | EU647857.1 |
| TF3* | <i>Cheilymenia coprinaria</i> voucherHMAS69605 18S rRNA gene, partial sequence | 83%* | DQ787818.1 |
| TFo1 | Uncultured fungus clone Nikos_253 18S rRNA gene, partial sequence | 99% | HM104530.1 |
| TFo2 | Uncultured fungus partial 18S rRNA gene, isolate 9 | 91% | FM202462.1 |
| TFo3 | Uncultured soil fungus isolate DGGE gel band 12060835(SF01)FF390 | 98% | EU647857.1 |
| BtoF1 | <i>Mortierella</i> sp. 20006 18S rRNA gene | 100% | EU710842.1 |
| BtoF2* | Uncultured fungus clone DC_H09F 18S rRNA gene | 88%* | EU726151.1 |
| BtoFo1* | <i>Knightiella splanchnirima</i> small subunit rRNA gene | 86%* | AF491856.1 |

* Sequence similarity values below 90% are not considered identical (Green *et al.*, 2006)

Soil fungal community structure under the different sugarcane management practices at Mount Edgecombe is shown in an NMS plot (Figure 5.10), which clustered the replicate soil DNA subsamples from the four trash managements on the basis of the presence or absence of bands (OTUs). Soil subsamples from under TFo were the most

closely clustered of all the treatments, indicating the structural similarity of their intrinsic fungal communities. Although the three subsamples from under BtoF were separated by a similar distance to those of BtoFo (MRPP, av. distance ~ 0.5), the two treatments were associated with different fungal OTUs, and were clearly separated in the NMS biplot. This indicates that the intraspecies similarity of the three subsample communities within the same management (Bto) was greater than the interspecies similarity between the fungal communities from the fertilized (BtoF) and unfertilized (BtoFo) soils.

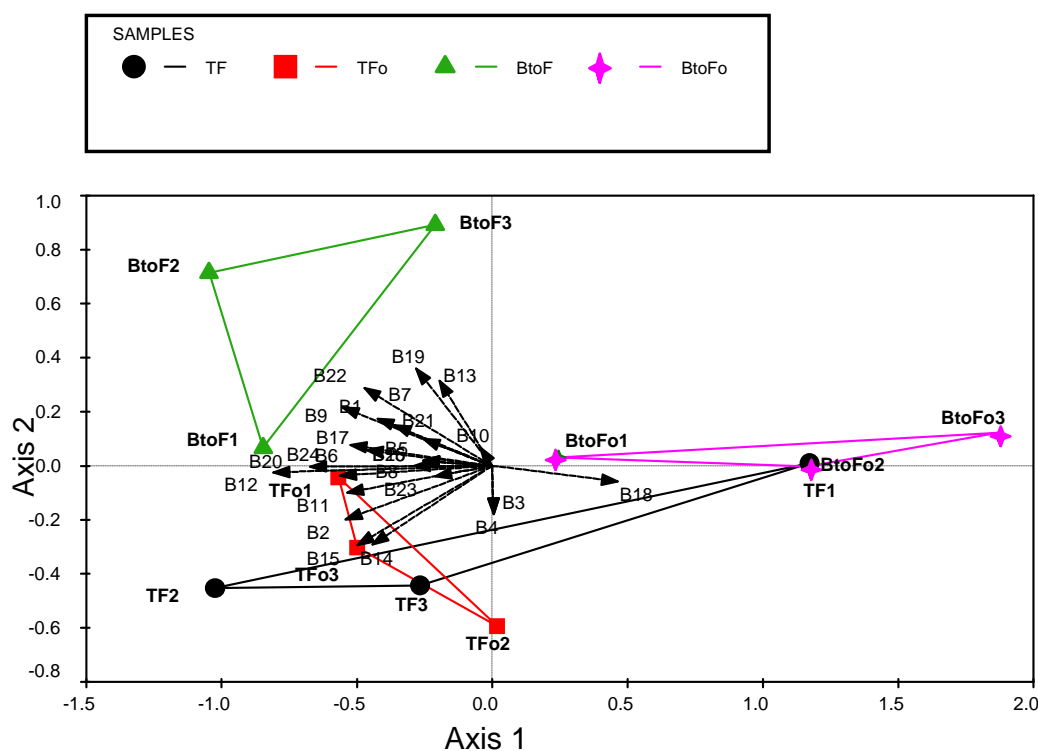


FIGURE 5.10 A NMS two-dimensional plot (rotated by PCA) of fungal communities (presence or absence of bands) at Mount Edgecombe. NMS stress = 0.07917.

Key: TF = green cane harvested with retention of crop residues, fertilized with N, P and K; TFo = green cane harvested with retention of crop residues but unfertilized; BtoF = pre-harvest burnt sugarcane with tops raked off, fertilized with N, P and K; BtoFo = pre-harvest burnt sugarcane with tops raked off but unfertilized; B = band.

MRPP analysis of this data showed that the different land management practices had a significant effect on soil fungal community composition at this site ($T = -1.853$; $p = 0.04$).

One-way ANOVA of soil fungal species richness (S) at Mount Edgecombe showed that the four different land management practices had an overall significant effect on the community species richness ($p = 0.02$) (Table 5.8). Comparisons of fungal species richness from soil treatments TF and TFo showed that they were not significantly different, nor did the richness of the soil fungi under these managements differ from that of the soil communities under BtoF or BtoFo. However, there was a significant difference in fungal species richness between the BtoF and BtoFo soils.

TABLE 5.8 ANOVA and land management means of soil fungal species richness (S) at Mount Edgecombe

| Source of variation | d.f | s.s. | m.s. | f-ratio | p-value |
|---------------------|-----|--------|-------|---------|---------|
| Land management | 3 | 163.58 | 54.53 | 5.95 | 0.02 |
| Residual | 8 | 73.33 | 9.17 | | |
| Total | 11 | 236.92 | | | |

| | | | | | |
|-----------------|------|------------------|-------------------|-------------------|--------------------|
| Grand mean | 8.08 | | | | |
| Land management | | TF ^{ab} | TFo ^{ab} | BtoF ^b | BtoFo ^a |
| Mean | | 6.67 | 9.62 | 13.00 | 3.00 |

Means with common superscript letters are not significantly different ($LSD_{5\%} = 5.7$).

Key: TF = green cane harvested, fertilized; TFo = green cane harvested, unfertilized; BtoF = burnt cane harvested, fertilized; BtoFo = burnt cane harvested, unfertilized.

No significant treatment effects were shown by one-way ANOVA on fungal community evenness (J) ($F_{3,8} = 1.74$, $p = 0.25$, grand mean = 0.85), nor did the Shannon Weaver diversity index (H') ($F_{3,8} = 3.82$, $p = 0.06$, grand mean = 1.52) show any significant treatment effects on soil fungal community structural diversity.

The results of a CCA showing the relationship between selected (non-collinear) soil physicochemical variables and the soil fungal community (genetic) structure under the different sugarcane trash management practices at Mount Edgecombe are presented in Figure 5.11. CCA 1 accounted for 49.1% of variance due to environmental effects and 18.5% of total variance, and CCA 2 for 29.6% of variance due to environmental

effects and 11.2% of total variance. ECEC and pH were strongly correlated with CCA 1 and organic C with CCA 2. Trashed treatments, particularly TF but also TFo, were associated with a high soil organic C content, in contrast to the Bto treatments where the soil organic C content was lower. Both TF and BtoF treatments were correlated with a lower soil pH than the equivalent unfertilized treatments. Low ECEC was associated with the BtoF subsamples and high ECEC with the TFo subsamples. Subsamples from the BtoF and BtoFo treatments were more closely clustered than those from the TF and TFo treatments.

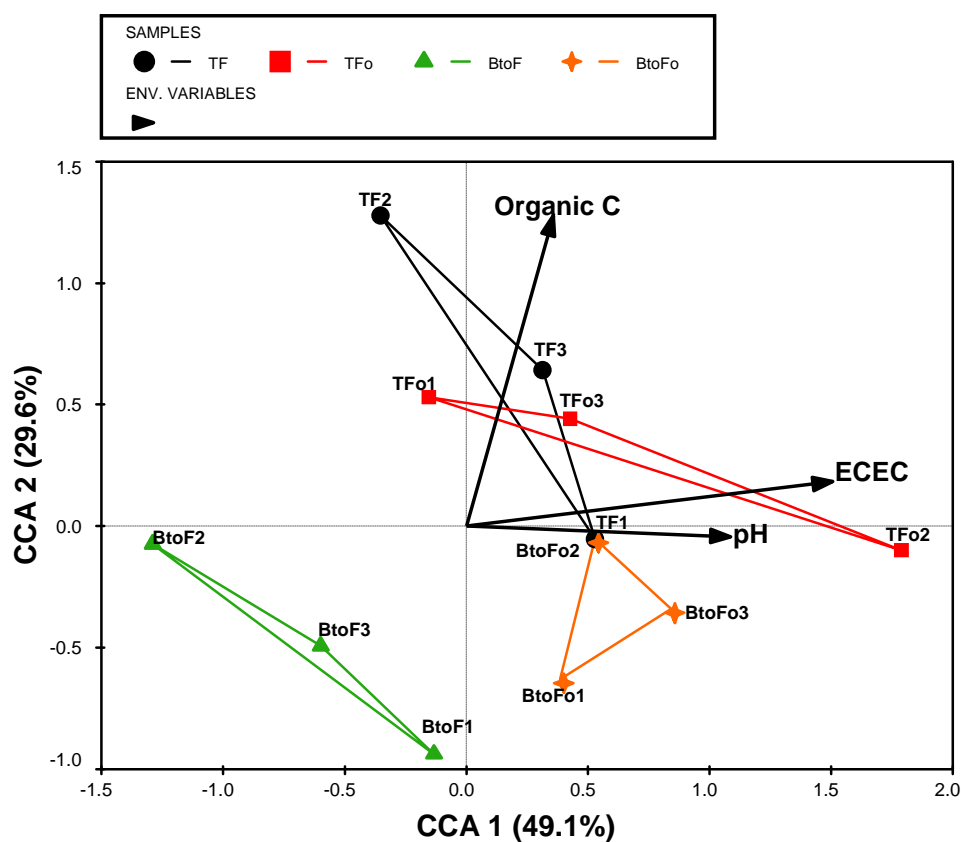


FIGURE 5.11 Plot of samples (classified by land management) and soil variables along the first two axes of a CCA of the effects of selected soil variables on fungal community composition (band presence) at Mount Edgecombe.

CCA 1 accounted for 49.1% of variance due to environmental effects and 18.5% of total variance. CCA 2 accounted for 29.6% of variance due to environmental effects and 11.2% of total variance.

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

A plot showing the presence of species centroids (bands = fungal OTUs) associated with the different management practice soils in the CCA (Figure 5.11), is shown in Figure 5.12.

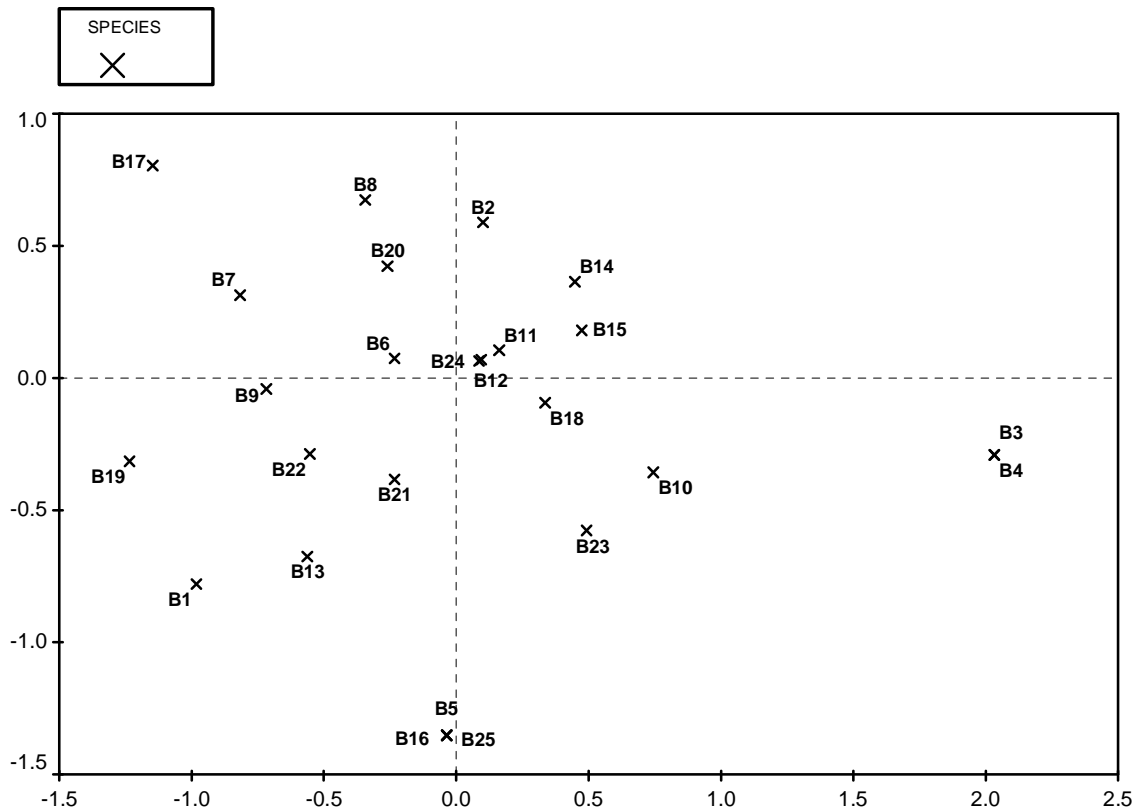


FIGURE 5.12 Plot of bands (centroids) in the CCA (Figure 5.11) showing the relationship of the different fungal OTUs to the land management soils at Mount Edgecombe.

Key: B = band.

Results of the Monte Carlo permutation test for significant relationships in the CCA data showed that along the first canonical axis $p = 0.01$ and along all canonical axes $p = 0.008$. This indicated that the selected soil variables had a significant effect on soil fungal community structure at this site (Appendix C, Table C2).

5.4 DISCUSSION

The accurate determination of diversity related to environmental processes is one of the central problems in microbial ecology (Mitchell and Zuccaro, 2006). However, the development and refinement of molecular tools for microbial analyses have prompted

a resurgence in interest, particularly for studies of fungal communities in the environment (Porter *et al.*, 2008). An efficient tool for identifying the species present in a given environmental sample is community fingerprinting, followed by modern sequencing methods. With this approach, by simply generating a list of the species present in the samples, a basis is provided for: (i) analyses of species-specific habitat requirements; (ii) models of single-species and community dynamics; and (iii) the assessment of the functional roles of species in an ecosystem (Kennedy and Clipson, 2003; Ovaskainen *et al.*, 2010).

Aguilera *et al.* (2006) reported that while sequences obtained by DGGE are usually short (< 500 bp), they are nonetheless very reliable for taxonomic identification to the genus level. However, when using this approach, preliminary studies have produced many ‘unclassified’ sequences, which could not be compared directly with known fungi (Hirsch *et al.*, 2010). This may be due to the study regions being too small (0.3–0.8 Kb) and divergent, or that non-overlapping rDNA regions such as SSU 18S rDNA, ITS regions or LSU 28S rDNA, are targeted (Porter *et al.*, 2008). Another problem with fungal sequence identification is that the genetic databases are under-represented for many fungal groups, which makes identification of environmental sequences difficult. This has resulted in a sequence misidentification level of 20% being recorded (Mitchell and Zuccaro, 2006).

In the present study, PCR-DGGE was used to determine the effects of various land uses and management practices on the structural diversity of soil fungal communities at two long-term experimental sites. As in section 4.2.5, universal fungal primers were used, as the objective remained to show soil fungal community response to various land uses and managements, without deliberate selection by specific PCR primers. However, by using a different universal primer pair to that used previously (section 4.2.5), by changing the DGGE conditions and by sequencing some of the dominant bands, a more comprehensive, broad-based analysis of the soil fungal communities at both sites was possible.

Migration in DGGE gels is dependent on sequence composition (Muyzer *et al.*, 1993) and although most DGGE bands contain many copies of a single amplicon (sequence type) others (particularly in the eukaryotes) may consist of multiple different

amplicons that co-migrate (Hirsch *et al.*, 2010). As the number of fungal OTUs obtained with the original universal fungal primer pair, namely, FR1GC/NS1 (section 4.3.2) was low, amplification of soil fungal DNA from both experimental sites using a different primer pair, FR1GC/FF390, was carried out. It was anticipated that the smaller fragments generated by the latter primer pair (~390 bp) would be more easily detected by DGGE, as the resolution of bands > 500 bp using this method is limited (Kennedy and Gewin, 1997). The results obtained were encouraging, as the number of bands produced at both sites was greater than when using FR1GC/NS1. However, this could have been a temporal effect, as the DNA used in the current study was isolated 4 years after that of the initial study. As indicated previously, the number of bands in these gels was still considerably lower than that of the soil bacteria from the same sites, suggesting the possibility of co-migration of different amplicons in single bands, thus leading to an underestimation of fungal community richness and diversity in the earlier study. This was found to be the case with both the Baynesfield and the Mount Edgecombe soil fungal samples. Another problem in DGGE gels was noted by Aguilera *et al.* (2006), where more than one band in the same sample showed different mobility but the same identity. This was attributed to the possible formation of heteroduplex molecules during PCR amplification, which would result in an overestimation of the number of community constituents.

In the present study, this problem was overcome by running consecutive DGGE gels (two for each site), as recommended by Aguilera *et al.* (2006). The first, used soil fungal DNA amplicons from the various sites, while the second used excised, re-amplified and re-eluted DNA from single dominant bands in gel one. Single bands were again excised from gel two, re-eluted, PCR-amplified and after purification, sequenced. This method enabled all the Baynesfield soil fungal DNA samples to be identified, although most could not be assigned to known phyla and so were designated 'unclassified' (Table 5.2). This was probably due to the small size (~300–350 bp) of the study region as well as the non-overlapping, SSU 18S rDNA target region (Porter *et al.*, 2008). The targeting of this region by the universal primer pair FR1GC/FF390 provided a low level of discrimination among the samples. Bands from longer PCR products give better identification when sequencing, but separation efficiency in DGGE is poor (Dahllöf, 2002). This indicates that in the present study, while the primer pair used is well-suited to DGGE, it is not as useful for specific

sequencing studies. This was regarded as unimportant, as the aim of this study was broad-based, and not intended to identify specific soil fungi but to determine the effects of different land uses and managements on the soil fungal communities. Nonetheless, by comparing microbial DNA, evolutionary relationships between microorganisms can be studied, which allows ecological and biodiversity questions to be answered (Mitchell and Zuccaro, 2006).

Sequencing of soil fungal DNA from the land managements at Mount Edgecombe was more problematic than that of the corresponding Baynesfield samples. This may largely be attributed to the fact that the yield of total genomic DNA from Mount Edgecombe was too low (< 2 ng) to be visualized in agarose gels until after PCR amplification. Even then, subsamples from under burnt cane harvesting, produced very light bands (or none at all) in agarose gels, indicating a low DNA concentration. Only after a two-step nested PCR (of 40 cycles each) was sufficient fungal DNA from all managements obtained, for subsequent DGGE. During PCR, if the amount of fungal DNA template is low, in spite of the highly enhanced selectivity for fungi of the reverse primer FR1, it may target non-fungal organisms (Vainio and Hantula, 2000). As a result of this, two sequences (bands 1 from both BtoFo2 and BtoFo3) were discarded having been identified as plant DNA. Sequencing of Mount Edgecombe soil fungal DNA samples also revealed the presence of chimeras and mixed DNA templates. This could possibly be as a result of the two-step, nested PCR approach used to amplify the DNA for DGGE, which may cause bias by increasing the chance of preferential amplification of specific sequences (Vainio and Hantula, 2000). Oros-Sichler *et al.* (2006) noted that care should be taken with the nested PCR approach as it might enhance 'noise signals', thus leading to a distorted image of the community under investigation.

5.4.1 Effects of different land uses on soil fungal community structure at Baynesfield Estate

Investigations involving soil microbes are hindered by the heterogeneity of the soil composition, the vast number and diversity of the microorganisms present in each gram of soil, and the lack of knowledge regarding the majority of the microbiota

(Hirsch *et al.*, 2010). At this site, the soil fungal communities from the replicate subsamples of a single land use were more similar to each other than they were to those of the other land uses, indicating greater inter- than intra-species variation. This was confirmed both by visual assessment of the DGGE gels and also by statistical analyses of the data. Low spatial variability of microbial fingerprints from soil samples collected within a single field has been reported in other PCR-DGGE studies (Johnson *et al.*, 2003b). In addition, the number of bands produced in the fungal gels was lower than that of the bacteria from the same site. Other workers have also reported obtaining fewer sequence types (bands) from fungal DGGE fingerprints compared to those of the bacteria (Johnson *et al.*, 2003b; Pennanen *et al.*, 2004). In the NMS analysis of the fungal communities at this site (Figure 5.4) based on the presence or absence of bands, the high NMS stress (0.19955) suggests that the apparent differences among these communities should be interpreted with caution. In this study, each band was inferred to represent a single OTU (Wakelin *et al.*, 2008a), without considering the reliability of the species identification (Ovaskainen *et al.*, 2010).

Continuous arable cropping is a land use that has distinctive effects on biological soil quality, with the type of cultivation exerting a greater influence than soil type on the soil biota (van Eekeren *et al.*, 2008). In the present study, under arable agriculture, replicate fungal communities from soil subsamples of sugarcane (burnt cane harvested) were very similar in structure. This was shown by NMS (Figure 5.4), which closely clustered the replicate subsamples on the basis of the presence of common groups of species (bands). By burning sugarcane at harvesting, the amount of organic matter and nutrients returning to the soil is reduced (Galdos *et al.*, 2009). This, together with the fact that the soils are under a long-term monoculture, restricted the variety of nutrient inputs to the soil, which were limited to root turnover and small amounts of leaf litter. This may account for the observed similarity of the fungal communities under sugarcane, as the homogeneous nature of the available nutrients would require a less diverse fungal population for their degradation.

A factor influencing the temporal dynamics of soil carbon is the degree of soil disturbance in preparation for replanting (Galdos *et al.*, 2009). Sugarcane soils at Baynesfield had an organic C content of 5%, which was greater than that of the maize

soils (see below). This was possibly due to the fact that, at the time of sampling, the sugarcane soil had not been tilled for two years and some plant litter had accumulated on the soil surface. PCA showed that these soils were also associated with high levels of exchangeable K and extractable P, as a result of annual applications of inorganic fertilizer. Analysis by CCA of the influence of soil variables on fungal community structure indicated that P had been the main influence on the composition of the sugarcane soil fungal communities. The values for community richness, evenness and the Shannon diversity index were slightly higher for the sugarcane fungal communities than those for maize, but were lower than those for all the other land use types. This shows the detrimental effects of excessive fertilization on the soil fungal communities.

The three maize (CT) soil fungal subsamples were grouped on the same side of the NMS biplot as those of sugarcane, as they shared some common DGGE bands, indicating the presence of similar fungal OTUs. However, as confirmed by MRPP, the individual replicate fungal communities under maize were the most dissimilar in structure of all the land uses. At the time of sampling, the maize had already been harvested, the field tilled and most of the crop residues removed. Consequently, the soil organic C level was the lowest (3.9%), of all the land uses at this site. Several authors have reported that in maize soils under conventional tillage, a concomitant decline in organic C and the level of soil microbial activity, occurred (Bell *et al.*, 2007; Bausenwein *et al.*, 2008). Oliveira *et al.* (2009) showed that more mycorrhizal bands were produced in DGGE gels from no-till maize soil samples than from maize soils under conventional tillage. Joergensen and Wichern (2008) reported that in arable soils, a reduction in tillage (as opposed to regular ploughing) usually promoted fungi, with the maximum increase in soil fungal C being 81% of microbial residue C under disc ploughing but 89% under no tillage.

In the present study, PCA showed that, like the sugarcane soils at this site, those under maize were characterised by high levels of P and K (from fertilizer applications). CCA analysis indicated that a high soil P level was the main influence on the genetic structure of the maize soil fungal communities, as was the case with the sugarcane soil fungi. Jangid *et al.* (2008) reported that soil microbial communities were significantly altered by long-term agricultural management systems, particularly

fertilizer amendment. Values for community richness, evenness and the Shannon diversity index for maize fungal communities were the lowest of all the land use soils. The removal of crop residues from, and annual tillage of, the experimental site under the long-term maize monoculture resulted in a low organic C level, which may account for the lower overall diversity of these soil fungal communities compared to those of the other land uses. Wakelin *et al.* (2008b) found that the return of maize residues to the soil resulted in a significant increase in the relative abundance and diversity of *Fusarium* genotypes present in the soil. The large differences between the individual maize subsample communities could be a short-term effect, since as mentioned above, at the time of sampling the maize field had recently been tilled, and repeated tillage mechanically disrupts and redistributes fungal mycelium (Pennanen *et al.*, 2004). It was recommended by van Eekeren *et al.* (2008) that for efficient nutrient use and soil recovery in maize cultivation, a ley-arable crop rotation was preferable to continuous arable cropping.

Understanding and managing soil microbial processes leads to increased crop production, with these processes also affecting the sustainability and environmental impact of production systems (Wakelin *et al.*, 2009). In addition, diversity of the soil microbial community is regarded as an important measure of sustainable land use (Yao *et al.*, 2006). Soil fungal community structure in replicate subsamples from under kikuyu (as shown by NMS) indicated that the individual communities were more dissimilar than those from under native grassland. However, the communities from both these land uses clustered together in the biplot, as a result of the presence of some similar fungal groups (Figure 5.2). Under improved high-yielding kikuyu cultivars, the level of soil organic C was the highest (9.4%) of all the land uses. This was probably due to a combination of the large amounts of dry matter produced by the irrigated, fertilized kikuyu pasture (Haynes *et al.*, 2003) and the no-till status of this land use.

Although the native grassland site had never been tilled or cultivated, the soil organic C level was much lower (5.1%) than under kikuyu and only slightly higher than that under sugarcane. In contrast to the continuous cover in kikuyu pasture, in native grassland, grasses grew in clumps, interspersed with bare soil and various widely-spaced angiosperms. Different C compounds are excreted by the roots of different

plant species, which may affect microbial community composition (Trinder *et al.*, 2009). Thus under native grassland, root exudates and plant litter were probably more varied but less abundant than under the kikuyu monoculture. Litter quality impacts on mineralization processes in pasture soils, with evidence of a preferential utilization of readily available organic substances in grass litter causing a shift in the microbial community and a lower relative abundance of fungi (Potthast *et al.*, 2010).

In this study, values for fungal community structural diversity (H') were higher under native grassland than under kikuyu, indicating a more structurally diverse fungal community in soils under the former land use than under the latter. Yao *et al.* (2006) reported that grasses that are managed as a monostand may result in reduced soil microbial diversity. However, in comparison with the arable crops, both types of grassland were more diverse, due to increased species richness in kikuyu and native grassland soil fungal communities compared to that of the sugarcane and maize communities. Wu *et al.* (2007) reported a greater fungal diversity under perennial bahiagrass than under various arable crops and that agricultural management practices significantly affected soil fungal communities.

Organic C and ECEC were shown by CCA to be the soil variables mainly influencing fungal community structure under both kikuyu pasture and native grassland (Figure 5.3). Lauber *et al.* (2008) suggested that the composition of fungal communities was most strongly correlated with specific soil properties, particularly changes in soil nutrient status. Carpenter-Boggs *et al.* (2003) indicated that soils managed under permanent grass contained approximately 160% more labile C and 50% greater microbial biomass than either no-till or conventionally tilled soils. In an experiment by Maharning *et al.* (2009), succession from former agricultural land and pastures to naturalized grasslands, was shown to be accompanied by changes in both plant biodiversity and the soil microbial community, with fungal biomass tending to be higher in older grassland communities. Wakelin *et al.* (2009) reported that the soil microbiota were highly responsive to pasture management practices, which altered the dominant soil fungi, and influenced the microorganisms involved in the key processes that underpinned productivity and environmental sustainability.

Soil fungal community structure under wattle was shown by ANOVA to have the greatest structural richness (S), evenness (J) and diversity (H') of all the land uses. Specific land use systems may lead to the adaptation of special soil microbial communities, which are responsible for degrading the respective recycled plant materials, for example, forest soil fungi are more resistant to condensed tannins than prairie soil fungi (Mutabaruka *et al.*, 2007). The observed greater structural diversity, richness and evenness of the wattle soil fungal communities than that of the other land uses, may be attributed to the variety of inputs to this soil from both the wattle trees (*Acacia mearnsii*, an exotic N₂-fixer) and also from the decaying stumps of the Eucalyptus plantation previously located on this site. Polyphenol-rich plant material decomposes slowly in soil and release of N from their tissues is restricted, even if the degrading materials contain a high N concentration. Certain soil fungi, such as ectomycorrhizal fungi, ericoid mycorrhizal and wood decomposing fungi, are more effective at degrading hydrolysable polyphenol-protein complexes, than other microorganisms (Mutabaruka *et al.*, 2007). In contrast, Allison *et al.* (2007) reported that when N availability exceeded biological demand in forested ecosystems, N saturation may occur. This led to soil pH changes, forest decline, large N losses and changes in fungal diversity and community structure, through altered plant C inputs. In the present study, PCA indicated that wattle soils were correlated with a pH of 4.6 and an organic C concentration of 4.8% (the second lowest value of all the land uses). Analysis by CCA of the effects of soil variables under this land use, showed that soil pH and ECEC were closely correlated with wattle soil fungal community structure. Marchante *et al.* (2008b) indicated that long-term occupation by invasive *Acacia longifolia*, increased levels of organic C, total N and ECEC, and resulted in a higher microbial biomass. As shown by NMS, the soil fungi from the replicate wattle subsamples were closely clustered, indicating that they were similar in structure. This suggests that while a greater richness and diversity of fungal OTUs was required to degrade the variety of plant and nutrient inputs to this soil, the different subsample communities were highly specialised and structurally similar to each other. This was confirmed by visual assessment of the duplicate DGGE profiles.

In South Africa, plantations comprising exotic pine species (such as *Pinus patula* at Baynesfield) have long been established for the production of timber and wood products. Most of these plantations occupy land that was formerly native forest, and

little is known of the effects of a plantation monoculture on the soil fungal community composition and diversity. The present study determined the effects of this land use on the resident soil fungal communities at Baynesfield. At this site, the soil pH (3.8) was lower under pine than under all the other land uses, while the concentration of organic C (6.3%) was higher than all the land uses except kikuyu pasture. Fungi play a key role in the formation, transformation and degradation of lignin (Alexander, 1977) and are the most efficient degraders of humic substances, particularly in forests, where large amounts of organic matter are present (Grinhut *et al.*, 2007). At Baynesfield, the combination of a low pH (< 4.0) and the recalcitrant litter inputs (from the thick layer of decaying pine needles and fragments of bark and twigs) would be expected to favour the soil fungi (Grinhut *et al.*, 2007). This was confirmed by ANOVA, with the values for soil fungal community structural evenness and diversity being greater under pine than those for all the other land uses except wattle. In nature, humic substances (mainly humic acids and humin) are highly resistant to biodegradation, as they comprise an extremely complex, amorphous mixture of chemically reactive, though heterogeneous refractory molecules (Grinhut *et al.*, 2007). This would require a structurally diverse, highly specialised fungal community to effect degradation into more readily metabolizable substrates (Alexander, 1977). Analysis by CCA at this site showed that the soil variable most closely associated with pine fungal community structure was pH. Edaphic properties are often consistent among similar land use types, with management practices having appreciable effects (Lauber *et al.*, 2008). The pine soil fungal communities were distinct from those of the other land uses as shown by NMS. This indicated that a diverse, specialized fungal community, adapted to the prevailing conditions, which differed markedly from those of the other land uses, was present in pine soils.

5.4.2 Effects of different management practices on soil fungal community structure at the Mount Edgecombe experimental site

That agricultural land management is one of the most significant anthropogenic activities, responsible for changes in soil physicochemical and biological properties (Jangid *et al.*, 2008), is particularly evident in the sugar industry. Sugarcane

production is an intensive system, involving a high-input monoculture, with high levels of fertilizer use, and few fallow periods (Bell *et al.*, 2007), leading to a decline in soil fertility. Traditionally, soils were evaluated according to their capacity to ensure the growth and reproductive efficiency of cropped plants. Only recently has soil quality also been assessed. Microorganisms play a key role in the maintenance of soil quality and health and may be used as early indicators of soil degradation (Aboim *et al.*, 2008).

In South Africa, the normal practice prior to harvesting sugarcane is to burn the standing cane, in order to remove dead leaves (trash). This results in the volatilization and consequent loss of large amounts of C, N and S present in the plant residues, and an accumulation in the soil of chemically inert C, in the form of charcoal (Graham *et al.*, 2002a, b). Moreover, conventional cultivation and replanting of the cane approximately every 7 years, promotes soil organic matter (SOM) decomposition (Haynes and Beare, 1996). This loss of SOM and concomitant loss of microbial activity under pre-harvest burnt sugarcane, is regarded as a major factor in the degradation of soil in the South African sugar industry, which has now been linked to a decline in sugarcane yields per hectare (Graham *et al.*, 2002a; Graham and Haynes, 2006). The impact of residue management on SOM, can be measured by the total carbon stocks in the soil (Galdos *et al.*, 2009).

As previously noted (section 3.4.2) at Mount Edgecombe, the main treatment effects are a progressive loss of organic matter under burnt cane harvesting, acidification due to applications of inorganic fertilizers, and a breakdown in soil structure (Graham *et al.*, 2002a). PCA (Figure 5.4) showed that higher levels of soil organic C were associated with soils under green cane harvested treatments retaining a trash mulch, both fertilized (6.6%) and unfertilized (6.0%), compared to burnt cane harvesting. Under the latter fertilized treatment, organic C concentrations were lower at 5.14% whereas, under the equivalent unfertilized treatment, the concentration was 4.93%. Razafimbelo *et al.* (2006) reported that C concentrations in topsoil (0–5 cm depth) were significantly greater in soils under green cane management with residue mulching than in soils under pre-harvest residue burning. In addition, changes in management not only influenced levels of soil organic C, but also affected its quality. In the present study, CCA (Figure 5.7) showed that the soil variables which had

significantly affected soil fungal community structure under green cane harvesting, were organic C and ECEC, particularly in the unfertilized, green cane management. On the other hand, both burnt cane treatments were associated with low organic C, while the fertilized treatment was also associated with a low pH and low ECEC level. These effects were significant ($p = 0.01$). Galdos *et al.* (2009) showed that soil total C concentration decreased under long-term sugarcane cropping. However, in unburnt treatments, the total C concentration was higher than in burnt treatments. These results support those obtained in the present study.

The profiles of the fungal communities from soils under the four different sugarcane trash managements at this site, were shown by DGGE to be very similar across the gels, regardless of the treatment, in contrast to those of the fungal communities under the different land uses at Baynesfield. However the number of bands (OTUs) varied per lane, indicating large differences in the fungal communities under the four land management practices. Analysis by NMS separated all the land management communities, and these differences were significant (MRPP, $p = 0.04$). Communities under both BtoF and BtoFo were associated with different fungal species, and were, therefore, separated from the other fungal communities in the NMS plot. In contrast, those from the greencane harvested trash managements were more closely associated, and shared some common fungal groups, although the fungal communities from the replicate soil subsamples under the TF treatments were the most dissimilar in structure. Fungal distribution is determined by the availability of oxidizable carbonaceous substrates, with organic matter status, hydrogen ion concentration, and fertilizers (organic and inorganic) being among the most important external influences on fungal flora (Alexander, 1977).

Soil pH was lower under fertilized treatments, (pH = 3.7 under BtoF) with those retaining a trash blanket having the lowest pH (3.5 under TF). Exchangeable acidity was also higher in fertilized managements. In an earlier study at this site, Graham *et al.* (2002a) reported a decrease in soil pH under fertilization, and to a lesser extent with trash retention. This was accompanied by an increase in exchangeable acidity. These conditions would tend to favour fungal communities in soil. In the present study, analysis of the soil fungal community structural richness, evenness, and the Shannon Weaver diversity index, showed that land management practices had a

significant effect on richness ($p = 0.02$) but not on evenness or diversity. Fungal community richness in soils under TF and TFo was not significantly different from that under BtoF or BtoFo. However community richness under BtoFo was significantly different from that under BtoF. Fertilizer applications to the burnt cane harvested treatment had increased the soil fungal community richness compared to the unfertilized treatment. This could have been due to the higher yields of sugarcane under fertilization and an associated increase in root turnover and in the amounts of root exudates, together with the lower pH in fertilized soils. At this site, applications of inorganic fertilizers were shown to have increased the concentrations of both extractable P and exchangeable K (Table 5.6). Phosphorus is routinely applied to sugarcane as a fertilizer, as it influences both crop yield and juice quality (Sundara *et al.*, 2002). In contrast, higher levels of Ca and Mg were seen in the unfertilized plots than in the fertilized plots. This was because acidification, induced by fertilizer and trash retention, was accompanied by a decrease in exchangeable Ca and Mg (Graham *et al.*, 2002a). All these factors may have contributed to the increased fungal richness observed under BtoF compared to BtoFo.

5.5 CONCLUSIONS

The use of the universal fungal primer pair FR1GC/FF390 for PCR-DGGE analysis of the effects of different land uses and management practices, on the autochthonous soil fungal communities at the two study sites, enabled a wider spectrum of sequences to be produced, which allowed for a more comprehensive analysis than was possible with primer pair FR1GC/NS1 (section 4.4). However, it was noted that this could have been a temporal effect, as the DNA used in the current study, was isolated from soils collected 4 years after those of the initial study. In the present study, communities at both sites were clearly separated, and banding profiles in replicate gels were consistently produced. The narrower denaturing gradient used in the DGGE gels showed that apparently 'single' dominant bands, actually contained from 2–7 bands (Baynesfield samples) and from 2–4 bands (Mount Edgecombe samples). Therefore, fungal community richness and diversity had previously been underestimated at both sites. However, sequencing revealed the presence of chimeras

and mixed DNA templates among the samples from site 2, leading these results to be interpreted with caution.

At site 1, the different land uses on the same soil type, had affected the soil fungal communities' structural diversity. Greater differences were found between the various land uses than within them. Soil variables influencing the different communities were organic C, soil pH, ECEC and extractable P. Fungal community structural richness, evenness and diversity were greatest under exotic forestry plantations and lowest under arable cropping. At site 2, the differences in the soil fungal communities under the four land management practices were less marked than at site 1. This was expected, as differences between the various land uses were greater than the differences in management within a single land use. Soil variables having a significant effect on the fungal communities at site 2 were organic C, ECEC and pH. Applications of the inorganic fertilizers N, P and K had a significant effect on the richness but not on the evenness or diversity of the soil fungal community structure in treatments under burnt cane harvesting.

Chapter 6

EFFECTS OF LAND USE AND MANAGEMENT ON SOIL BACTERIAL CATABOLIC DIVERSITY

6.1 INTRODUCTION

Functional diversity of soil prokaryote communities cannot be determined by community composition, as microbes may be present in soil in a resting or dormant stage and not be functionally active (White and MacNaughton, 1997; Govaerts *et al.*, 2007). Therefore, after Garland and Mills (1991) used BIOLOG™ plates (section 2.4.1.4.1) successfully to characterize the functional potential of mixed heterotrophic microbial communities, this method became widely used. The protocol is based on patterns of community-level carbon source utilization (catabolic diversity) that generate community-level physiological profiling (CLPP) data for relative comparison of environmental samples (O'Connell *et al.*, 2000). Ros *et al.* (2008) reported that by determining the diversity of microbial heterotrophic functions related to C utilization, more relevant information on the roles of microorganisms in the ecosystem can be obtained. This method has proved useful as a fast and highly reproducible means of studying soil microbial functional diversity (Kirk *et al.*, 2004) and of differentiating between microbial communities in natural soils, such as those under different land uses or those subjected to degradation or contamination (Ros *et al.*, 2008).

The BIOLOG system detects microbial metabolism of specific C sources, with differences in substrate utilization allowing different species to be distinguished. To quantify the degree of utilization of a particular C source, the colour change intensity of a tetrazolium dye is measured. Colour development in each well shows both species density and activity, while the number and categories of substrates utilized, provide a data set from which bacterial functional diversity can be assessed (Zak *et al.*, 1994).

Graham (2003) used BIOLOG GN microplates and substrate-induced respiration (SIR) techniques to estimate the catabolic diversity of bacterial communities in soils under different agricultural land uses and various management practices in South Africa. Substantial differences in the ability of the microbial communities to metabolize a range of C substrates were observed with both methods in soil from different land uses. BIOLOG data of soils under various managements showed large differences in microbial community catabolic capability between sugarcane soils under trashing and those under preharvest burning, but also revealed that fertilizer applications had little effect.

As PCR-DGGE using universal primers (which don't target specific genes) provides no information on soil microbial function, CLPP analysis of the soil bacteria at the two experimental sites, was used as an adjunct to the genetic diversity analyses in this study. For this purpose, BIOLOG EcoPlates were chosen to determine how the different long-term land uses and management practices had affected the catabolic diversity of the resident soil bacterial communities at the study sites. EcoPlates contain 31 C substrates consisting of ten carbohydrates, seven carboxylic acids, four polymers, six amino acids, two amines and two phenolic compounds, replicated three times on each microplate (Choi and Dobbs, 1999) (Appendix D, Table D1). Patterns of substrate utilization are compared between the different soil bacterial communities, to determine whether their catabolic (functional) diversity has been affected by the various land uses and treatments. These data are then used to establish bacterial functional diversity using CLPP assays which provide substantial information on communities in a variety of ecosystems (Calbrix *et al.*, 2005). As CLPP of bacteria requires fresh inoculum from soil (within 24 hours of collection), the same sites were re-sampled four years after collecting the original soil samples, but at the same time of year to minimise seasonal differences (section 6.2.1).

6.2 MATERIALS AND METHODS

6.2.1 Study sites and soil sampling

Details of the study sites were previously given in section 3.2.1. Re-sampling of soils from both sites was as described in section 5.2.1 and followed the same procedures in sections 3.2.2.

6.2.2 Chemical analysis

Chemical analysis of the fresh soil samples was according to the methods described in section 3.2.3.

6.2.3 Soil moisture

As the soil moisture content is critical for bacterial activity, the field soil moisture content at the time of sampling at both Baynesfield Estate and Mount Edgecombe, was determined by weighing approximately 10 g of each field-moist soil sample into a separate glass beaker and air-drying at 105°C for 24 hours. Each soil subsample was then reweighed, and the percentage soil moisture content and mean moisture content calculated.

6.2.4 CLPP using BIOLOG microplates

In this study, BIOLOG EcoPlates were chosen in preference to the GN microplates used by Graham (2003) as they are a more recent development, created specifically for environmental studies. The three replications of the 31 substrates and control well across each plate, increase the likelihood that the CLPP's obtained are representative of the experimental soils. Furthermore, EcoPlates avoid functional redundancies and concentrate on substrates that are known to occur in plant root exudates. The number and diversity of substrates in these plates is sufficient to distinguish between different microbial communities in environmental samples (Ros *et al.*, 2008).

A method adapted from Govaerts *et al.* (2007) was used to determine the CLPP of the bacterial communities in the experimental soils. A short incubation period at high inoculum density was chosen to reduce both the enrichment effect (Garland, 1997) and the risk of contamination (Govaerts *et al.*, 2007). Within 24 hours of sample collection, 10 g of field-moist soil from each of the replicate bulked samples of the various land uses at Baynesfield Estate and the different management practices at Mount Edgecombe, were added separately to 500 ml Erlenmeyer flasks containing 90 ml of a sterile saline solution (0.85 % w/v NaCl). The resultant soil suspensions were shaken at 150 r.p.m. for one hour at room temperature, and thereafter allowed to settle for 30 minutes.

When using the BIOLOG system to determine microbial catabolism of C substrates over time, increasing optical density is measured. However, the presence of soil particles in the bacterial suspensions is problematic as it increases optical density. Therefore, to reduce and standardize the initial inoculum OD₅₉₀, a serial dilution of the soil suspensions to 10⁻³ was carried out (Calbrix *et al.*, 2005; Ros *et al.*, 2008). Each soil suspension was first diluted to 10⁻² in 9 ml aliquots of sterile saline and finally to 10⁻³ by adding 5 ml of the 10⁻² dilution to 45 ml sterile saline. Immediately prior to microplate inoculation, the 10⁻³ dilutions were thoroughly mixed and poured into separate, sterile, glass Petri dishes. Aliquots (150 µl) of the soil suspensions were simultaneously inoculated into each 8-well row in the EcoPlates, using an Eppendorf multi-channel pipettor, such that each microplate contained 3 replicate subsamples of a single soil. The plates were prepared in duplicate. A Versamax tunable microplate reader was set at OD₅₉₀ nm, the peak absorbance for tetrazolium dye. The rate of substrate utilization is indicated by the reduction of tetrazolium, which changes from colourless to purple. Readings were taken over 5 days at 0, 24, 48, 72, 96 and 120 hours. Between readings, the microplates were incubated in the dark at 25°C. A single time point (in this study, 120 h) was selected for statistical analysis. This was determined by the rate of colour development in the wells and the time by which most of the substrates were used (Garland, 1996).

6.2.5 Data analysis

Raw difference data for each BIOLOG plate were obtained by subtracting the colour response of the control wells (without a C source) from that of each of the substrate-containing wells. Values were calculated separately for each plate at each time point (Govaerts *et al.*, 2007). Wells showing very little colour response sometimes gave negative absorbance values after normalisation. Since this is physically meaningless, such negative numbers were set to zero as recommended by several authors (Hu *et al.*, 2007; Liu *et al.*, 2007a; Weber *et al.*, 2007).

Prior to statistical analysis, to eliminate variation in well colour development caused by different cell densities, the average well colour development (AWCD) value for each sample at each time point, was calculated according to Garland and Mills (1991), as follows:

$$\text{AWCD} = \sum (C - R) / n$$

where C is the colour production within each well (optical density measurement), R is the absorbance value of the plate's control well and n is the number of substrates (EcoPlates, $n = 31$) (Choi and Dobbs, 1999; Liu *et al.*, 2007b).

Prior to PCA, the data were transformed by dividing, for each substrate, the difference in optical density at 120 h relative to the control wells, by the AWCD of the plate at 120 h, that is, $(C - R) / \text{AWCD}$. This further reduced the bias between samples caused by different initial inoculum densities, and resulted in standardized patterns being compared rather than absolute values (Garland and Mills, 1991).

An OD_{590} threshold value of 0.1 (significant catabolic activity) was chosen for analysis, as lower values could not reliably be ascribed to bacterial activity but rather to 'noise' (no catabolic activity) (Heuer and Smalla, 1997b).

6.2.6 Statistical analysis

BIOLOG substrate utilization patterns were statistically analyzed (Zak *et al.*, 1994; Garland, 1996; Liu *et al.*, 2007a) to determine the catabolic diversity of the soil bacterial communities at both experimental sites. To visualize the relationship among samples, PCA was carried out on the transformed AWCD data from the microbial communities, using CANOCO v 4.5 (Microcomputer Power, Ithaca, NY) (ter Braak and Šmilauer, 1997). Logarithmic [X+1] transformation was used to normalize skewed data (Weber *et al.*, 2007). PCAs of soil physicochemical data from both sites were also carried out. The effect of ordination is to arrange samples of multidimensional space into a low-dimensional space so that similar samples were grouped closer together whereas dissimilar samples were spaced further apart. PCA reduced complex multidimensional data into a smaller number of interpretable variables (principal components) representing a subset of the original variables (Garland, 1996). The angles between variables expressed their correlation (Govaerts *et al.*, 2008).

Soil data from the PCAs were tested by MRPP standardized to standard deviation = 1, using PC-ORD v 4.25. Tests were carried out on standardised data where the original value for each soil variable from each site was divided by its standard deviation (across samples) to allow comparisons of variables on a common scale. Dissimilarity between samples was measured by Sørensen (Bray-Curtis).

Land use and treatment effects on soil microbial communities were also tested by MRPP using PC-ORD v 4.25. Tests were done on log-transformed data using the Bray-Curtis algorithm. Distance between samples was measured using the Sørensen index (Šmilauer, 2002).

Redundancy analysis (RDA) (CANOCO v 4.5) was used to correlate selected environmental variables with microbial community substrate utilization at both experimental sites. Sets of soil variables having relatively independent effects (low collinearity) were chosen for the analyses. Testing for significant relationships was by

means of the Monte Carlo permutation test. Least significant differences were calculated at the 5% level.

Species catabolic richness (S) (the number of substrates utilized), evenness (J) (distribution of colour development) and Shannon's diversity index (H') (a composite measure of both richness and evenness) were determined by the nonparametric Kruskal-Wallis ANOVA by Ranks and Median test, using STATISTICA v 7.1, as residuals were skewed (non-normal). These tests are nonparametric alternatives to between-groups one-way ANOVA, and their interpretation is almost identical except that they are based on ranks rather than means (STATISTICA, StatSoft Inc., 2006).

6.3 RESULTS

Soil physicochemical factors that can potentially affect community structure and function were used as environmental data.

6.3.1 Analyses of soils at the Baynesfield experimental site

The soil physicochemical variables selected for analysis at this site were previously presented in Table 5.1 (section 5.3.1).

The results of calculations of field soil moisture content (%) are shown in Table 6.1.

TABLE 6.1 Mean soil moisture content (%) of soils (0-5cm depth) under the different land uses at Baynesfield Estate

| Land-use | SC | M | KIK | NAT | PF | W |
|-----------------|-------|-------|-------|-------|-------|-------|
| Soil moisture % | 14.88 | 11.93 | 23.14 | 12.02 | 20.93 | 11.76 |

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = permanent kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

6.3.2 Bacterial catabolic (functional) diversity at Baynesfield

BIOLOG substrate-utilization patterns, as shown by plotting mean AWCD values for each sample over time, increased in the order: NAT < M < W < PF < SC < KIK (Figure 6.1). In the BIOLOG plates, an initial lag phase in colour development was observed for all the land use soil bacterial communities. This is the time required for the bacterial cells to reach a density of 10^8 ml^{-1} , at which colour is observed (Konopka *et al.*, 1998). The AWCD values for the first 48 h showed little if any change for any of the land use communities except KIK, which showed a sharp increase after 24 h. Values for SC, PF and W only increased after 48 h incubation, and M after 72 h, whereas NAT showed no response. The highest AWCD value at 120 h was associated with the soil bacterial community under KIK (Figure 6.1).

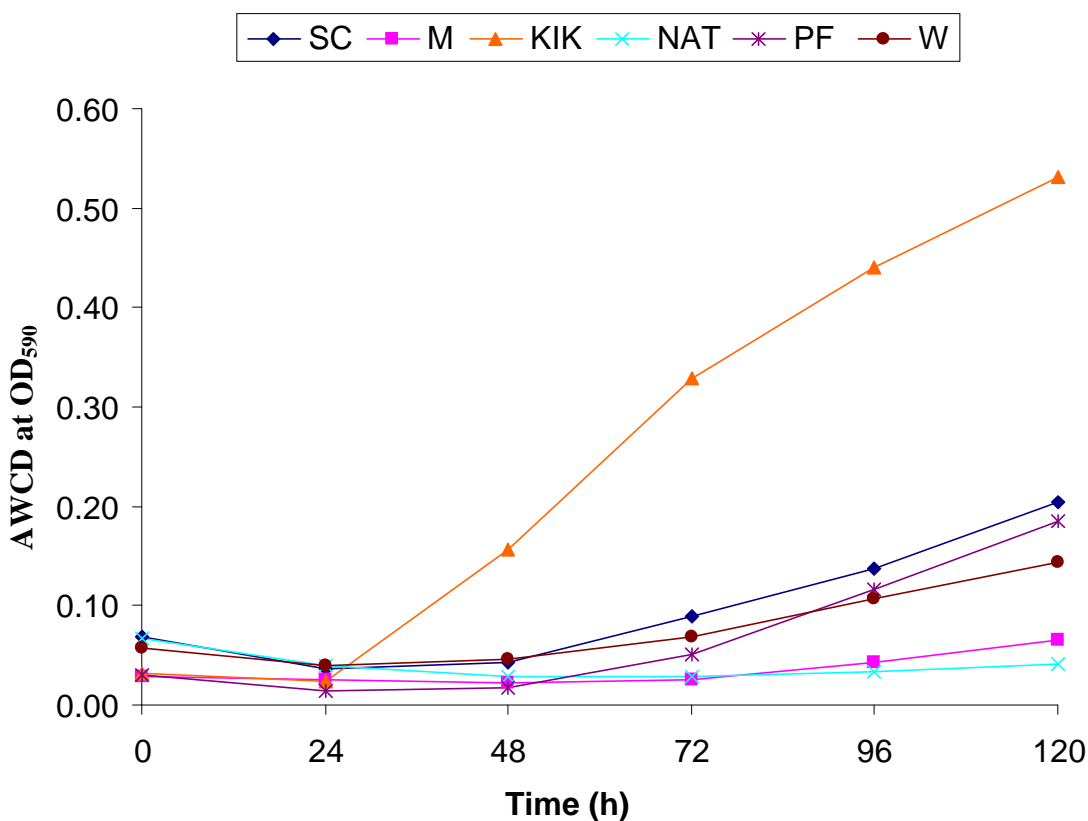


FIGURE 6.1 Variation over time in AWCD of Baynesfield soil bacterial communities under six different land uses.

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = permanent kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

A PCA plot of log [X+1]-transformed data shows the distinctive bacterial catabolic community profiles among the land use subsamples, based on substrate utilization after 120 h incubation (Figure 6.2). The PC1 axis accounted for 20.3% and the PC2 axis for 16.1% (cumulatively 36.4%) of the variance in bacterial catabolic diversity, therefore these results should be interpreted with caution. Subsample profiles under NAT were most dissimilar, followed by M. On the other hand, PF and W subsamples clustered closely together in the biplot, an indication of their similarity. Profiles of KIK subsamples also clustered closely together, whereas those under SC were more widely separated.

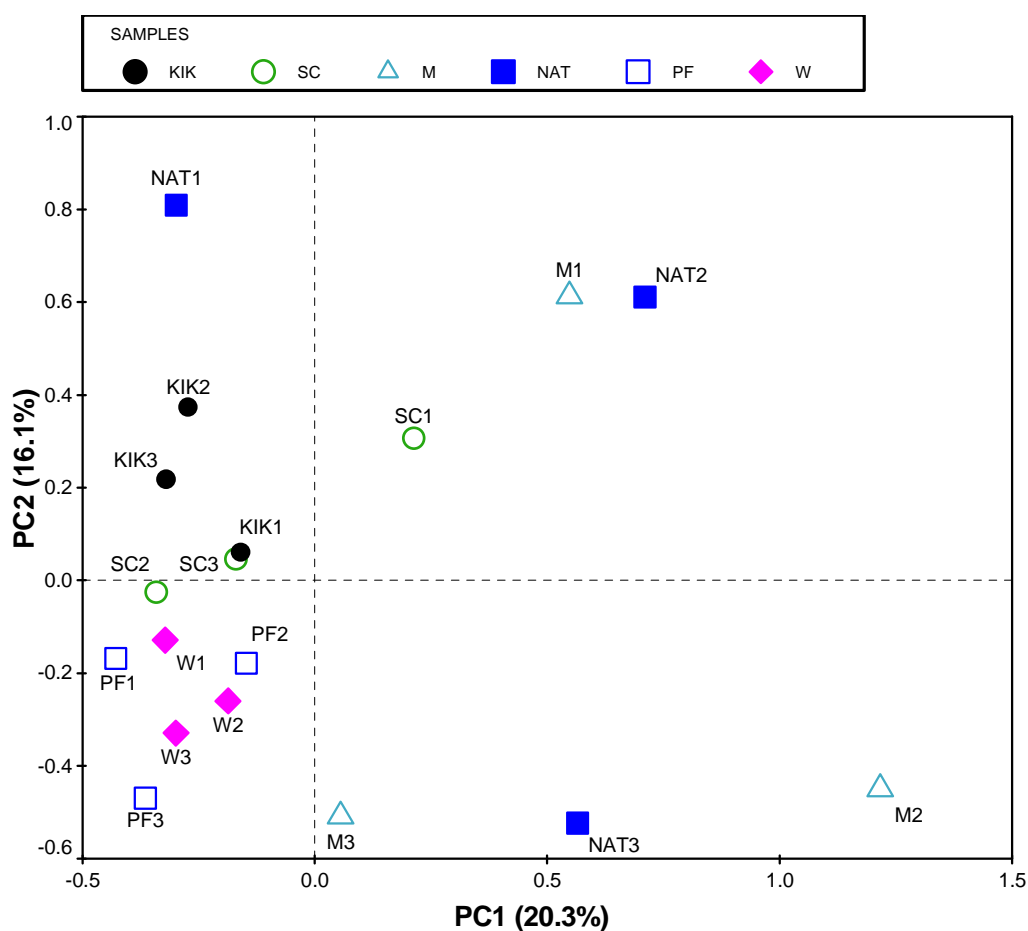


FIGURE 6.2 A PCA plot of samples (log [X+1]-transformed values) along gradients of bacterial functional diversity for subsamples of fields with various land uses at Baynesfield.

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = permanent kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

Testing by MRPP of the PCA data of the functional diversity of the soil bacterial communities from the different land uses (Figure 6.2), showed that land use had affected bacterial functional diversity at this site ($T = -2.762$, $p = 0.005$). The results of the MRPP are summarised in Appendix D (Table D2).

A PCA plot (Figure 6.3) shows those substrates in the BIOLOG EcoPlates having at least 50% of their variance accounted, along the gradients (Figure 6.2) of bacterial community functional diversity at Baynesfield. A biplot (Figure D1) of all the substrates, and Table D1 listing all substrates in the EcoPlates are presented in Appendix D.

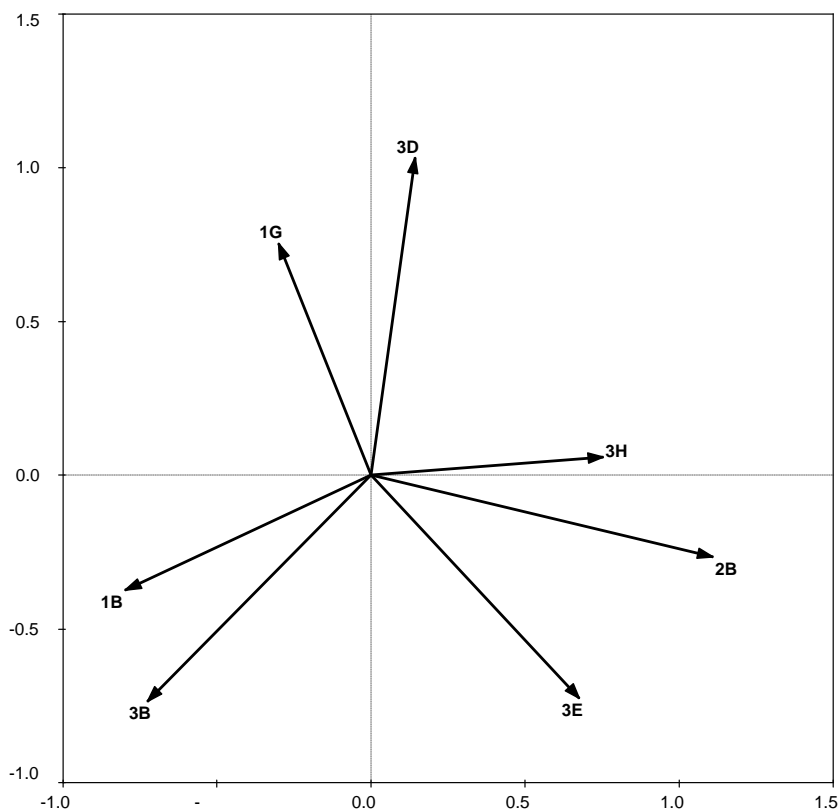


FIGURE 6.3 A PCA plot of C sources with at least 50% of their variance accounted, along gradients of bacterial functional diversity (Figure 6.2) at Baynesfield.

Key: 3D = 4-hydroxy benzoic acid; 3H = D-malic acid; 2B = D-xylose; 3E = γ hydroxybutyric acid; 3B = D-galacturonic acid; 1B = pyruvic acid methyl ester; 1G = D-cellobiose.

The non-parametric Kruskal-Wallis ANOVA by Ranks and Median test used to determine soil bacterial community catabolic richness (S) ($p = 0.133$), evenness (J)

($p = 0.071$) and the Shannon Weaver diversity index (H') ($p = 0.113$), showed that land use at Baynesfield had not affected these parameters (Appendix D, Tables D3, D4, and D5).

RDA results of the relationship between selected (non-collinear) soil physicochemical variables and soil bacterial community substrate utilization patterns at Baynesfield are shown in Figure 6.4. The RDA1 axis accounted for 46.0% of fitted and 14.6% of total variation in data along the axis, while the RDA2 axis accounted for 27.5% of fitted and 8.7% of total variation in the data. Organic C, P and ECEC were aligned with the RDA1 axis, and pH with the RDA2 axis. The PF subsamples were associated with a low pH. The KIK, SC and NAT 1 and 2 subsamples were correlated with high levels of organic C. NAT 3 was dissimilar from NAT 1 and 2, having a higher ECEC level than the other NAT subsamples. Subsamples of M were clustered on the basis of high P levels.

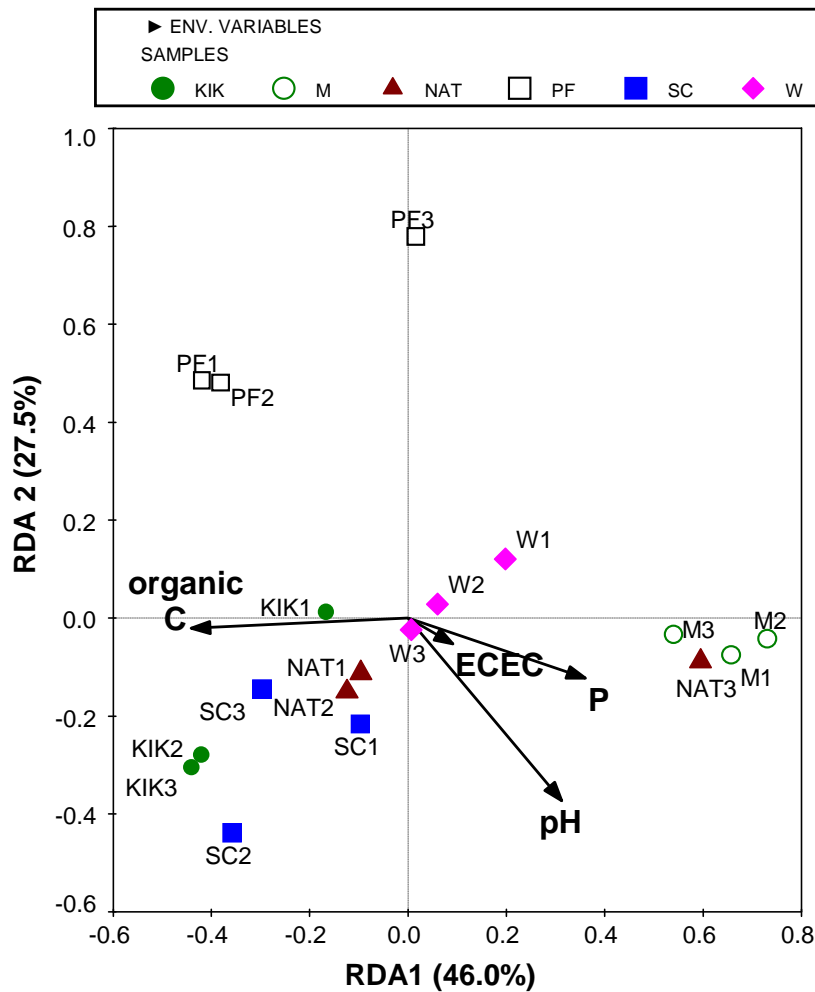


FIGURE 6.4 RDA ordination biplot of Baynesfield bacterial BIOLOG data (log-transformed) using selected (non-collinear) soil variables, showing the relationship between the variables and the soil bacterial communities under the different land uses. The RDA1 axis accounted for 46.0% of fitted and 14.6% of total variation in the data and the RDA2 axis for 27.5% of fitted and 8.7% of total variation.

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = permanent kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

Substrates (C sources) with at least 25% of their variance accounted in the RDA ordination biplot (Figure 6.4) are shown in Figure 6.5. The relationship between the substrates and the selected soil variables is explained in the key below Figure 6.5.

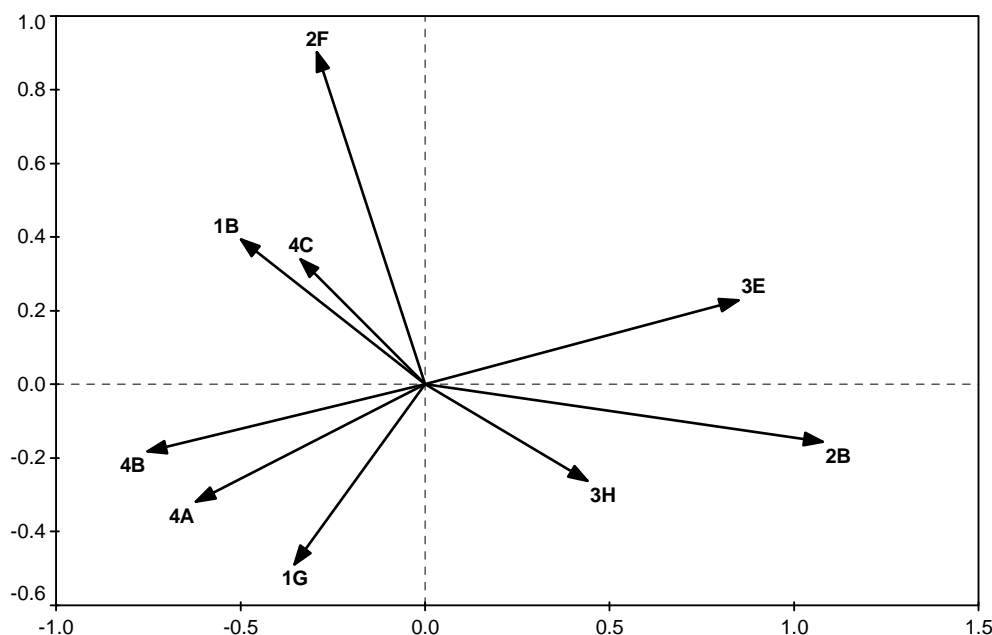


FIGURE 6.5 Carbon sources utilized by soil bacterial communities under the different land uses at Baynesfield, with at least 25% of their variance accounted in the RDA ordination plot (Figure 6.4).

Key: 3E= γ hydroxybutyric acid is correlated with lower soil organic C levels;
 2B =D-xylose is associated with high levels of P and ECEC;
 3H = D-malic acid is associated with a higher soil pH;
 1G = D-cellobiose, 4A = L-arginine, 4B = L-asparagine, are correlated with higher soil organic C levels;
 1B = pyruvic acid methyl ester, 4C = L-phenylalanine, 2F = D-glucosaminic acid are associated with a low soil pH and low levels of P and ECEC.

The Monte Carlo Permutation test for significant relationships within the RDA data showed that soil variables had affected diversity at this site (Appendix D, Table D6).

6.3.3 Analyses of soils at the Mount Edgecombe site

The soil physicochemical properties selected for analysis at this site were previously summarised in Table 5.7 (section 5.3.4).

The results of calculations of soil moisture content (%) at the time of sampling are presented in Table 6.2.

TABLE 6.2 Mean soil moisture content (%) of soils (0–5cm depth) under the different land management practices at Mount Edgecombe

| Land management | TF | TFo | BtoF | BtoFo |
|-----------------|-------|-------|-------|-------|
| Soil moisture % | 22.35 | 24.62 | 22.77 | 22,71 |

Key: T = green cane harvested with trash retention; Bto = burnt cane with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

6.3.4 Bacterial catabolic (functional) diversity at Mount Edgecombe

Substrate utilization patterns in the BIOLOG EcoPlates as shown by plotting mean AWCD values at A_{590} (OD) over time were, in increasing order: BtoF < TF < BtoFo < TFo. In the BIOLOG plates, colour development for the soil bacterial communities under all the land management practices did not commence until after 24 h incubation (the lag period until the cell density reached 10^8 ml⁻¹) (Konopka *et al.*, 1998), after which it increased to different levels (Figure 6.6). Higher AWCD values (activity levels) after 120 h incubation were reached by bacteria under the unfertilized treatments (Fo) than by those under the fertilized treatments (F).

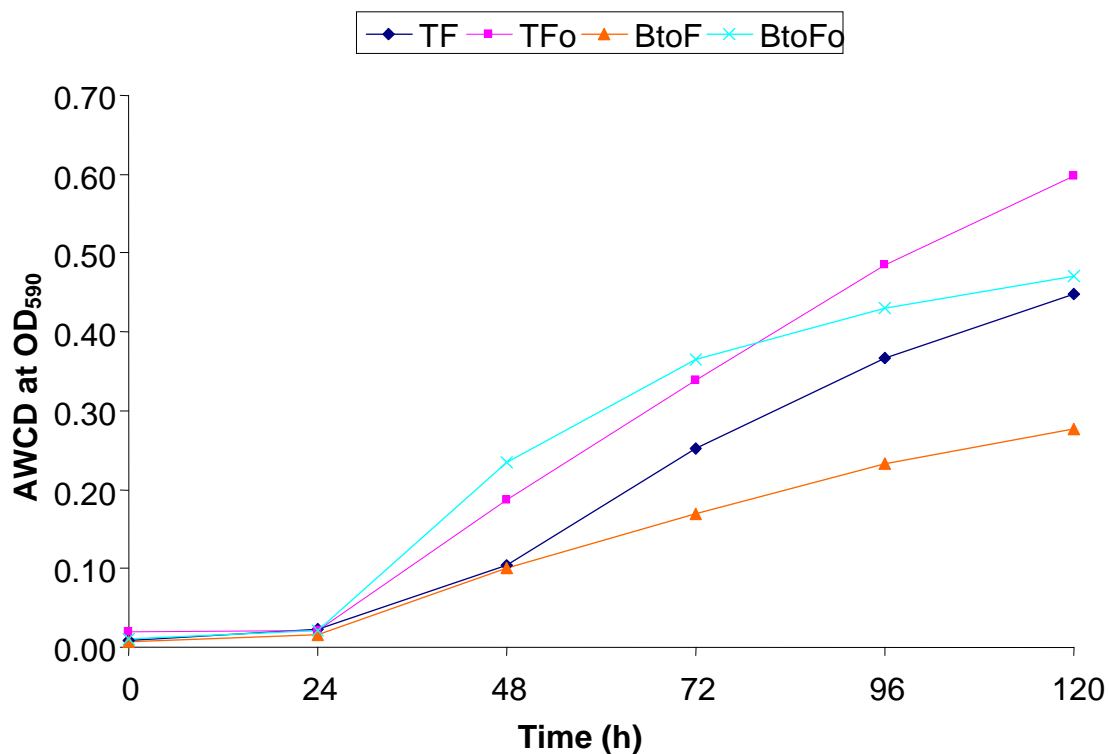


FIGURE 6.6 Variation over time in AWCD of Mount Edgecombe soil bacterial communities under four different sugarcane residue treatments.

Key: T = green cane harvested with trash retention; Bto = burnt cane with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

A PCA plot of log [X+1]-transformed data showed soil bacterial community catabolic profiles among samples from the different sugarcane trash managements at Mount Edgecombe (Figure 6.7). The different treatments were clustered along the X- and Y-axes according to residue management and fertilizer practices. The PC1 axis accounted for 28.8%, the PC2 axis for 17.3% and, cumulatively, the axes accounted for 46.1% of the variance in bacterial functional diversity at this site. The TFo subsamples were the most closely clustered in the biplot, followed by the TF subsamples. The Bto soil subsamples were further apart, with the fertilized (F) soils being the most dissimilar.

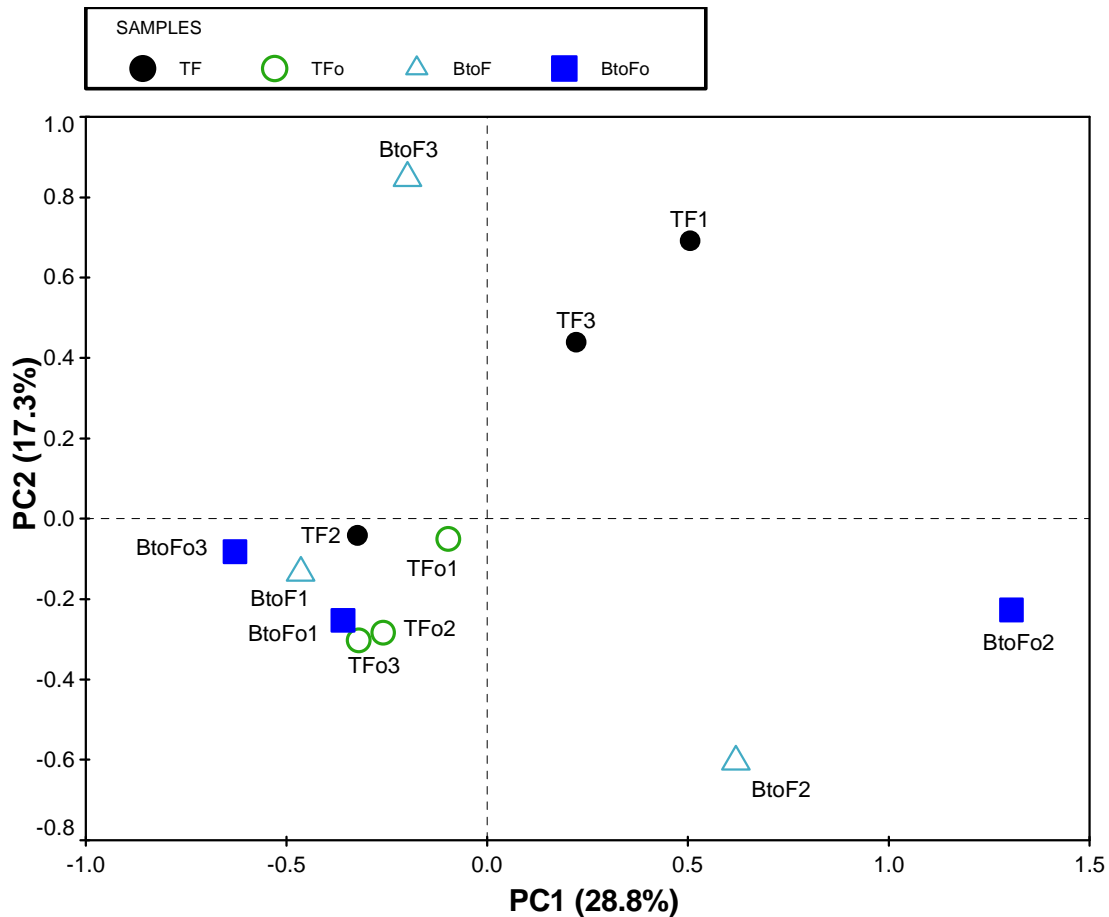


FIGURE 6.7 A PCA plot of samples (log [X+1]-transformed values) along gradients of bacterial functional diversity, for subsamples of plots with various land managements at Mount Edgecombe.

Key: T = green cane harvested with trash retention; Bto = burnt cane with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

Tests of the PCA data by MRPP, of the functional diversity of the soil bacterial communities under the different land managements (Figure 6.7) indicated that the four experimental treatments did not have a significant effect on bacterial catabolic diversity at this site ($T = -0.288$; $p = 0.361$). (Appendix D, Table D7).

A PCA plot showing those C sources in the BIOLOG EcoPlates having at least 50% of their variance accounted along the gradients of bacterial functional diversity at Mount Edgecombe (in the ordination plot, Figure 6.7) appears in Figure 6.8. A PCA plot (Figure D2) and Table D1, showing all the substrates in the EcoPlates, are presented in Appendix D.

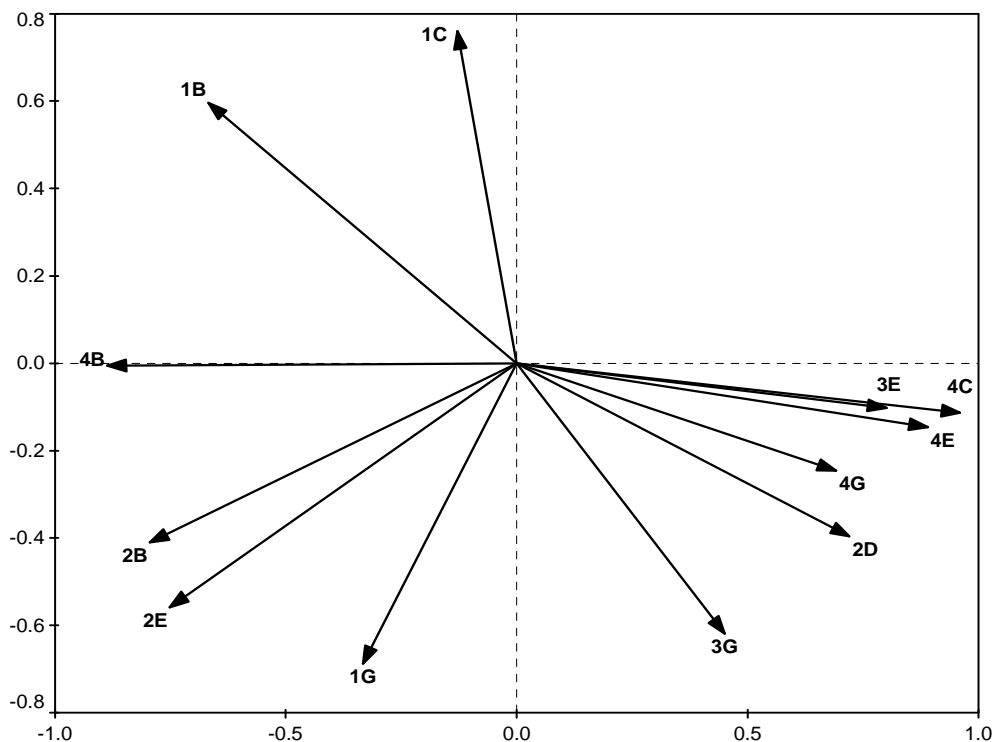


FIGURE 6.8 A PCA plot of C sources with at least 50% of their variance accounted, along gradients of bacterial functional diversity at Mount Edgecombe.

Key: 4C = L-phenylalanine; 3E = γ hydroxybutyric acid; 4E = L-threonine; 4G = phenylethylamine; 2D = D-mannitol; 3G = α -ketobutyric acid; 1G = D-cellobiose; 2E = N-acetyl-D glucosamine; 2B = D-xylose; 4B = L-asparagine; 1B = pyruvic acid methyl ester; 1C = Tween 40.

Trends for soil bacterial catabolic richness at this site were: BtoF < BtoFo < TF < TFo. The non-parametric Kruskal-Wallis ANOVA by Ranks and Median test showed that land treatments at Mount Edgecombe had a significant effect on catabolic richness (S) ($p = 0.046$) (Table 6.3).

TABLE 6.3 A non-parametric one-way analysis (Kruskal-Wallis test) of variation in catabolic richness under different treatments at Mount Edgecombe

| Species richness (S) | | Significant (p = 0.046) | | |
|--|---------------|--------------------------------|-----------------|------------------|
| Multiple comparisons p values (2-tailed); S (BIOLOG) Mount Edgecombe bacterial diversity | | | | |
| Independent (grouping) variable: TREATMENT | | | | |
| Kruskal-Wallis test: H (3, N = 12) = 7.992 p = 0.046 | | | | |
| Depend: S | TF R:7.167 | TFo R:11.000 | BtoF R:3.500 | BtoFo R:4.333 |
| TF | | 1.000000 | 1.000000 | 1.000000 |
| TFo | 1.000000 | | 0.065076 | 0.141240 |
| BtoF | 1.000000 | 0.065076 | | 1.000000 |
| BtoFo | 1.000000 | 0.141240 | 1.000000 | |

Key: T = green cane harvested with trash retention; Bto = burnt cane with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

Catabolic evenness (J) increased in the order: BtoF < TF < TFo < BtoFo, but the non-parametric Kruskal-Wallis ANOVA showed that land treatments had no significant effect (p = 0.740) on soil bacterial community catabolic evenness (Appendix D, Table D8). Similarly, although trends for catabolic diversity (H') followed the same order as those for richness, the Kruskal-Wallis ANOVA showed that land management effects on the Shannon diversity index of the soil bacterial communities at Mount Edgecombe were not significant (p = 0.135) (Appendix D, Table D9).

The results of an RDA of the relationship between selected (non-collinear) soil physicochemical variables and the substrate utilization patterns of the Mount Edgecombe soil bacterial communities are shown in Figure 6.9. The RDA1 axis accounted for 43.1% of fitted and 12.5% of total variation along the axis, and the RDA2 axis for 36.9% of fitted and 10.6% of total variation in the data. Organic C and pH were correlated with the RDA1 axis, and ECEC with the RDA2 axis. All the soil variables had low inflation factors (< 10) so multicollinearity was not severe. Those treatments with retention of a trash blanket (T) were associated with higher organic C than burnt cane harvested managements (Bto). Fertilized plots (F) had a lower pH than unfertilized treatments (Fo), which were associated with higher pH and ECEC.

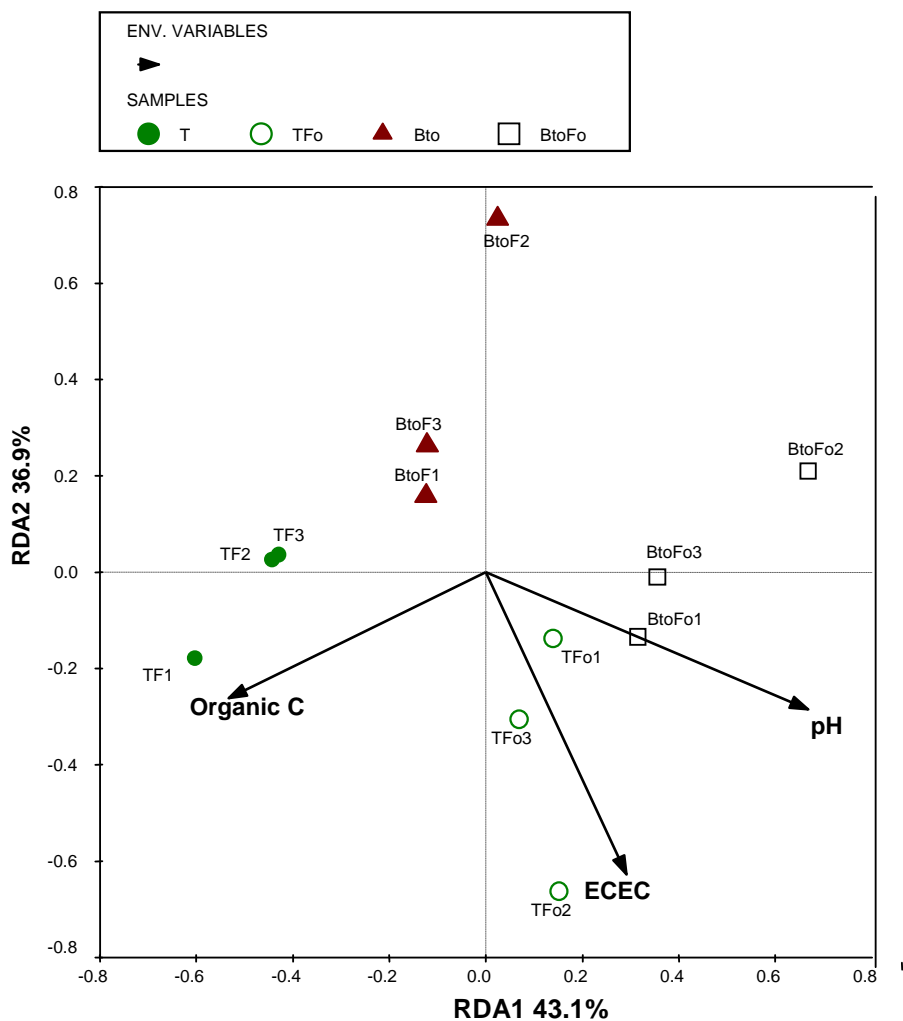


FIGURE 6.9 RDA ordination biplot of Mount Edgecombe bacterial BIOLOG data (log-transformed) using selected (non-collinear) soil variables, showing the relationship between the variables and the soil bacterial communities under the different land treatments.

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

Carbon sources having at least 25% of their variance accounted in the RDA ordination plot (Figure 6.9) are shown in Figure 6.10. The relationship between the substrates and the selected soil variables in Figure 6.9 is explained in the key below Figure 6.10.

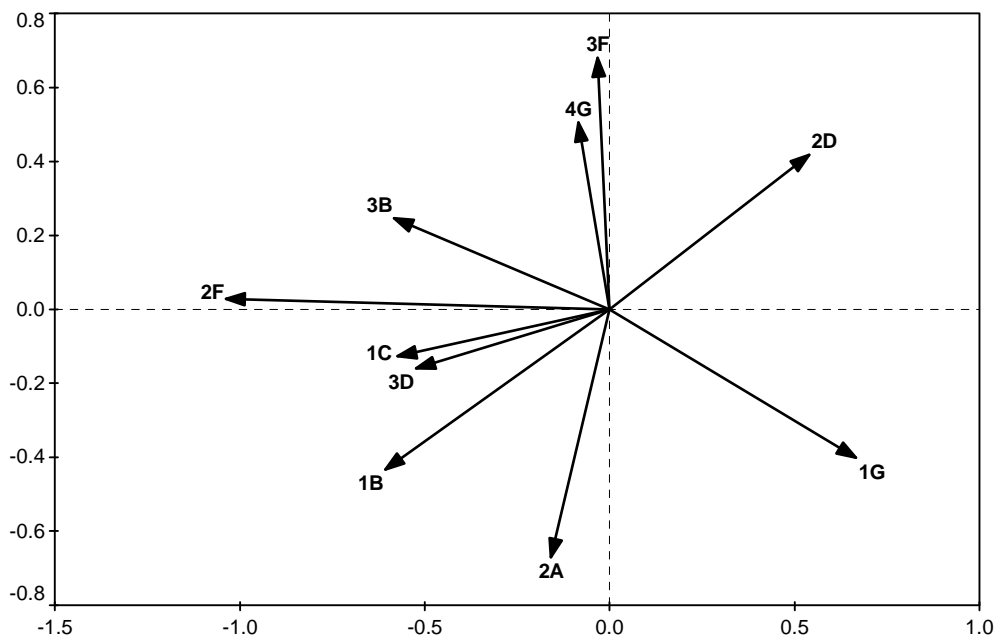


FIGURE 6.10 Substrates utilized by soil bacterial communities under the different land managements at Mount Edgecombe, with at least 25% of their variance accounted for in the ordination plot (Figure 6.9).

Key: 2D = D-mannitol, is associated with low soil organic C levels;
 1G = D-cellobiose, is correlated with a higher soil pH and ECEC levels;
 2A = β -methyl-D-glucoside, 1B = pyruvic acid methyl ester, 3D = 4-hydroxy benzoic acid,
 1C = Tween 40, are correlated with high levels of soil organic C;
 2F = D-glucosaminic acid, 3B = D-galacturonic acid, 4G = phenylethylamine, 3F = itaconic acid are associated with low soil pH and low levels of ECEC.

A Monte Carlo Permutation test for significant relationships within the RDA data showed that soil variables had no significant effect on bacterial community functional diversity at Mount Edgecombe (first canonical axis, $p = 0.708$; all canonical axes, $p = 0.338$). (Appendix D, Table D10)

6.4 DISCUSSION

In soil ecological studies, the concept of prokaryotic functional diversity has recently been proposed as an alternative to structural diversity determinations. Measurements of functional diversity may provide a more sensitive and meaningful approach to

detect the effects of changing environmental factors on soil prokaryotic communities (Tebbe and Schloter, 2007).

In this study, bacterial community-level catabolic profiling with BIOLOG EcoPlates was used to determine differences in the functional capabilities of the soil bacterial communities under different agricultural land uses and management practices. However, a disadvantage of the BIOLOG system is that it is questionable whether the inoculum is representative of the numbers and diversity of soil microbes present in the original sample (Ros *et al.*, 2008). This is because it is not possible to determine whether substrate utilization responses represent the selective enrichment of fast-growing microorganisms, or relative species numbers. Also, the efficiency of microbial cell release from soil is low (Konopka *et al.*, 1998). A further limitation is that changes in microbial community composition occur in the wells during incubation, therefore substrate utilization may not adequately reflect the capacity to utilize these substrates *in situ* (Smalla *et al.*, 1998). Hatzinger *et al.* (2003) studied the applicability of tetrazolium salts for measuring bacterial viability and respiratory activity in groundwater. They concluded that a reasonable overall estimate of viable cell numbers was possible but that certain bacterial strains might be falsely considered nonviable, due to poor uptake and reduction of the salts. Despite these shortcomings, BIOLOG assays are less time-consuming and less labour-intensive than other methods, as a large number of soil samples can be screened in a short time (Govaerts *et al.*, 2007). Zhang *et al.* (2008) reported that the BIOLOG microplate method was an effective tool in the assessment of soil microbial function and health. However, because of the limitations of BIOLOG CLPP's, Ros *et al.* (2008) suggested that complementary techniques should be used in conjunction with this approach. Accordingly, in the present study a combination of CLPP and PCR-DGGE was used to determine soil microbial diversity.

6.4.1 Bacterial catabolic (functional) diversity at Baynesfield Estate

BIOLOG analysis of catabolic diversity of the soil bacterial communities under the various land uses at this site showed substantial differences among the various

populations. This was confirmed by MRPP ($p = 0.005$), and agrees with the findings of Graham (2003) and Graham and Haynes (2005).

As indicated by AWCD, bacterial communities under kikuyu showed a much higher level of activity over time than those from the other land uses (Figure 6.2). This was attributed to the fact that the kikuyu pasture was the only land use at Baynesfield that had been irrigated at the time of sampling (Table 6.2). The importance of soil moisture in regulating microbial activity and diversity is well known, affecting the physiological status of bacteria and their ability to decompose organic substrates (Chen *et al.*, 2007). The bacteria-based channel of the detritus food web requires a water film for growth (Pankhurst, 1997; Haynes and Graham, 2004), therefore, as collection of the soil samples had taken place at the end of the dry season, the AWCD of the bacterial communities was considerably higher under kikuyu than under those land uses that had not been irrigated at the time of sampling. In addition, the greater dry matter production under improved (irrigated and fertilized) kikuyu pasture resulted in high levels of organic C inputs to the soil. This is reflected in the high organic C content of the soil under this land use (Table 5.1, section 5.3.1) and the strong association of the bacterial communities under kikuyu with soil organic C. This may also have contributed to the high level of heterotrophic bacterial activity observed. Below-ground energy channels with a strong bacterial component mainly occur in nutrient rich soils containing readily decomposable substrates that promote rapid growth, turnover of C and rapid nutrient cycling (Joergensen and Wichern, 2008). Increasing nutrient availability to plants often results in their producing higher amounts of organic matter with a lower C/N ratio which promotes bacterial biomass, as do management practices, e.g. fertilization, tillage and livestock grazing (Joergensen and Wichern, 2008). The PCA analysis of the bacterial communities showed that the subsamples under kikuyu clustered closely together, indicating their catabolic similarity (Figure 6.3). This agrees with the findings of Graham and Haynes (2005), that catabolic richness was lowest under kikuyu pasture at this site. In the present study, D-cellobiose, a degradation product of cellulose, was the substrate utilized, with at least 50% of its variance accounted along gradients of functional diversity (Figure 6.5).

In contrast to the kikuyu soil bacterial communities, those from native grassland soils, that had never been cultivated, irrigated or fertilized, had a lower cell density and activity level than any of the other land uses. This was largely attributed to the dry condition of the soil at the time of sampling. Bacterial community substrate utilization profiles of the replicate subsamples under native grassland were the most dissimilar from each other, as indicated by PCA. The most actively utilized substrates were 4-hydroxy benzoic acid, an intermediate in the breakdown of cellulosic compounds and D-xylose, a degradation product of hemicelluloses, which are commonly found in grasses (Alexander, 1977; Ratering *et al.*, 2007). Under native grassland, the observed metabolic differences in bacterial communities from the different subsamples may have reflected the more heterogeneous above-ground plant cover than that of the kikuyu monoculture. The greater diversity of plants may have produced a greater variety of C inputs to the soil and a greater biochemical diversity of root exudates, thus selecting for a more diverse soil microbial community to effect degradation (Chen *et al.*, 2007). The findings in the present study agree with those of Graham and Haynes (2005) who, using GN microplates, found greatest catabolic diversity under native grassland at Baynesfield. In the present study, PCR-DGGE analysis of soil bacterial community composition showed that communities under kikuyu were less structurally diverse than those under native grassland (section 3.4.1).

Soil bacterial communities under pine were shown by AWCD to be slightly more active than those under wattle. In the PCA biplot (Figure 6.3), soil bacterial communities from pine clustered together with those from wattle, indicating a low but very similar functional (catabolic) diversity. Both communities utilized D-galacturonic acid and pyruvic acid methyl ester, which are intermediates in the bacterial metabolism of plant structural components (Brock *et al.*, 1994). The MRPP analysis of this data confirmed that pine bacterial communities from the three subsamples were slightly more functionally similar to each other than were those under wattle. However under pine, the soil pH (3.8) was the lowest of all the land uses, which would tend to favour the fungal over the bacterial communities. RDA confirmed that a low pH was strongly associated with bacterial communities under pine but not under wattle (Figure 6.5). Pine litter breakdown into simple aromatic substances and, finally, into low molecular weight organic/humic acids (Alexander, 1977; Grinhut *et al.*, 2007; Ratering *et al.*, 2007), is a likely cause of the high acidity

observed in pine soil. Less litter was present on the soil surface under wattle than under pine, causing soil organic C levels to be lower. However, the pH was higher in wattle soils than in those of pine. As both these land uses are long-term monocultures, the bacterial communities are likely to have become adapted to a limited number of substrates, and are probably highly specialised, thus reducing functional diversity. This agrees with the findings of an earlier study in the KwaZulu-Natal midlands by Nsabimana *et al.* (2004), who concluded that greatest catabolic diversity occurred under native grassland, and lowest diversity under exotic forests.

Under maize (conventional tillage), soil bacterial communities were markedly less active than those under kikuyu, sugarcane, wattle or pine. This could partly be attributed to the sampling taking place at the end of winter, after the maize had been harvested and irrigation had ceased. The drier soil conditions would have had a negative impact on the soil bacteria. In addition, most of the crop residues had been removed from the soil surface after harvesting. Govaerts *et al.* (2008) reported that the effect of crop residue retention was more pronounced in maize than in wheat, as removal of residues was far more detrimental to the beneficial soil microflora populations under maize. Agronomic management of both maize stubble and N-fertilization were shown by Wakelin *et al.* (2007) to have a significant and long-term effect on the size and structure of the soil microbial communities, with stubble retention being the primary driver of change. At Baynesfield Estate, levels of organic C in the maize soil samples were low, due to the removal of most of the harvest residues and also to annual conventional tillage, which causes loss of organic C by increasing rates of C mineralisation (Li *et al.*, 2007). Improved soil health is often related to the retention of sufficient crop residues, particularly in maize production (Govaerts *et al.*, 2008). However, the bacterial communities from the different maize soil subsamples showed considerable differences in substrate utilization patterns, and were widely separated in the PCA biplot ordination space (Figure 6.3). The substrates utilized were mainly D-malic acid, D-xylose and γ hydroxybutyric acid, which are either intermediates or breakdown products in bacterial metabolism of plant material in soil (Figure 6.4). Govaerts *et al.* (2007) reported that tillage (zero or conventional tillage) and residue management (retention or removal) systems resulted in microbial communities of different catabolic diversity, but that the most utilized C-substrates would depend on local conditions and would differ from one system to another. The

RDA of selected soil physicochemical properties and their influence on the bacterial communities at this site showed that high levels of P and a higher soil pH were correlated with the maize soil bacteria (Figure 6.5). A PCA biplot of the relationship between land uses and soil properties (Figure 6.1) showed that high levels of P and K were associated with maize soils as a result of fertilizer applications.

The sugarcane soil bacterial communities, although less active than those under kikuyu, were slightly more active than those under the other land uses, but only marginally more active than those from pine soils. As climatic conditions at Baynesfield are sub-optimal for sugarcane cultivation, cane is harvested every two years and only replanted approximately every six years, whereas the maize is harvested and the soil tilled annually. This reduces soil disturbance under sugarcane and creates greater organic C inputs, which has led to a higher organic C content in sugarcane soils than under maize. Furthermore, as the sugarcane is irrigated during the winter months, the higher soil moisture content (14.8%) could also account for the greater bacterial community activity observed compared to that in the maize soils. PCA of bacterial community profiles clustered the subsamples on the basis of substrate utilization and showed there was greater similarity between the sugarcane soil communities than those of maize or native grassland. This was confirmed by the MRPP analysis. The most used substrates were 4-hydroxy benzoic acid and D-cellobiose, both of which are degradation products of cellulosic and hemicellulosic materials. The RDA analysis indicated that soil organic C was the main soil property influencing bacteria under sugarcane. Soil PCA (Figure 6.1) showed that sugarcane soils were associated with high levels of P and K, which were as a result of annual fertilizer applications. Graham and Haynes (2005), who used BIOLOG GN plates, also found lowest catabolic diversity under sugarcane.

Chen *et al.* (2007) reported significant differences in CLPP richness and evenness in response to different treatments and soil moisture content. Microbial functional diversity from BIOLOG data is usually quantified using two analytical methods, which was the approach used in this study. One procedure measures total activity such as AWCD (Garland and Mills, 1991; Garland, 1996) and the other (as used by Zak *et al.*, 1994), focuses on the number of different C sources utilized (substrate richness) together with the Shannon index (H'), which is a composite measure of both richness

and evenness. In this study, the results obtained in the Kruskal-Wallis ANOVA analysis of soil bacterial catabolic richness (S), evenness (J) and Shannon's diversity index (H') under the different land uses at Baynesfield Estate, showed that land use had not influenced any of these parameters. This was probably due to the low catabolic evenness observed at this site. Yao *et al.* (2006) also reported that, based on the Shannon index, calculations from their CLPP data set showed no significant change in population diversity due to land use (pine or turf).

6.4.2 Bacterial catabolic (functional) diversity at Mount Edgecombe

As previously noted in section 3.4.2, a major treatment effect at this site is an accumulation of organic matter in the surface soil under green cane harvesting with retention of crop residues, in contrast to a loss of organic matter after burning and trash removal. Another major effect is soil acidification under fertilizer applications. The PCA (Figure 6.7) of the various soils at Mount Edgecombe supported these earlier findings and showed that a high level of organic C was associated with those treatments retaining crop residues, and a high acidity and low pH with the fertilized soils. As may be expected, higher amounts of K and P were also associated with the fertilized managements. These differences were shown to be highly significant by MRPP ($T = -4.976$; $p < 0.001$). Application of P is an essential part of sugarcane fertilizer programmes, as P increases cane yield, sustains productivity and improves juice quality (Sundara *et al.*, 2002). After addition of soluble P to soil, it reacts rapidly with soil constituents to form less soluble or non-labile compounds, which, in acid soils (such as those at Mount Edgecombe) are likely to be Al and Fe phosphates. Sundara *et al.* (2002) suggested that fixed P reserves in soil could be made available to crops by means of appropriate land management involving the addition of organic matter and/or the use of P-solubilising microorganisms. Graham *et al.* (2002a) reported that changes in soil chemistry and aggregate stability were induced by fertilizer applications, sugarcane burning and trash retention at the Mount Edgecombe experimental site.

BIOLOG analysis of the functional diversity of the soil bacterial communities under the various land managements showed marked differences in catabolic capability.

Substrate utilization over time, expressed as AWCD, showed the highest level of bacterial community activity in sugarcane soils that had not been fertilized with N, P, and K (Figure 6.8). Ratering *et al.* (2007) reported that agricultural practices such as fertilization may initiate changes in soil microbial metabolism. In the present investigation, communities under unfertilized, trashed treatments (TFo) were the most active, followed by unfertilized, burnt cane treatments (BtoFo). In the fertilized managements, green cane harvested (TF) soil bacterial communities were more active than those from burnt cane (BtoF) soils, although neither of these communities was as active as those from unfertilized soils. This may indicate that while the absence of fertilizer was the main factor influencing bacterial activity, retention of crop residues within treatments, resulted in a more active community than burnt cane harvesting without trash retention. This could be attributed to greater amounts of available organic C under the former than under the latter management practice.

The PCA of substrate utilization patterns (Figure 6.9) indicated that the profiles of the three replicate bacterial communities under fertilized, burnt cane harvesting managements (BtoF) were the most widely separated (most variable) of the soil communities at Mount Edgecombe. This was confirmed by MRPP analysis. In this study, the amount of soil organic C was lowest under burnt cane treatments, with the primary organic matter returns being in the form of root turnover. These findings are similar to those of Graham and Haynes (2006). In the BIOLOG plates, the substrates utilized by these communities with at least 50% of their variance accounted along gradients of functional diversity, were Tween 40 (a surfactant) and D-mannitol, a sugar alcohol, many of which are metabolised by a wide range of bacteria (Figure 6.10). Under unfertilized, burnt cane managements (BtoFo), the profiles of two of the replicate bacterial communities (BtoFo1 and BtoFo3) clustered closely together in the ordination plot, indicating they were very similar, whereas the community under BtoFo2 was quite dissimilar and widely separated from the other two BtoFo replicates. N-acetyl-D-glucosamine, (a major component of bacterial cell walls) and D-cellobiose, a degradation product of cellulose, a major component of plant cell walls, were the substrates mainly utilized. As expected, soil bacterial community profiles from subsamples under fertilized, green cane harvested (TF) managements were spread more widely in biplot ordination space, indicating a greater dissimilarity between communities than among the unfertilized TFo communities. The latter were

more closely clustered together than those under TF, and had the most similar profiles of all the soil bacterial communities at this site. These results indicate that large differences in bacterial catabolic profiles exist within and between the various sugarcane soils, both under preharvest burning and under green cane harvesting with trash retention, and also that fertilization had influenced the bacterial communities. These findings are similar to those of Graham (2003) except this earlier study found fertilizer applications had little effect on catabolic diversity.

RDA of the relationship between non-collinear soil physicochemical variables and bacterial functional diversity at Mount Edgecombe, showed that treatments retaining a full trash cover were more closely correlated with organic C than those under burnt cane managements. In sugarcane production, burning the trash (dry leaves and tops) is a common practice, as it facilitates stalk harvest and transportation. However, burning reduces the amounts of organic matter and nutrients that are returned to the soil (Graham, 2003; Galdos *et al.*, 2009). In addition, burning of plant residues has raised environmental concerns regarding greenhouse gas emissions (e.g. CO₂, CH₄ and N₂O) and release of particulates into the atmosphere (Galdos *et al.*, 2009), hence the interest in green cane harvesting and the return of plant residues to the soil surface (Graham, 2003). Recent studies have shown that retention of cane residues affects both the physicochemical and biological soil properties, and influences soil temperature and water content (Galdos *et al.*, 2009). In the present study, RDA showed that unfertilized treatments, regardless of trash management practices, were associated with higher pH and ECEC than were the fertilized treatments. This analysis also showed that soil pH was lowest under the trashed, fertilized treatments. However, a Monte Carlo permutation test for significant relationships in the RDA data showed the selected soil variables had not had a significant effect on bacterial catabolic diversity at Mount Edgecombe.

Analyses of catabolic richness by the non-parametric Kruskal-Wallis ANOVA by Ranks and Median test (based on the number of substrates utilized) indicated that land treatments at Mount Edgecombe had a significant effect on soil bacterial community catabolic richness ($p = 0.04$). Trends for richness (BtoF < BtoFo < TF < TFo) showed that the most functionally rich bacterial communities were under green cane harvested, trashed treatments (T), followed by the burnt cane harvested treatments

(Bto). Again, the unfertilized plots (TFo and BtoFo) showed greater catabolic richness than the equivalent fertilized managements (TF and BtoF). These results were possibly due to the higher levels of organic C observed in plots retaining a full trash cover. Govaerts *et al.* (2007) reported that residue retention on the soil surface resulted in accumulation of organic C in the top soil, and provided microbial substrates of different quality and quantity. This affected soil C and nutrient cycling dynamics, and increased microbial abundance. As previously mentioned in section 3.4.2, fertilizer applications in long-term sugarcane monocultures are known to lower the soil pH, which would tend to favour fungi over bacteria under these managements. Soil acidification of fertilized plots is primarily attributed to nitrification of the annually applied fertilizer NH_4^+ (Graham, 2003). Fertilizers could also reduce functional diversity as, for example, applications of nitrogen fertilizers would tend to select against nitrogen-fixing bacteria in the soil (Demoling *et al.*, 2008). Papatheodorou *et al.* (2008) reported that lowest values of catabolic activity and diversity were recorded in fields under conventional cultivation, which they attributed in part, to the addition of nitrogen fertilizers. Wakelin *et al.* (2007) showed that nitrogen application had a significant effect on the total soil microbial community structure under maize, but that stubble retention was the strongest driver affecting species composition.

Trends for catabolic evenness in ascending order were BtoF < TF < TFo < BtoFo, the respective values for these managements being 4.7, 6.3, 7.3 and 7.7. This indicated that replicate soil bacterial communities under unfertilized treatments showed a greater catabolic evenness (distribution of colour in the microplate wells) than did those under fertilized managements, although the differences were not statistically significant. Trends for catabolic diversity, as calculated by the Shannon diversity index (H'), followed the same order as those for richness (BtoF < BtoFo < TF < TFo) and had values of 3.7, 5.3, 6.7 and 10.3, respectively. This indicates that, as expected, soil bacterial communities under green cane treatments retaining a trash cover (T) were more diverse than those under burnt cane harvesting with trash removed (Bto). This could again be as a result of the greater amounts of organic matter inputs to the soil due to plant residue retention on the soil surface (Graham, 2003; Govaerts *et al.*, 2007). In contrast, Papatheodorou *et al.* (2008) suggested that a correlation between organic C and microbial diversity exists only if land uses induce large differences in

soil organic C content. In the present study, unfertilized managements (Fo) were more diverse than the equivalent fertilized (F) managements. Soil acidification as a result of fertilization led to a lower pH, lower amounts of exchangeable Mg and Ca, and higher exchangeable acidity. In addition increased amounts of extractable P and K were observed. These significant differences in the soil chemical environment between fertilized and unfertilized managements could result in the observed differences in bacterial catabolic diversity. However, the differences in diversity (H') were not significant. Similarly, analysis of the PCR-DGGE data from this site using H' also showed no significant difference in the structural diversity of the soil bacterial communities, although this was due to a low evenness.

6.5 CONCLUSIONS

It is generally accepted that a combined approach is preferable when analysing microbial diversity in soil, as no single approach is without its drawbacks. Therefore, this study has the advantage of using both PCR-DGGE to determine the structural diversity, and CLPP (using BIOLOG EcoPlates) to determine the catabolic diversity, of the soil bacterial communities at two long-term experimental sites.

In order to determine the catabolic diversity of the bacterial communities under the different land uses (Baynesfield Estate) and sugarcane trash management practices (Mount Edgecombe), soil was re-sampled in 2008, four years after the initial sampling. This was considered acceptable as a comparison between the results generated by PCR-DGGE and CLPP was not being made; rather the aim was to determine whether or not the different land uses or managements had caused distinguishable structural and functional changes in the soil bacterial communities.

Considerable differences in catabolic capability were observed in the different soil bacterial communities under the various land uses at Baynesfield Estate and the sugarcane trash managements at Mount Edgecombe. A combination of factors such as long-term land use, tillage, irrigation, crop plants and soil physicochemical factors (including organic C and pH) were found to have had a considerable effect on the functional diversity of the soil bacterial communities at the former site. Land uses

under exotic plantations had bacterial populations that were functionally similar, whereas those under native grassland were markedly different. Levels of catabolic activity were highest under irrigated, fertilized kikuyu pasture and lowest under native grassland. At Mount Edgecombe, the main influences on bacterial functional diversity were the retention of plant residues, applications of inorganic fertilizers and soil pH. However, while the soils at this site were significantly different, the soil bacterial communities under the four managements were not, and were more similar to each other than those at Baynesfield.

The BIOLOG EcoPlates used for the CLPP analysis successfully differentiated the catabolic capabilities of the various bacterial communities at both sites, although it was noted that the communities selected for were probably those fast-growing microbes that could survive under the microplate conditions. However, the advantages of the BIOLOG method outweigh the disadvantages, as the procedure is simple, and large numbers of samples can be screened in a relatively short time. Although the Shannon Weaver diversity index (H') is a favoured approach among environmentalists for determining microbial diversity, as noted in the analysis of the PCR-DGGE data, the results were not entirely satisfactory. Despite visual assessment of the microplates, as well as data from the plate reader, showing large differences both in the degree of substrate utilization and in the variety and numbers of substrates used by the bacterial communities, the Shannon index indicated that these differences were not significant. As previously mentioned in section 3.2.8, neither the richness nor evenness parameter can be determined with certainty for prokaryotes in soil samples. Consequently, the Shannon index, which is a composite measure of both richness and evenness, cannot be determined accurately for these microorganisms either (Tebbe and Schloter, 2007). This suggests that the H' index is not ideal for the statistical analysis of bacterial BIOLOG data, even after log-transformation.

Chapter 7

EFFECTS OF LAND USE AND MANAGEMENT ON SOIL FUNGAL CATABOLIC DIVERSITY

7.1 INTRODUCTION

Agricultural land management practices such as tillage, organic soil amendments and crop rotation, are known to affect plant disease outbreaks caused by soil fungi. Their effects on soil pathogens are well documented but an understanding of their impact on soil fungal communities is vital if crop production systems that minimise agroecosystem disruption and control disease outbreaks are to be created (Wu *et al.*, 2007). Ecosystem function may be influenced by the impact of environmental change on fungal diversity, through decomposition of organic substrates (Deacon *et al.*, 2006). Decomposer (saprotrophic) fungi are primary, secondary and tertiary decomposers, feeding on and recycling large amounts of C and other nutrients in the ecosystem (Grinhut *et al.*, 2007). These fungi are sensitive to changes in the environment such as soil disturbance and pollution (Deacon *et al.*, 2006) although their response to these perturbations differs. In soils exposed to elevated CO₂ levels, a higher relative abundance of fungi and a higher level of soil C-degrading enzyme activity were observed (Grinhut *et al.*, 2007). In addition, Allison *et al.* (2007) reported that increased N availability in boreal ecosystems, due to anthropogenic disturbance, reduced fungal diversity and changed community structure.

Soil fungi are an important functional group in forestry and other terrestrial ecosystems. While they are integral in mediating nutrient cycling, litter and organic matter decomposition and bioremediation, as well as soil aggregate stabilisation, establishing their functional roles can be difficult (Hobbie *et al.*, 2003; Cullings *et al.*, 2005). Fungal communities can alter both spatially and temporally and replace one another, with each species adapted to a particular niche, including the utilization of specific substrates (Deacon *et al.*, 2006). However, as soil fungi can grow on a wide

variety of complex media *in vitro* and can utilize many different C sources, it is difficult to ascertain which of these are preferred. Community level physiological profiling (CLPP) using BIOLOG assays, which measure fungal community metabolic activity, can be used for this purpose (Hobbie *et al.*, 2003).

Accurate determination of the effects of disturbance on soil fungal communities requires a combination of genetic and physiological assessments (Cullings *et al.*, 2005). Therefore, as PCR-DGGE provides no information on microbial functional diversity and as very little is known about fungal catabolic diversity in South African soils, this study incorporated CLPP of soil fungal communities in addition to the genetic analysis. Buyer *et al.* (2001) described a method for fingerprinting fungal communities in environmental samples such as soil, the rhizosphere, the phylloplane and compost, using BIOLOG SF-N plates. They reported that the method was far less time-consuming and technically much simpler than either isolate identification or phylogenetic analysis, and provided a useful tool for analysing community structure and function. Therefore, soils under the different land uses and sugarcane trash managements at the two study sites were analysed using 96-well BIOLOG SF-N2 MicroPlates™ (section 2.4.1.4.1) which are suitable for fungal studies requiring a high degree of replication (Deacon *et al.*, 2006). As previously noted (section 6.1) CLPP requires fresh inoculum from soils (Wakelin *et al.*, 2007), therefore the subsamples used in this fungal analysis were taken from the same bulk samples (section 5.2.1) as those used for the bacterial CLPP.

7.2 MATERIALS AND METHODS

7.2.1 Study sites and soil sampling

The study sites are the same as described in section 3.2.1. Re-sampling of soils from both sites (0–5 cm depth) took place in August/September 2008, at the end of the dry winter season (section 5.2.1), and followed the same procedure described in section 3.2.2.

7.2.2 Chemical analysis

Chemical analysis of the fresh soil samples followed the methods described in section 3.2.3.

7.2.3 Soil moisture

Soil moisture content was calculated as described in section 6.2.3.

7.2.4 CLPP using BIOLOG microplates

Soil fungal communities were analysed by sole C source utilization profiles using a protocol adapted from the fingerprinting method of Buyer *et al.* (2001). The protocol was modified (described below) so that the plates could be inoculated using a multi-channel micropipettor. Moreover, as the 96-well BIOLOG SF-N plates used by Buyer *et al.* (2001) are no longer readily available, the more recently developed SF-N2 plates were used in the present study. These contain identical C sources to BIOLOG GN 2 plates (section 2.4.1.4.1), but lack the tetrazolium dye that is highly toxic to certain fungi. Substrates in the wells include polymers, carbohydrates, carboxylic acids, amines and amides, amino acids, and substrates classified as ‘miscellaneous’ (Carrera *et al.*, 2007) (Appendix E, Table E1). In this method, growth is measured turbidimetrically. Increasing optical density (OD) gives an indication of fungal growth and activity, whereas the number and categories of utilized substrates provide a data set from which to establish fungal metabolic diversity (Zak *et al.*, 1994; Buyer *et al.*, 2001).

Sieved, bulked, soil samples were stored in plastic bags for 8 days at 4°C before use, to allow time for any fungal spores present to germinate. Each soil subsample (10 g field-moist weight) from both sites was added to separate 500 ml Erlenmeyer flasks and diluted 1:10 in 90 ml sterile distilled ultrapure MilliQ water.

The following procedures were carried out at 10 minute intervals, thus avoiding the necessity of storing molten, antibiotic-supplemented agar in a waterbath for long

periods, which would have caused antibiotic degradation. Each flask containing a soil suspension, was shaken at room temperature for 10 minutes at 200 r.p.m, after which it was allowed to settle for 30 minutes. Immediately prior to inoculating each SF-N2 plate, the soil suspension was diluted to 10^{-3} by pipetting 600 μl of soil suspension into a bottle containing 36.4 ml of sterile, molten 0.2% water agar, to which three filter-sterilized antibiotics (1 ml $50 \mu\text{g ml}^{-1}$ gentamycin, 1 ml $100 \mu\text{g ml}^{-1}$ rifampicin and 1 ml $100 \mu\text{g ml}^{-1}$ streptomycin) had been added to prevent bacterial growth. The dilutions had the effect of standardising and reducing the initial inoculum density, and of removing any soil particles that could interfere with subsequent plate readings. Before inoculating a BIOLOG microplate, the contents of the bottle were thoroughly mixed and poured into a sterile glass Petri dish from which 100 μl aliquots were simultaneously added to each microplate well using an eight-channel Eppendorf micropipettor. Each soil suspension was inoculated into a separate 96-well plate, that is, three plates per land use or management. Any bubbles that had formed in the wells during pipetting were carefully removed before the agar set, as these would interfere with subsequent plate readings.

As soon as the agar had set, the microplates were read (time zero) at OD_{650} (Buyer *et al.*, 2001; Carrera *et al.*, 2007), using a Versamax tunable microplate reader. Thereafter, they were incubated at 22°C in the dark and checked visually for turbidity on a daily basis. Turbidity became apparent only after 72 hours. Accordingly, readings were taken at 72, 96 and 120 h. During the incubation period, after the readings had been taken, any wells which were very opaque or white were inoculated with 50 μl of a sterile 2% CuSO_4 solution. This inhibited further fungal growth, thus preventing hyphae from invading adjacent wells in the BIOLOG plates. Subsequent absorbance readings remained unchanged after the addition of the CuSO_4 solution, as the absorbance of these wells had already reached the upper limit of the plate reader (Buyer *et al.*, 2001).

7.2.5 Data analysis

Raw difference data for each soil subsample at each time point, were calculated by subtracting the OD_{650} reading of the control well (containing water) from the readings

obtained from the substrate-containing response wells (after Garland and Mills, 1991). As described in section 6.2.5, wells giving negative absorbance values after data normalization, were set to zero (Hu *et al.*, 2007; Liu *et al.*, 2007a; Weber *et al.*, 2007).

The same procedure detailed in section 6.2.5 was used to calculate the AWCD of each plate at each time point (Garland and Mills, 1991):

$$\text{AWCD} = \sum (C - R) / n$$

However, in this experiment, C is the increase in turbidity within each well, and $n = 95$ in the SF-N2 microplates (Garland and Mills, 1991; Buyer *et al.*, 2001). As described in section 6.2.5 standardized patterns rather than absolute values were compared (Garland and Mills, 1991).

Generally, a reference AWCD (threshold value) from the range 0–2.5 absorbance units for the majority of plate readers, is used for most samples. The choice of a reference AWCD is based on experience with given samples (O’Connell *et al.*, 2000). In the present study a threshold value of $\text{OD}_{650} = 0.25$ (after blanking against the control well) was considered positive. Therefore, wells giving readings of 0.25 or greater after 120 h incubation (the time at which most wells in all the microplates exceeded the threshold value) were chosen to compare BIOLOG patterns and for statistical analysis (Garland, 1996; Hu *et al.*, 2007; Plassart *et al.*, 2008). A single time point (120 h) was preferred to multiple time points for statistical analysis, as Buyer *et al.* (2001) had reported that better separation of data was achieved by using a single time point than by using combined time points.

7.2.6 Statistical analysis

Statistical analysis of BIOLOG substrate utilization patterns by the soil fungal communities followed the procedures previously described in section 6.2.6. Species catabolic richness (S) (the number of substrates utilized), evenness (J) (distribution of turbidity) and Shannon’s diversity index (H') were determined as described in section 6.2.6.

7.3 RESULTS

Variations in the physicochemical properties of the experimental soils may act as potential selection factors affecting both plants and soil fungal communities. Therefore, the relationships between selected soil variables and fungal communities under the different land uses and sugarcane residue managements, were investigated.

7.3.1 Analyses of soils at the Baynesfield experimental site

Soil analyses of the various land uses at Baynesfield were described previously in section 5.3.1.

7.3.2 Soil fungal catabolic (functional) diversity at Baynesfield

Fungal substrate utilization patterns in the BIOLOG SF-N2 plates were shown by plotting mean AWCD values at OD₆₅₀ over time, and increased in the order: KIK < NAT < M < W < SC < PF. In the microplate wells, fungal growth from all the land use soil subsamples was seen only after 72 h incubation (an initial lag period) and thereafter increased to different levels (Figure 7.1). The lag phase was longer than that observed for soil bacteria from the same site as fungi are generally slower growing. This had necessitated the addition of antibiotics to the water agar used for fungal microplate inoculation, to prevent bacterial growth in the microplate wells interfering with results. The highest AWCD value (level of activity) at 120 h was associated with fungal communities under PF. Fungal communities under KIK had the lowest value (Figure 7.1), in contrast to the KIK soil bacterial communities at this site, which were the most active (Figure 6.1).

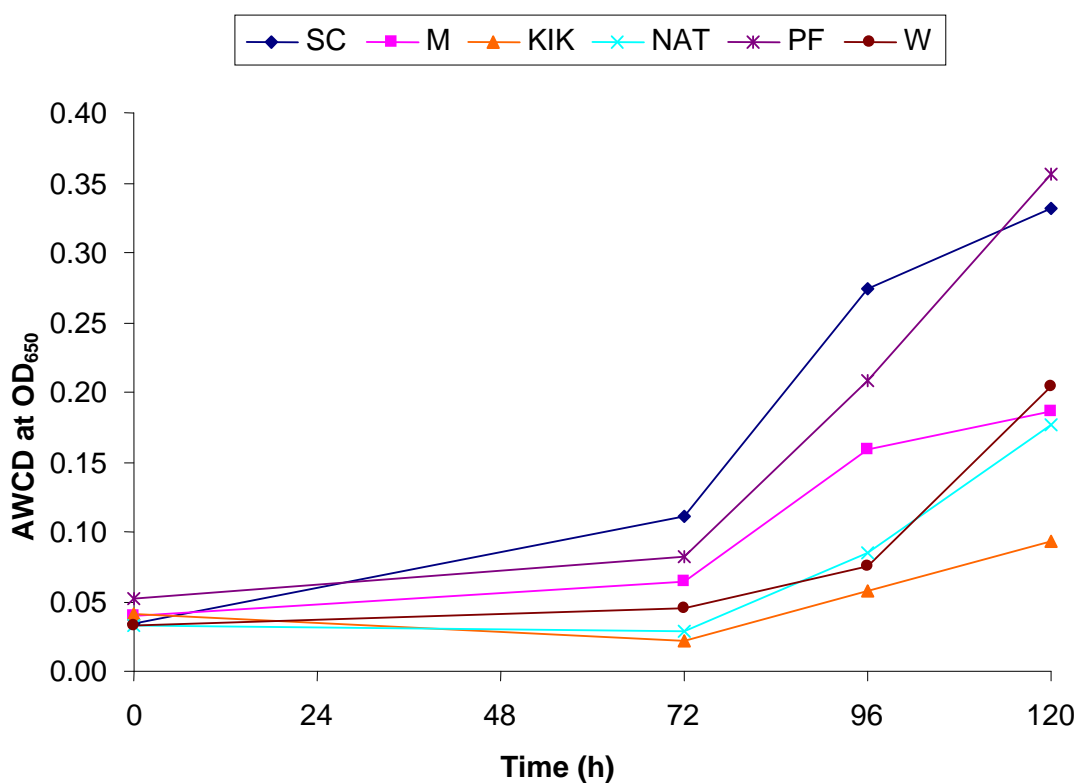


FIGURE 7.1 Variation over time in AWCD of Baynesfield soil fungal communities under the different land uses.

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = permanent kikuyu pasture; NAT = native, undisturbed grassland; PF = pine plantation; W = wattle plantation.

A PCA plot of log [X+1]-transformed data separated the various land use subsamples and shows differences in community substrate utilization profiles (after 120 h incubation) by the soil fungi from under the various land uses (Figure 7.2). The PC1 axis accounted for 21.7% and the PC2 axis for 15.0% (cumulatively, 36.7%) of the variance in fungal catabolic diversity, therefore these results should be interpreted with caution. Subsample catabolic profiles under KIK were most dissimilar, followed by W and M, whereas SC subsamples clustered together in the biplot, indicating their similarity. Profiles of PF also clustered closely together, whereas those under NAT were more widely separated, indicating their greater variability compared to PF.

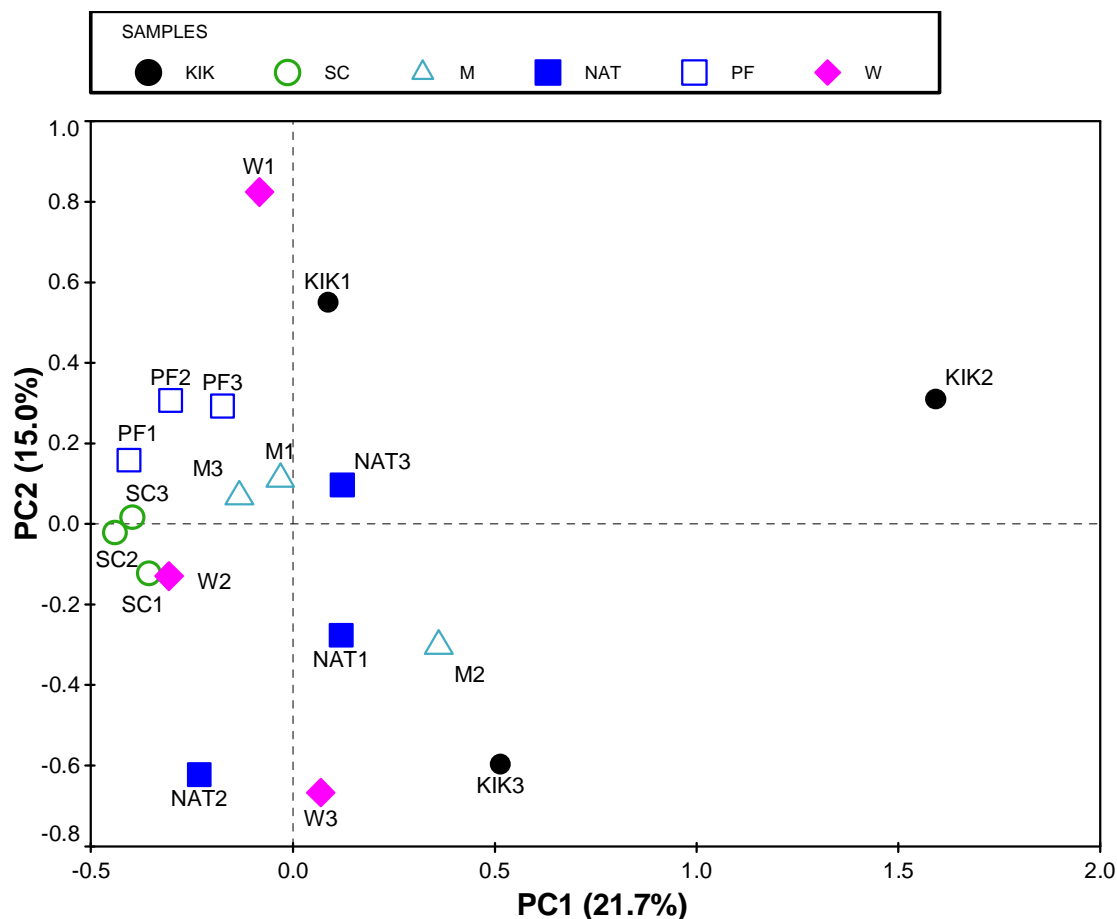


FIGURE 7.2 A PCA plot of samples (log [X+1]-transformed values) along gradients of fungal functional diversity for subsamples of fields with various land uses at Baynesfield.

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = permanent kikuyu pasture; NAT = native, undisturbed grassland; PF = pine plantation; W = wattle plantation.

Analysis of the PCA data (Figure 7.2) by MRPP, confirmed that land use had affected the overall functional (catabolic) diversity of the soil fungal communities at Baynesfield (Appendix E, Table E2).

A PCA plot of those C sources in the BIOLOG SF-N2 plates with at least 50% of their variance accounted, along the gradients of fungal functional diversity (Figure 7.2) at Baynesfield, is shown in Figure 7.3. All substrates in the SF-N2 plates are shown in Appendix E, Table E1 and Figure E1.

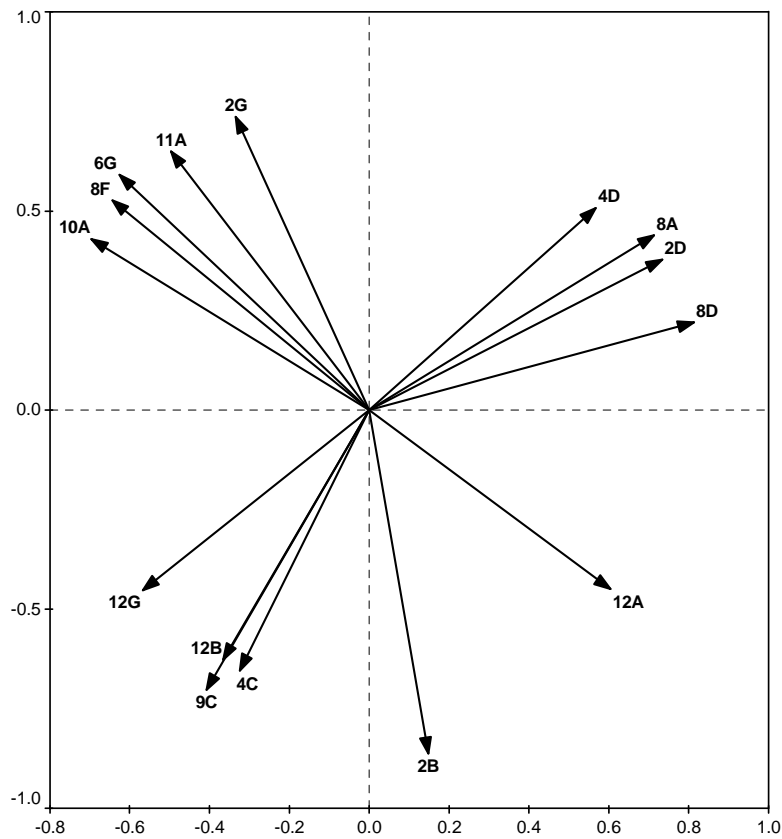


FIGURE 7.3 A PCA plot of C sources with at least 50% of their variance accounted, along gradients of fungal functional diversity at Baynesfield.

Key: 4D = formic acid; 8A = n-acetyl-D-glucosamine; 2D = cis aconitic acid; 8D = D-glucosaminic acid; 12A = D-cellobiose; 2B = D-fructose; 4C = D-raffinose; 9C = turanose; 12B = D-mannose; 12G = γ -aminobutyric acid; 10A = L-arabinose; 8F = L-asparagine; 6G = L-proline; 11A = D-arabitol; 2G = hydroxy-L-proline.

Trends for fungal catabolic richness increased in the order: KIK < W < NAT \leq M < SC < PF and values were 3.33, 6.5, 9.3, 9.5, 12.67 and 15.67 respectively. The non-parametric Kruskal-Wallis ANOVA by Ranks and Median test, showed that as with the soil bacterial communities, land use had not influenced fungal community richness (S) at this site (Appendix E, Table E3).

The trends for soil fungal community catabolic evenness (J) (distribution of turbidity) under the different land uses were: KIK = NAT < M < W < PF < SC. The Kruskal-Wallis ANOVA showed the different land uses had influenced catabolic evenness (Table 7.1).

TABLE 7.1 A non-parametric one-way analysis (Kruskal-Wallis test) of variation in catabolic evenness (J) (turbidity distribution) of soil fungal communities under different land uses at Baynesfield Estate

| Species evenness (J) | | Significant (p = 0.037) | | | | |
|---|---------|--------------------------------|---------|----------|----------|---------|
| Multiple comparisons p values (2-tailed); J (BIOLOG) Baynesfield fungal diversity | | | | | | |
| Independent (grouping) variable: LAND USE | | | | | | |
| Kruskal-Wallis test: H (5, N = 18) = 11.830 p = 0.037 | | | | | | |
| Depend: | KIK | M | NAT | PF | SC | W |
| J | R:5.000 | R:7.000 | R:5.000 | R:14.333 | R16.000: | R:9.667 |
| KIK | | 1.000 | 1.000 | 0.484 | 0.174 | 1.000 |
| M | 1.000 | | 1.000 | 1.000 | 0.584 | 1.000 |
| NAT | 1.000 | 1.000 | | 0.484 | 0.174 | 1.000 |
| PF | 0.484 | 1.000 | 0.484 | | 1.000 | 1.000 |
| SC | 0.174 | 0.584 | 0.174 | 1.000 | | 1.000 |
| W | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | |

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = permanent kikuyu pasture; NAT = native, undisturbed grassland; PF = pine plantation; W = wattle plantation.

Functional diversity trends followed the order: KIK < NAT = W < M < SC < PF. The non-parametric Kruskal-Wallis ANOVA showed that land use had affected the Shannon Weaver diversity index (H') (catabolic diversity) of the soil fungal communities (Table 7.2). This was in contrast to the bacterial communities at this site.

TABLE 7.2 A non-parametric one-way analysis (Kruskal-Wallis test) of variation in catabolic diversity (H') of soil fungal communities (a composite of substrate richness and evenness) under different land uses at Baynesfield Estate

| Shannon index (H') | | Significant (p = 0.028) | | | | |
|---|---------|--------------------------------|---------|----------|---------|---------|
| Multiple comparisons p values (2-tailed); H (BIOLOG) Baynesfield fungal diversity | | | | | | |
| Independent (grouping) variable: LAND USE | | | | | | |
| Kruskal-Wallis test: H (5, N = 18) = 12.509 p = 0.028 | | | | | | |
| Depend: | KIK | M | NAT | PF | SC | W |
| H | R:3.333 | R:8.333 | R:7.333 | R:16.333 | R14.333 | R:7.333 |
| KIK | | 1.000 | 1.000 | 0.043 | 0.174 | 1.000 |
| M | 1.000 | | 1.000 | 0.997 | 1.000 | 1.000 |
| NAT | 1.000 | 1.000 | | 0.584 | 1.000 | 1.000 |
| PF | 0.043 | 0.997 | 0.584 | | 1.000 | 0.584 |
| SC | 0.174 | 1.000 | 1.000 | 1.000 | | 1.000 |
| W | 1.000 | 1.000 | 1.000 | 0.584 | 1.000 | |

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = permanent kikuyu pasture; NAT = native, undisturbed grassland; PF = pine plantation; W = wattle plantation.

RDA results, showing the relationship between selected (non-collinear) soil physicochemical variables and soil fungal community substrate utilization patterns at Baynesfield Estate, are given in Figure 7.4. The RDA1 axis accounted for 44.9% of fitted, and 15.3% of total variation in data, and the RDA2 axis for 25.4% of fitted and 8.6% of total variation in the data. ECEC, P and organic C were aligned with the RDA1 axis, while pH was associated with the RDA2 axis.

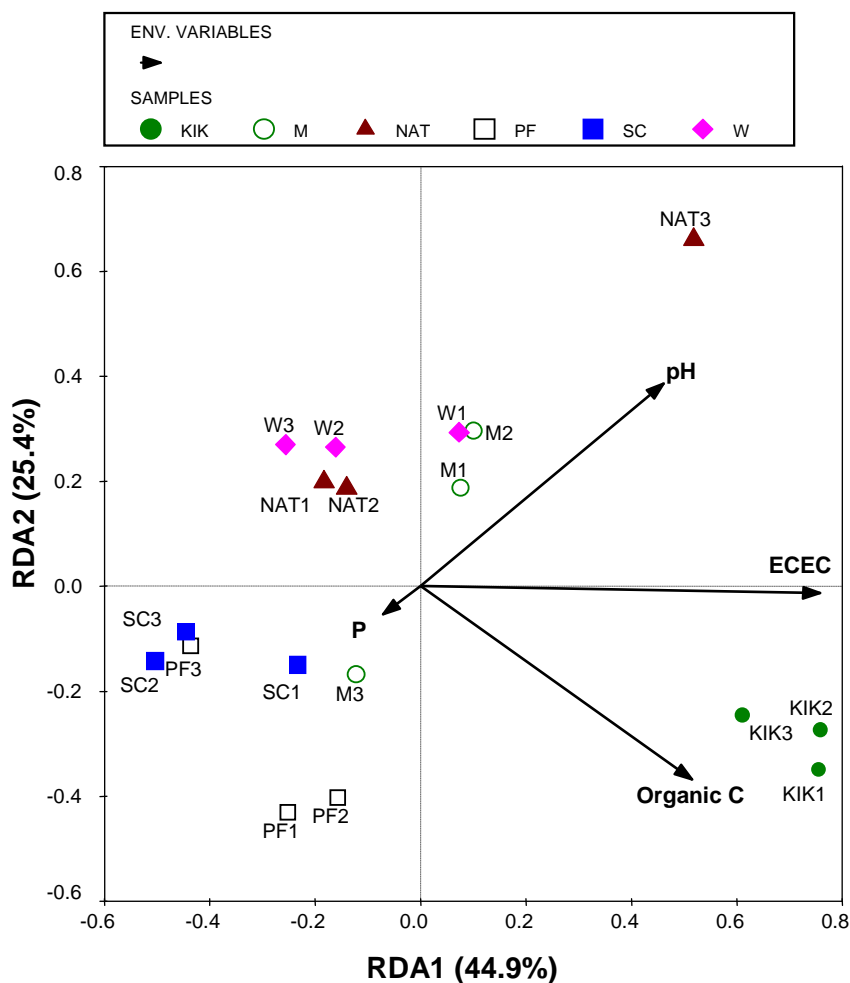


FIGURE 7.4 RDA ordination biplot of Baynesfield fungal BIOLOG data (log-transformed) using selected (non-collinear) soil variables, showing the relationship between the variables and the soil fungal communities under the different land uses.

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = permanent kikuyu pasture; NAT = native, undisturbed grassland; PF = pine plantation; W = wattle plantation.

Substrates (C sources) having at least 25% of their variance accounted in the RDA ordination plot (Figure 7.4) are shown in Figure 7.5. The relationship between the substrates and the selected soil variables is summarised in the key below Figure 7.5.

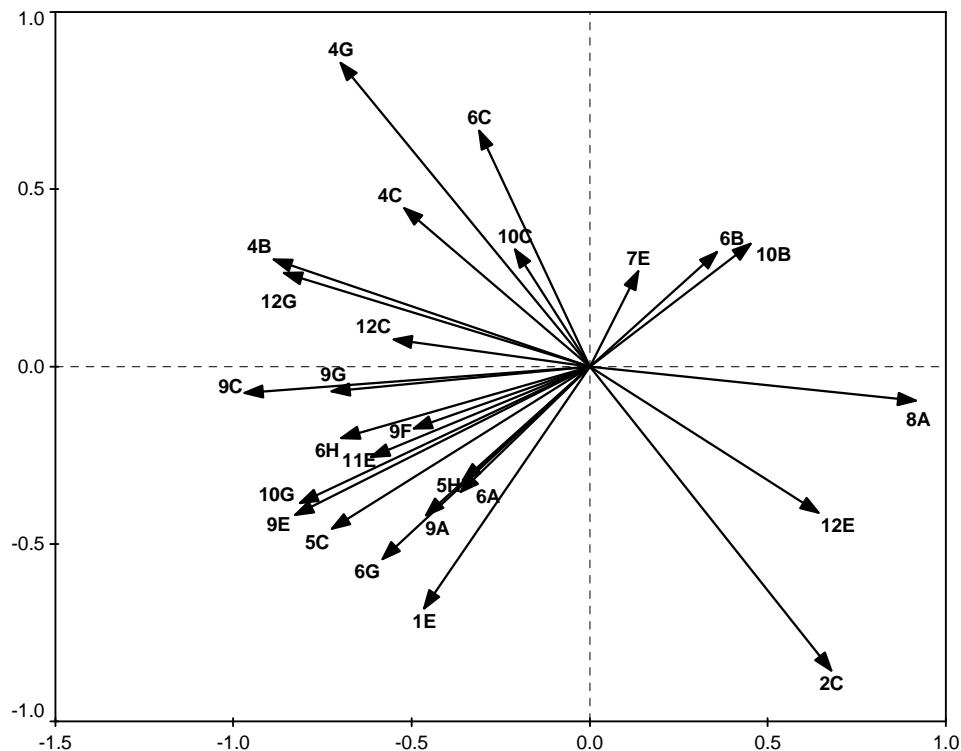


FIGURE 7.5 C sources utilized by soil fungal communities under the different land uses at Baynesfield, with at least 25% of their variance accounted in the ordination RDA plot (Figure 7.4).

Key: 7E = malonic acid, 6B = α -D-glucose, 10B = maltose, are closely associated with higher pH;

8A = N-acetyl-D-glucosamine, is closely correlated with high ECEC;

12E = succinic acid, 2C = β -methyl-D-glucoside, are closely associated with high organic C;

1E = p -hydroxy-phenylacetic acid, 6A = Tween 80, 6G = L-proline, 9A = adonitol, 5H = phenylethylamine, 5C = L-rhamnose, 9E = quinic acid, 10G = L-threonine, 11E = sebacic acid, 9F = L-aspartic acid, 6H = putrescine, 9G = L-serine, 9C = turanose, are associated with P and low pH;

12C = succinic acid monomethyl ester, 12G = γ -aminobutyric acid, 4B = D-galactose, 4C = D-raffinose, 4G = L-ornithine, 10C = xylitol, 6C = D-sorbitol are correlated with low organic C.

The Monte Carlo Permutation test for significant relationships in the RDA data showed that soil variables had affected fungal catabolic diversity at this site (Appendix E, Table E4).

7.3.3 Analyses of soils at the Mount Edgecombe site

The analyses of soils at this site are presented in detail in section 5.3.4.

7.3.4 Soil fungal catabolic (functional) diversity at Mount Edgecombe

Patterns of substrate utilization in BIOLOG SF-N2 plates by the soil fungal communities under the different land managements at Mount Edgecombe (as shown by mean AWCD values at OD₆₅₀, plotted over time) followed the order: BtoF < BtoFo < TFo < TF. After the initial 72 hour lag period, AWCD values of the soil fungal communities from under the four land management regimes increased to different levels. Higher values were recorded for treatments retaining a trash blanket (T) than for those with harvest residues removed (Bto) (Figure 7.6). As with the Baynesfield microplates, antibiotics were added to the water agar to inhibit the faster-growing bacteria and prevent them from interfering with the plate readings.

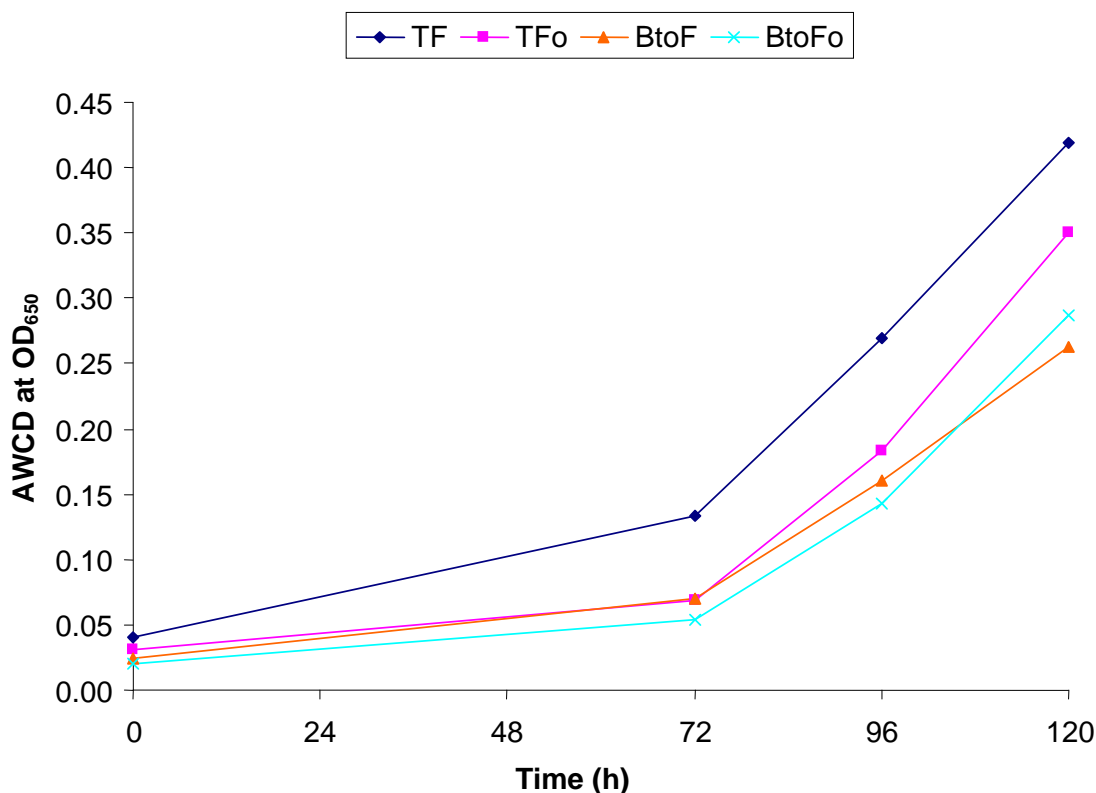


FIGURE 7.6 Variation over time in AWCD of Mount Edgecombe soil fungal communities under four different sugarcane residue treatments.

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

A PCA plot of log [X+1]-transformed data showed differences in fungal substrate utilization profiles (after 120 h incubation) by the soil fungal communities under the various sugarcane trash management regimes. PC1 accounted for 21.1%, and PC2 for 15.1% (cumulatively, 36.2%) of the variance in fungal functional diversity at this site (Figure 7.7). Soil subsamples from the green cane harvested with trash retention managements (T) were more closely correlated (av. distance 0.31) than the burnt cane harvested managements (Bto) (av. distance 0.38), with the fertilized (F) subsamples being more similar to each other than the unfertilized (Fo) samples under both trash managements.

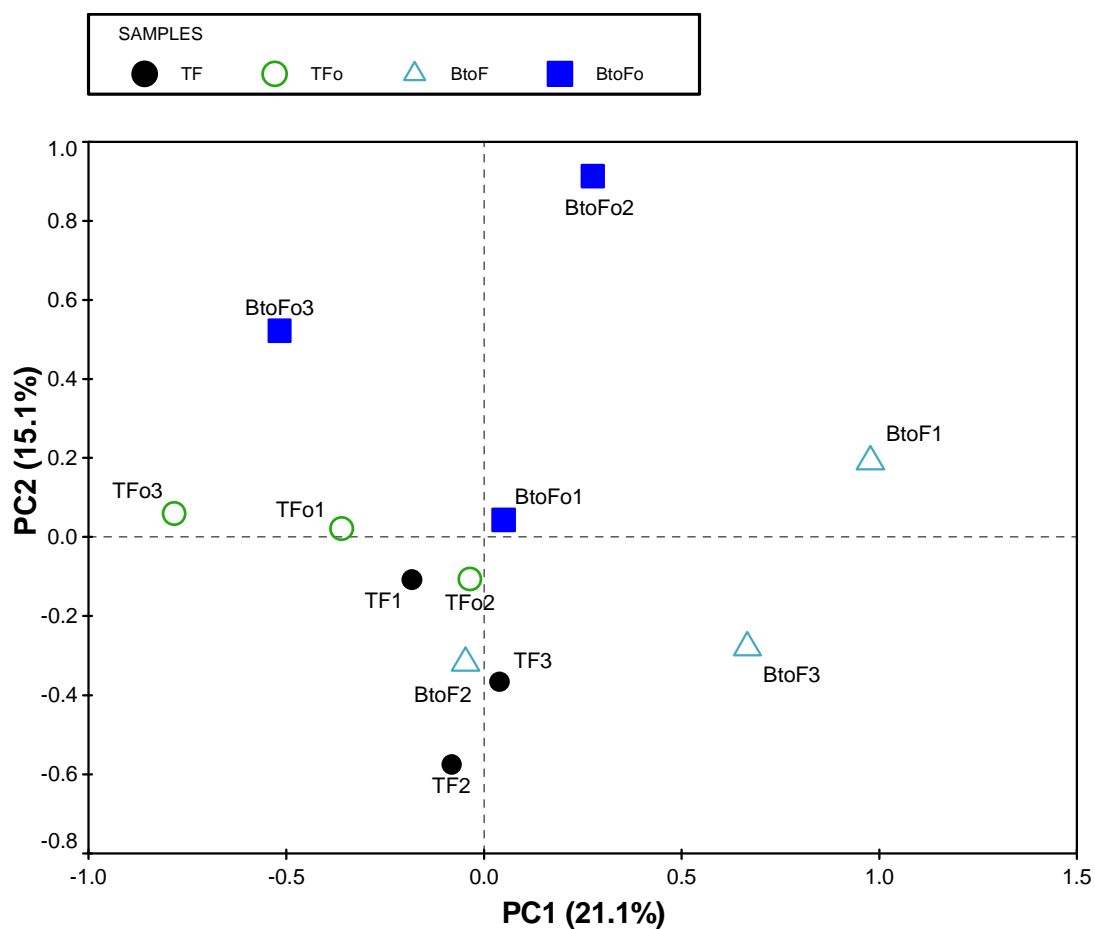


FIGURE 7.7 A PCA plot of samples (log [X+1]-transformed values) along gradients of fungal functional diversity, for subsamples of fields with various land managements at Mount Edgecombe.

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

Log [X+1]-transformed PCA data (Figure 7.7) tested by MRPP, indicated that the four experimental treatments had a small but significant overall effect on fungal catabolic diversity at this site ($T = -1.896$; $p = 0.038$), (Appendix E, Table E5).

Figure 7.8 is a PCA biplot of those C sources (substrates) with at least 50% of their variance accounted along gradients of fungal functional diversity at Mount Edgecombe (Figure 7.7). (Appendix E, Figure E shows all C sources along the gradients of fungal functional diversity in Figure 7.7).

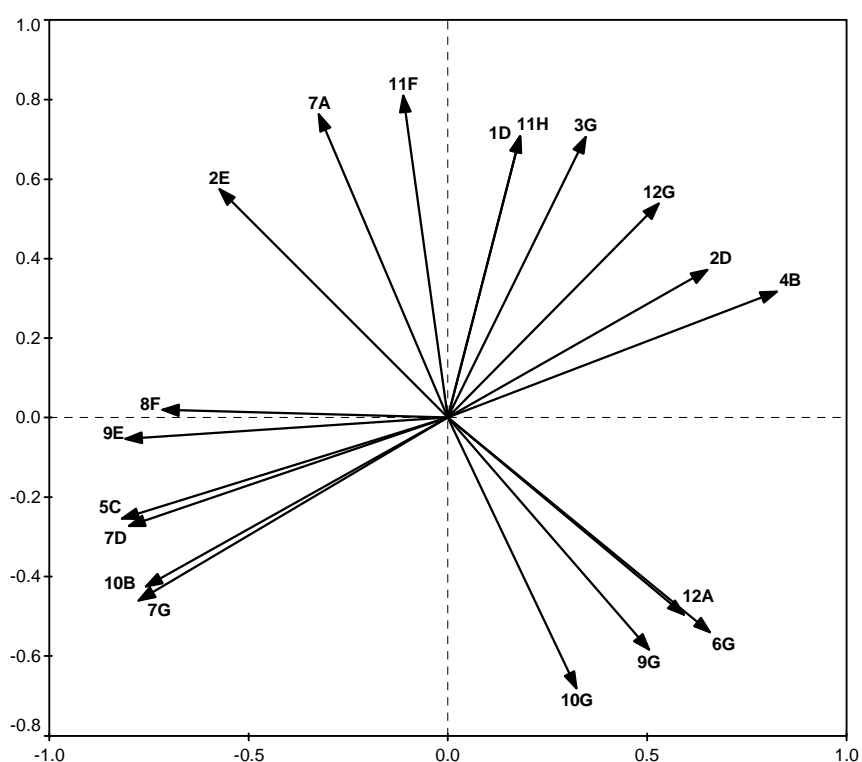


FIGURE 7.8 A PCA plot of C sources with at least 50% of their variance accounted, along gradients of fungal functional diversity at Mount Edgecombe (Figure 7.7).

Key: 1D = acetic acid; 11H = α -D-glucose-1-phosphate; 3G = L-leucine; 12G = γ -aminobutyric acid; 2D = cis-aconitic acid; 4B = D-galactose; 6G = L-proline; 12A = D-cellobiose; 9G = L-serine; 10G = L-threonine; 7G = L-pyroglutamic acid; 10B = maltose; 7D = D-gluconic acid; 5C = L-rhamnose; 9E = quinic acid; 8F = L-asparagine; 2E = itaconic acid; 7A = N-acetyl-D-galactosamine; 11F = glycyl-L-aspartic acid.

The trends for richness were: BtoF < TFo < BtoFo < TF, with values of 4.8, 5.8, 7.5 and 7.8, respectively. The trends for evenness were BtoF < BtoFo < TFo < TF, with values of 4.7, 5.3, 7.7 and 8.3, respectively. The trends for diversity followed the same order as those for richness although values were 5.0, 6.3, 7.0 and 7.7, respectively. The non-parametric Kruskal-Wallis ANOVA showed that land managements at Mount Edgecombe had no significant effect on the catabolic richness (S) ($p = 0.706$), evenness (J) ($p = 0.536$) or the Shannon Weaver diversity index (H') ($p = 0.826$) of the soil fungal communities (Appendix E, Tables E6, E7, and E8).

The results of an RDA analysis of the relationships between selected (non-collinear) soil physicochemical variables and the soil fungal community substrate utilization patterns at Mount Edgecombe, are shown in Figure 7.9. All soil variables have low inflation factors (< 10) so multicollinearity is not severe. RDA1 accounted for 47.1% of fitted and 15.3% of total variation, and RDA2 for 35.8% of fitted and 11.7% of total variation. ECEC were more closely aligned with RDA1, whereas pH and organic C were more closely aligned with RDA2. Those treatments retaining a trash blanket (T) were associated with a higher organic C content than burnt cane harvested with plant residues removed (Bto). Fertilized plots (F) were correlated with a lower pH than unfertilized plots (Fo), and the latter were associated with higher ECEC.

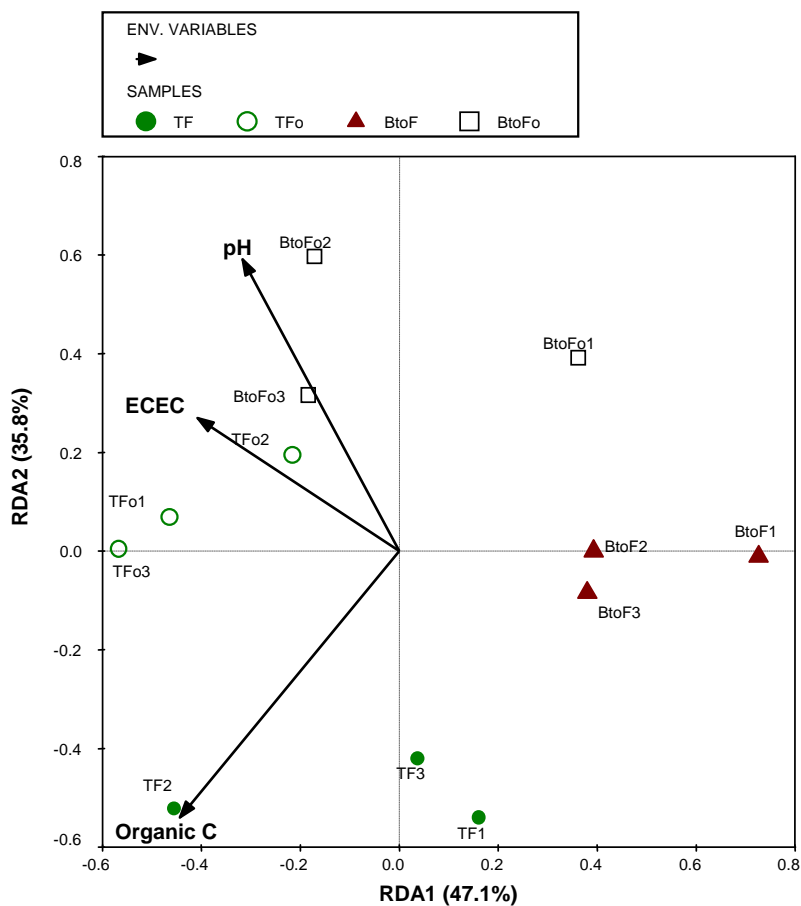


FIGURE 7.9 RDA ordination biplot of Mount Edgecombe fungal BIOLOG data (log-transformed) using selected (non-collinear) soil variables, showing the relationship between the variables and the soil fungal communities under the different land managements.

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

Carbon sources having at least 25% of their variance accounted in the RDA ordination plot (Figure 7.9) are shown in Figure 7.10. The relationships between the substrates and the selected soil variables are summarised in the key below Figure 7.10.

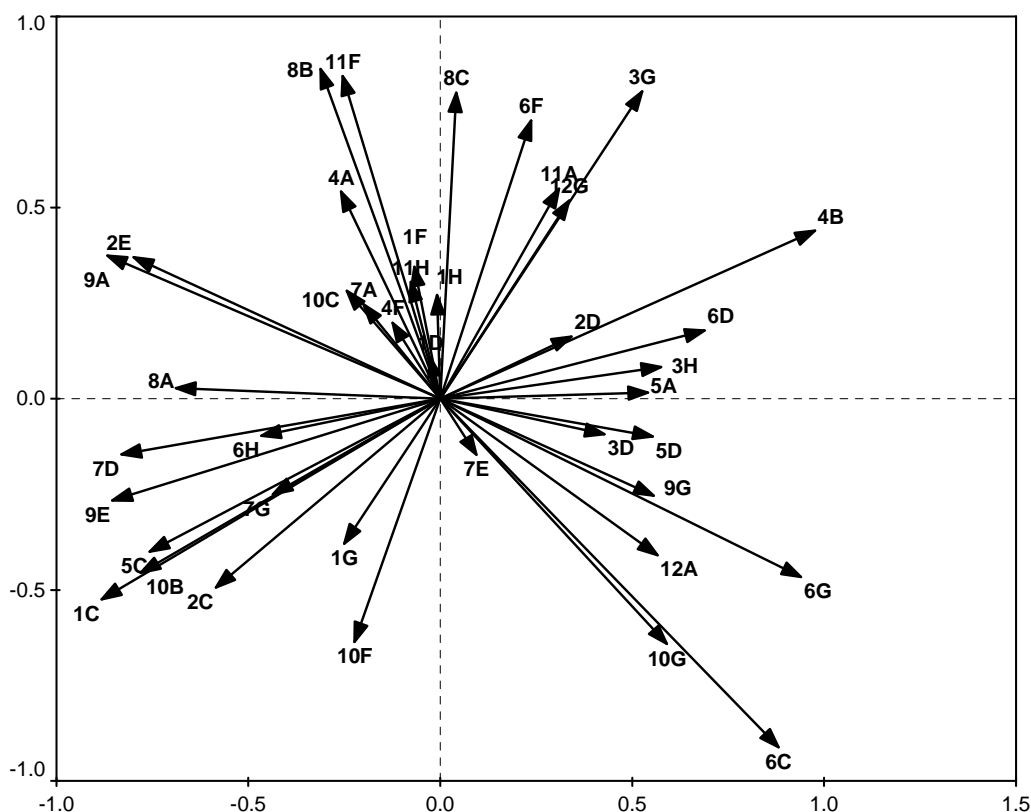


FIGURE 7.10 C sources utilized by soil fungal communities under the different land managements at Mount Edgecombe, with at least 25% of their variance accounted for in the ordination plot (Figure 7.9).

Key: 8C = D-trehalose, 6F = L-alanine, 11A = D-arabitol, 3G = L-leucine, 12G = γ -aminobutyric acid, 2D = cis-aconitic acid, 4B = D-galactose, 6D = D-galacturonic acid, 3H = uridine, 5A = Tween 40, are associated with low soil organic C levels;
 5D = D-galactonic acid lactone, 3D = citric acid, 9G = L-serine, 6G = L-proline, 12A = D-cellobiose, 6C = D-sorbitol, 10G = L-threonine, 7E = malonic acid, are correlated with low soil pH and ECEC;
 10F = L-glutamic acid, 1G = L-histidine, 2C = β -methyl-D-glucoside, 1C = D-melibiose, 10B = maltose, 7G = L-pyroglutamic acid, 5C = L-rhamnose, 9E = quinic acid, 6H = putrescine, 7D = D-gluconic acid, are associated with high soil organic C levels;
 8A = N-acetyl-D-glucosamine, 9A = adonitol, 2E = itaconic acid, 10C = xylitol, 7A = N-acetyl-D-galactosamine, 4F = L-alaninamide, 4A = glycogen, 8B = α -D-lactose, 11F = glycyl-L-aspartic acid, 1F = bromosuccinic acid, 11H = α -D-glucose-1-phosphate, 1H = urocanic acid, are correlated with a higher soil pH and ECEC.

A Monte Carlo Permutation test for significant relationships of fungal RDA data from Mount Edgecombe showed a near significant relationship between the selected soil variables and the soil fungal communities (first canonical axis, $p = 0.062$ and all canonical axes, $p = 0.060$) (Appendix E, Table E9).

7.4 DISCUSSION

It is important to target soil organic matter (SOM) as part of soil management, as it affects the physicochemical and biological properties as well as the long-term productivity of soil (Bausenwein *et al.*, 2008). It is, therefore, essential to elucidate the role of microbes in the degradation and transformation of SOM, which is the main reservoir of C in the biosphere, estimated to be 1600×10^{15} g C. In particular, the fungal contribution to these processes should be evaluated, as this is still not well understood (Grinhut *et al.*, 2007).

Changes in below-ground C allocations as a result of above-ground disturbances may change both the structural and physiological diversity of soil fungal communities (Cullings *et al.*, 2005). Therefore, in the present study, soil fungal communities under different agricultural land uses and managements in KwaZulu-Natal, were characterised in order to determine how these agronomic disturbances had affected the functional diversity of this class of soil microorganisms. Absorbance (OD) values from the BIOLOG plates were used to assess the relative preference of different soil fungi for specific compounds (Hobbie *et al.*, 2003), notwithstanding the fact that it is difficult to relate each substrate to specific soil functions or processes (Marchante *et al.*, 2008a). As previously noted in section 6.4, BIOLOG assays have certain limitations. In the case of fungi, an additional shortcoming is that basidiomycetes may not successfully be screened using this method, as they do not grow well after their mycelia have been fragmented (Deacon *et al.*, 2006).

7.4.1 Fungal catabolic (functional) diversity at Baynesfield Estate

As the soils for which functional diversity of the fungal communities was measured had been subjected to a wide range of different long-term land uses (arable agriculture, pastures and exotic plantations), considerable differences in their catabolic capabilities were shown by PCA. Ratering *et al.* (2007) reported that factors affecting soil microbial metabolic diversity include: climate (rainfall and temperature), soils (aeration and texture), the availability and degradability of substrates, and agricultural practices (e.g. pesticide and fertilizer applications or

cultivation methods). Also, plants can change the soil physicochemical environment, (for example by rhizodeposition) and can regulate net decomposition rates by directly affecting litter quality (Trinder *et al.*, 2009).

Substrate utilization patterns after 120 hours incubation indicated that the soil fungi under pine were the most active of all the land use communities at this site (Figure 7.1). In addition, these fungal communities had the greatest catabolic richness and diversity of all the land uses. As shown by PCA (Figure 5.1), pine soils were strongly correlated with high acidity and low pH (3.8). RDA (Figure 7.4) of the effects of selected soil physicochemical variables on the resident fungal communities confirmed that a low pH but relatively high P levels, were the properties most closely associated with pine. As many soils in forest ecosystems are acidic and receive recalcitrant litter inputs, conditions will tend to favour fungi over bacteria (Allison *et al.*, 2007). In acid soils, cellulose degradation is mainly performed by filamentous fungi (Alexander, 1977). In the organic layers of forest and woodland soils, in decomposing litter, fungal hyphal networks dominate the microbial protoplasm (Joergensen and Wichern, 2008).

In the present study, the soil surface in the pine plantation was covered by a thick layer of decaying pine needles, which are rich in phenolic and lignocellulosic compounds. In the upper layers of soils, the primary lignocellulose-degrading fungi, which occur widely in soil or in plant litter, are saprotrophs. These actively decompose humic substances, and include ascomycetes and basidiomycetes. The former are mainly involved in modifying and polymerising humic substances, whereas the latter degrade and mineralize refractory organic matter (Grinhut *et al.*, 2007). In addition, the level of soil organic C under pine was 6.3%, which was higher than all the other land use soils except that under kikuyu. As fungi are heterotrophic, their distribution is determined by the availability of oxidizable carbonaceous substrates (Alexander, 1977), with the quality and quantity of available organic matter an important influence on their activity, community structure and function (Bending *et al.*, 2002). These factors could all have contributed to the greater activity and functional diversity of the soil fungi under pine compared with those under the other land use types (section 6.3.2). As conditions under pine favoured soil fungal communities, the soil bacteria were unable to compete successfully for available

nutrients, which could account for the observed lower bacterial functional activity and diversity under this land use. Replicate subsamples of the pine soil fungal communities were closely clustered by PCA, reflecting the similarity in their substrate utilization profiles. This indicated that the soil fungi under pine were functionally similar, probably highly specialized, and that an adapted microbial community, dominated by fungi, had developed. Substrates in the BIOLOG microplates utilized by these communities, with at least 50% of their variance accounted along gradients of functional diversity, were the amide (L-asparagine), the amino acid (L-proline) and the carbohydrates (L-arabinose and D-arabitol). The active use by the fungal community of the pentose arabinose, and its sugar alcohol, arabitol, is interesting. These substrates were not utilized by the pine bacterial communities as not many bacteria can metabolise pentose sugars (Brock *et al.*, 1994).

In the wattle (*Acacia mearnsii*) plantation (an exotic N₂-fixing monoculture), the litter on the soil surface was much less dense than that under pine, consisting of fallen leaves, twigs and bark, together with rotting tree stumps from a eucalyptus plantation, formerly situated on this site. The N-rich wattle litter may thus have contained a mixture of substrates, including tannins and polyphenols. Little is known of the fate of litter tannins except that a large and rapid decline occurs ($\pm 80\%$) during the first year of decomposition (Bradley *et al.*, 2000). Polyphenols contained within wattle form complexes with proteins, those with the highest molecular weight being most resistant to microbial attack and remaining longest in soils. Condensed wattle tannins, when complexed with proteins, are less readily degraded than hydrolysable tannins and tannic acid (Mutabaruka *et al.*, 2007). It was reported that compared to native areas, litter under *A. longifolia* contained higher levels of N and had lower C/N ratios, and substantially altered soil microbial catabolic diversity (Marchante *et al.*, 2008a). It has also been reported that a N₂-fixing invasive plant *A. saligna*, influenced inputs of C and N and soil microbial processes (Yelenik *et al.*, 2004).

In the present study, the wattle soils were characterised by a pH of 4.6 and a lower organic C level (4.8%) than the pine soils. RDA confirmed that wattle soils were correlated with a low level of organic C. Fungal energy channels are regarded as slow cycles, as they are favoured by acidic soils containing recalcitrant organic materials that are low in available nutrients, leading to relatively long generation times

(Joergensen and Wichern, 2008). Mutabaruka *et al.* (2007) showed that high polyphenol-containing systems favoured the development of fungal communities that were well adapted to high acidity and phenol-rich soil conditions and, particularly, to the more recalcitrant, condensed tannin-protein complexes. Complexation had an apparent protective or masking effect, which led to reduced C availability.

Under the wattle plantation, the soil moisture content (11.8%) was the lowest of all the land uses. As the fungi-based channel does not require a continuous water film for growth (Haynes and Graham, 2004), fungal communities under wattle were much more active than the bacterial communities from this land use. Catabolic profiles of the replicate samples of wattle soil fungal communities were shown by PCA to be highly dissimilar, despite the similarity of the physicochemical profiles of the soil subsamples from this land use. The substrate utilized with 50% of its variance accounted along gradients of fungal functional diversity was D-fructose, a common soil carbohydrate. Although fungal catabolic richness was lower under the wattle monoculture than under all the other land uses except kikuyu, community functional diversity was greater than that of kikuyu and equal to that of native grassland. The low richness of the wattle fungal populations, coupled with a slightly higher catabolic diversity than occurred under kikuyu, could indicate that, in the wattle soils, a limited number of fungal species were carrying out a variety of functions, and were probably highly adapted to the conditions under this land use.

Under arable agriculture at this site, as shown by AWCD, the sugarcane soil fungal communities were more metabolically active than those of maize as well as those of all the other land uses except pine. As the cane is harvested only every 2 years and the soil was undisturbed at the time of sampling, this contrasted with the maize (CT) soils, which had recently been tilled and the plant residues removed. Govaerts *et al.* (2007) reported that AWCD was significantly higher for maize with residue retention, compared to residue removal. In the sugarcane soil, organic C inputs would have been in the form of fallen leaves and decaying roots. These factors could account for the higher levels of organic C (5.0%) in sugarcane soils compared to those under maize (3.9%). Additions of easily degradable organic matter lead to an increase in catabolic activity in soils (Degens *et al.*, 2000). This could partly explain the greater catabolic activity of the sugarcane fungal communities compared to those of maize. The

sugarcane soils were more acidic (pH 4.4) than all the other land uses except pine, a condition which tends to favour fungi. Mutabaruka *et al.* (2007) reported that soils from agricultural systems such as sugarcane, maize and grasslands had low polyphenol contents, which had implications for the soil microbes. In the PCA biplot (Figure 7.2), sugarcane soil fungal catabolic profiles of the replicate samples were the most closely clustered and, therefore, the most similar of all the land uses. The carbohydrate, L-arabinose and the carboxylic acid, γ -amino-butyric acid, were the substrates preferentially utilized. Carboxylic acids are among the most easily utilized C compounds by microbial communities (Marchante *et al.*, 2008a).

Under maize, two of the subsamples (M1 and M3) were closely clustered by PCA, whereas the third subsample (M2) was highly dissimilar and distant from the other two (Figure 7.2). Seventeen substrates were utilized by both M1 and M3 but not M2. Those substrates preferentially utilized by fungi from the three subsamples were L-proline (amino acid) and the carbohydrates, L-arabinose and D-cellobiose. The latter is a glucose-containing disaccharide and primary constituent of hemicellulose, the preferential uptake of which, suggests the potential ability to utilize cellulose (Hobbie *et al.*, 2003).

At this site, both maize and sugarcane soils were correlated with low organic C and Mg but high levels of P and K, although the levels under the two crops varied. The high soil P and K levels were as a result of annual inorganic fertilizer applications. RDA showed that the soil physicochemical property most closely associated with sugarcane was a high P level. A lower pH was associated with the sugarcane soil subsamples than with those of maize. Maize soil fungal community activity was lower than that of pine, sugarcane and wattle but greater than native grassland and kikuyu. In addition, maize fungal communities had a lower catabolic richness, evenness and diversity than the sugarcane fungal communities at this site. Govaerts *et al.* (2008) reported that maize under conventional tillage with removal of plant residues induced a reduction in populations of the soil micro-flora, including fungi.

Among the soil fungal communities from the various land uses, the least active were those under kikuyu, followed by those under native grassland. The kikuyu soil fungi were also the least catabolically rich, even and diverse of all the land use types.

Native grassland fungal communities, on the other hand, showed a much greater richness and diversity than those of the kikuyu monoculture. Litter in grasslands is in the form of plant detritus, which is relatively less recalcitrant than that under forest (Lauber *et al.*, 2008). The greater plant biodiversity in native grassland would supply a greater variety of C substrates and root exudates than the kikuyu monoculture, thus leading to the greater fungal catabolic richness and diversity observed in the former. However, catabolic evenness was the same under the two land uses indicating that, although the fungal communities are functionally different, they are nonetheless very similar in terms of the evenness of substrates utilized. Substrates utilized with 50% of their variance accounted along gradients of kikuyu fungal diversity were the carbohydrates, D-fructose and D-cellobiose, whereas those utilized by the native grassland fungal communities were D-fructose and D-raffinose (Figure 7.3). The kikuyu fungal communities were the least active of all the land use types and had the most dissimilar subsample catabolic profiles. This was in contrast to the kikuyu bacterial communities, which were the most metabolically active, albeit closely clustered by PCA, indicating their catabolic similarity (Figure 6.2). As the soil conditions, namely the high soil moisture content (23.14%), the availability of readily decomposable nutrients (the highest organic C level of 9.4%), and a higher pH, favoured the bacteria, the soil fungi could be expected to be less active. In comparison, soil fungi under native grassland were much more active than the bacterial communities under this land use. In addition, the fungal catabolic profiles of the replicate samples were much more closely clustered in the PCA biplots (Figure 7.2) than the bacterial profiles, which were highly dissimilar (Figure 6.2).

Analyses by the Kruskal-Wallis ANOVA showed that while the various land uses at Baynesfield had not affected the soil fungal community catabolic richness (S), they had affected evenness (J) and the Shannon diversity index (H'), with the greatest functional diversity shown by the pine soil fungi, and the lowest diversity by the fungal communities under the kikuyu monoculture.

7.4.2 Fungal catabolic (functional) diversity at Mount Edgecombe

An imbalance of different nutrients in the farming system is a concern for the long-term sustainability of the sugarcane industry. The combination of a sugarcane monoculture and the intensity of sugarcane production has a negative effect on soil health (Bell *et al.*, 2007). As previously noted (section 6.4.2), the degradation of soils under sugarcane production results in a progressive loss of organic matter, a decline in fertility, acidification, and a breakdown in soil structure, particularly in the surface layers (Graham *et al.*, 2002a). While changes in soil health and fertility and the loss of productivity under sugarcane are well documented, little is known of the effects of these parameters on soil microbes, particularly on fungal community functional diversity. A well-functioning soil microbial community is essential for soil fertility and resistance to disturbance (Ros *et al.*, 2008). Soil microbial community function reflects the interaction of many biotic and abiotic factors, with both crop residues and SOM quality affecting the functional diversity (Bending *et al.*, 2002). In the present study, CLPP (using BIOLOG SF-N2 microplates) of the soil fungal communities under the four different land management practices at this site, clearly separated the various communities and showed differences in their catabolic capabilities.

As indicated by AWCD, higher levels of substrate utilization (activity) were observed in the soil fungal communities under greencane harvesting treatments retaining a trash blanket (T) than under burnt cane harvesting with removal of plant residues (Bto) (Figure 7.6). The soil organic C level was highest (6.6%) under the trashed, fertilized treatments (TF) followed by the trashed, unfertilized treatments (TFo) (6.0%). Under the burnt cane treatments, organic C levels were lower (5.14%) in the fertilized (BtoF) and lowest (4.93%) in the unfertilized (BtoFo) plots. RDA (Figure 7.9) confirmed these results. Soil management practices, such as mulching with plant residues, have been reported to promote storage of C originating from the atmosphere and to constitute potential C sinks. Sugarcane leaves represent a considerable biomass that is either burned or mulched depending on the harvesting procedure. Burning leads to a loss of organic C (and nutrients through particulate dispersion) and emissions of greenhouse gases. In contrast, greencane harvesting with residues mulched on the soil surface increases the concentration of soil organic C (Razafimbelo *et al.*, 2006). As

previously mentioned, soil fungi are involved in the decomposition of crop residues and in the humification of cellulosic material in the soil (Boopathy *et al.*, 2001). At Mount Edgecombe, the higher levels of organic C in soils under trash retention and the lower pH in fertilized soils compared to unfertilized soils, are conditions favouring fungi, which may explain the observed higher levels of fungal activity under green cane than under burnt cane harvesting. Bell *et al.* (2007) reported that all residue retention treatments, irrespective of subsequent management, increased the ratio of fungi to bacteria in the soil microbial biomass.

Differences in the substrate utilization profiles of the soil fungal communities were clearly shown by PCA (Figure 7.7). Testing of this data by MRPP showed that the different treatments had a small but significant overall effect on fungal catabolic diversity ($p = 0.037$). This was in contrast to the non-significant effect on bacterial functional diversity at this site (section 6.3.4). Subsample profiles of those treatments retaining a trash mulch were more closely correlated (indicating a greater similarity in their catabolic capabilities) than those of the subsamples from under burnt cane harvesting. In addition, the fertilized subsample profiles were more similar to each other than were the unfertilized subsample profiles under both trash management practices. Soil PCA (Figure 5.4) indicated that those soils, fertilized annually with N, P and K, were associated with higher acidity, and had higher levels of P and K than unfertilized soils. RDA indicated that a higher pH and a higher ECEC level, were strongly correlated with soil fungal communities from the unfertilized burnt cane harvested management. ECEC were also closely correlated with unfertilized greencane harvested soil fungal communities. Soil fungi are known to differ in their response to NPK fertilizers, with N fertilization changing the abundance of fungal species (Marschner *et al.*, 2003). Lauber *et al.* (2008) reported that relatively P-rich cultivated soils contained more Ascomycota and fewer Basidiomycota than those with lower extractable P concentrations. In the present study, the results suggest, that at the long-term (69 years) trash management site at Mount Edgecombe, annual applications of N, P and K had possibly exerted a selective pressure on the resident fungal communities. However, there is not necessarily a correlation between changes in community composition and catabolic diversity, as functional redundancy is thought to be common in soil microbial communities (Hollister *et al.*, 2010). It seems likely that soil fungal communities under the different treatments have become adapted to

the conditions prevailing under a sugarcane monoculture and as such, are functionally highly specialized.

The substrates utilized by TF fungal communities with 50% of their variance accounted along gradients of fungal functional diversity were the amino acid, L-threonine, and the carboxylic acids, quinic acid and L-pyroglutamic acid. The latter is not surprising since, as stated previously, carboxylic acids have been reported to be among those C compounds most easily utilized by microbial communities (Marchante *et al.*, 2008a). The substrates utilized by TFo communities were the amide, L-asparagine and the carboxylic acid, L-pyroglutamic acid. The fertilized, burnt cane harvested (BtoF) soil fungal communities mainly utilized L-proline (amino acid) and the carbohydrates, D-galactose and D-cellobiose. The latter two substrates are co-produced during degradation of structural plant components, including hemicellulosic compounds which may comprise over 50% galactose sub-units. D-cellobiose is a primary constituent of hemicellulose and the fundamental disaccharide unit of cellulose. High metabolic activity in substrates such as galactose and cellobiose could indicate the presence of fungi involved in degradation of plant structural carbohydrates (Hobbie *et al.*, 2003). The unfertilized burnt cane harvested soil fungal communities mainly utilized the amino acid L-leucine, the carbohydrate D-galactose and the carboxylic acid, γ aminobutyric acid.

Although fertilized soils under green cane harvesting with retention of a trash mulch, were found to have a higher organic C content and a higher pH (possibly due to NH_3) than unfertilized soils under burnt cane harvesting with plant residues removed, soil fungal catabolic richness, evenness and the Shannon diversity index (H') (based on the number of substrates utilized) were not significantly different. This could suggest that the organic C in the mulched soils was of a low quality or may be unavailable for decomposer fungal communities (Marchante *et al.*, 2008a). Also, the inorganic fertilizer applications may possibly have altered the soil nutrient balance, with consequent effects on the fungal catabolic diversity.

7.5 CONCLUSIONS

As very little is known about the functional diversity of soil fungal communities in South Africa, the present study examined the effects on this, of different land uses and sugarcane trash management practices at two long-term experimental sites in KwaZulu-Natal. It was again noted that the fungal communities selected for in the BIOLOG SF-N2 plates would only be representative of those fungi able to grow under the plating conditions, and not necessarily of the soil fungal communities as a whole. However, by using a single time point for the statistical analyses of this data, an indication of heterotrophic fungal population density and of the total potential heterotrophic activity, was successfully obtained.

At Baynesfield Estate, BIOLOG analyses showed marked differences in the catabolic capabilities of the resident soil fungal communities under the different land uses. AWCD showed that, except under kikuyu, the soil fungal communities from the other land uses were more active than their bacterial counterparts. This was most likely due to sampling taking place at the end of the dry winter season, during which only the kikuyu pasture had been irrigated. As the different land uses at this site are all on the same soil type, the overall results of this investigation suggest that a combination of the different land uses and their associated plant cover, together with cultivation practices (irrigation, tillage and fertilizer applications), and not soil type, were the main influences on the soil fungal community catabolic diversity.

At Mount Edgecombe, CLPP clearly separated the soil fungal communities under the different treatments, on the basis of their activity levels and catabolic profiles. However, the results obtained indicated that the soil fungal communities under the four treatments were more catabolically similar to each other than the fungal communities under the various land uses at Baynesfield. The experimental plots at Mount Edgecombe are all on the same soil type and under a sugarcane monoculture. However, differences between the soils under the four management practices were highly significant ($p < 0.001$), whereas differences in the catabolic diversity of the various soil fungal communities were not as marked. This suggests that, rather than the soil type, the trash management practices and inorganic fertilizer applications

(which affected the pH and the levels of P, K, and organic C), were the main influences on soil fungal catabolic diversity at this site.

Chapter 8

GENERAL CONCLUSIONS

8.1 RATIONALE FOR THE RESEARCH

The worldwide search for cost effective, sustainable agricultural practices, which also have minimum impact on the environment, is an ongoing process. The way in which land use and management practices affect the soil microbiota, which are indicators of soil *health* and *quality*, must therefore be assessed. As traditional, culture-dependent techniques sample only a very small proportion of the microbial diversity in soils, alternative methods are being sought to address this shortcoming. A combined approach to the analysis of microbial diversity in soil is generally considered preferable, as no single method is without its drawbacks. Accordingly, the current study used a combination of molecular and community level physiological profiling techniques to investigate the effects of a wide range of commercial land uses (arable crops, forestry and pastures), and different trash management practices within a single land use (sugarcane production), on the soil microbial diversity at two long-term experimental sites in KwaZulu-Natal. This work is of particular relevance in the South African context, as it is the first time that a dual approach of this kind has been used. Also, few local studies have simultaneously examined both soil bacterial and fungal structural and catabolic diversity across multiple land uses and management practices, in a given landscape. Hitherto, little was known of the effects of agricultural practices in South Africa on the microbial structural diversity in these experimental soils, especially that of the fungi.

8.2 MEETING THE RESEARCH OBJECTIVES

This broad-based study successfully determined the effects of a range of land uses and different management practices on both the bacterial and fungal communities, resident in the various soils at the Baynesfield and Mount Edgecombe sites.

Notwithstanding the generally accepted viewpoint that soil type and location are the main influences on soil microbial composition and diversity, with land use and management of lesser importance, this study has demonstrated that differences in long-term land use and/or management practices within the same site, and on the same soil type, can cause substantial differences in the structural and catabolic diversity of the resident bacterial and fungal communities.

The realisation of each of the objectives outlined in Chapter 1 is summarised below.

Objective 1. To establish and optimize suitable protocols to profile soil bacterial and fungal communities, using a dual approach.

Protocols were established for the molecular profiling of both soil bacterial and fungal communities to determine their genetic (structural) diversity using PCR-DGGE. The initial study (Chapter 4) showed that the fungal procedures required refinement, which led to the extensive adaptation and optimisation of the protocols in the follow-up work (Chapter 5). All PCR procedures were modified/optimized for local conditions, with respect to: (i) the primers used; (ii) the concentrations of template DNA, Taq DNA polymerase and MgCl₂ used; and (iii) the number of cycles in the PCR reaction. For the Mount Edgecombe samples, the number of cycles was increased in both the initial and follow-up studies because of the difficulty in obtaining adequate DNA yields. In addition, in the latter work, a nested PCR approach was also adapted for use with these samples, to increase the DNA concentration still further. All these modifications enabled reproducible results to be obtained.

The optimization of the DGGE procedures, through adjustment of the denaturing gradients in the gels, and alterations to the running conditions, resulted in greatly improved band separation. The adaptation of an existing protocol for excising and eluting the fungal DGGE bands, enabled the sequencing and identification of some of the bands to be carried out successfully.

The modifications made to the CLPP protocols, to determine bacterial and fungal catabolic diversity using BIOLOG EcoPlates and SF-N2 plates respectively, allowed

for an improved characterisation of the microbial communities in the study soils. The adaptations allowed the use of a multi-channel pipettor for microplate inoculation, thereby enabling the 8 wells in each microplate row to be inoculated simultaneously. This considerably reduced the time required for the otherwise laborious plate inoculation process, and significantly minimised the risk of contamination.

Objective 2. Determining the effects of land use and management practices on the soil bacterial and fungal community structural and catabolic diversity, and relating these findings to selected soil variables.

Agricultural land use and management practices were shown to have a considerable influence on the genetic (structural) and catabolic (functional) diversity of the soil bacterial and fungal communities at the two study sites. However, notable differences were observed at each site.

Baynesfield Estate

At this site, large differences in both bacterial and fungal community structural and catabolic diversity were noted under the different land uses. In the initial study, land use had affected both bacterial species richness and diversity, but only the species richness of the fungal communities. However, the follow-up study, with the improved protocols, identified not only differences in fungal community structural richness, but also in evenness and diversity between the various land uses. It was found that land use did not affect soil bacterial catabolic richness, evenness, and diversity, or fungal catabolic richness, but did affect fungal evenness and diversity.

In the initial study, analyses of the data relating selected soil physicochemical properties to microbial structural diversity showed that organic C, Mg, ECEC, K, P, and exchange acidity had affected the genetic diversity of both the bacterial and fungal communities. In the follow-up study, organic C, ECEC, pH and P were found to be most influential on the soil fungal structural diversity, and also on both the bacterial and fungal catabolic diversity, showing a shift in the soil variables influencing the microbial communities. These results clearly show that over time, the major drivers of both structural and functional diversity at this site were organic C,

ECEC and P, with exchange acidity and pH being less closely correlated, possibly as an effect of acidity. Another temporal effect was the loss of Mg and K as drivers, possibly as a result of the fertilization of some of the sites shifting the soil chemical balance. It was also shown that the soil bacterial and fungal communities under the various land uses differed substantially in their overall nature, community composition and function.

Mount Edgecombe

At this site, the initial study revealed that bacterial species richness was greatest in the trashed/fertilized plots, and lowest in the burnt/unfertilized plots. This was not due to trash retention or to the interactions of trash and fertilizer, but rather to the fertilizer applications. In the follow-up study, a significant effect on fungal species richness, but not on evenness or diversity, was caused by fertilizer additions to the burnt cane, but not to the green cane managements. As in the earlier study, this showed that fertilization, and not trash retention, was the most important single influence on the soil fungal communities at this site. Thus, even within a single land use, the various soil management practices were found to affect the structure of the soil microbial community differently. In contrast to the general findings that soil type is the main influence on microbial diversity, at this site, the retention/removal of plant residues and applications of inorganic fertilizers were the main influences on soil bacterial and fungal community functional diversity.

In the initial study at this site, CCA showed that differences in organic C and Mg were the main soil variables influencing the genetic composition of both the bacterial and the fungal communities. In the follow-up study, organic C remained one of the main soil variables affecting the soil fungal community structure; however ECEC and pH, were also found to be influential. While the catabolic diversity of both the bacterial and the fungal communities was not affected significantly by the different management practices, bacterial catabolic richness was. Here, in contrast to the findings on structural diversity, retention of a trash mulch and the absence of fertilizer were the main influences on bacterial catabolic richness, suggesting that soil variables such as organic C and pH had played a role. This was confirmed by RDA, which

showed that organic C, ECEC and pH had a significantly effect on both the bacterial and the fungal communities' catabolic diversity.

Objective 3. To determine if the results obtained, with the profiling techniques used in this study, supported those of previous researchers on the same experimental soils.

Previous researchers at the two study sites focused on the organic matter status of the soils, and on determining the size, activity and catabolic diversity of the soil microbial biomass in crop soils, concentrating almost exclusively on the bacterial communities. In contrast, the present study provides a wider, more balanced and holistic view of the total microbial soil communities' diversity, since both the bacterial and the fungal communities are considered. This is important as the fungi are usually primary colonisers of plant litter, which is broken down into smaller molecules that provide suitable nutrients for the bacterial communities.

At Baynesfield, land use was shown to have affected soil bacterial catabolic diversity considerably, which agreed with the findings of earlier research at this site. At Mount Edgecombe, bacterial catabolic diversity was highest under green cane harvested, unfertilized treatments, and lowest under fertilized, burnt cane harvested treatments, showing that both trash retention and fertilizer applications had an effect. As trash is a natural substance, whereas inorganic fertilizers are not, the former is less likely to cause adverse long-term effects on agricultural soils. A previous study by Graham (2003) indicated large differences in bacterial catabolic capability between soils under trashing and those under preharvest burning, but in contrast to the present study, fertilizers were found to have little effect.

As the present study undertook pioneering work on the effects of land use and management practices on the genetic diversity of both the soil bacterial and fungal communities at these sites, there are no appropriate earlier data for comparison. This applies also to this study's unique fungal catabolic diversity data.

8.3 CRITICAL EVALUATION OF THE RESEARCH

This study was designed as a broad-based analysis of the effects on the autochthonous soil bacterial and fungal communities, of a wide range of agricultural land uses and management practices. As soil is a highly complex, dynamic system, constantly changing in terms of its physicochemical, spatial and temporal dimensions, no single experimental approach can possibly determine the total microbial diversity supported at any given location, over time. The current work has addressed some of these difficulties, by using a combination of molecular and physiological profiling methods. Nonetheless, given the complexity of the task, the approach taken in this study still provides only limited information.

As variations in soil physicochemical properties may act as potential selection factors affecting plant growth and soil microbial communities, the relationship between selected soil variables [organic C; pH (KCl); exchangeable acidity; total cations (ECEC); exchangeable K, Ca and Mg; and extractable P] and the microbial communities was determined. As previously mentioned, although N (specifically the C/N ratio) plays an important role in determining the structure and function of the soil microbial communities, it was not considered in this study because none of the current extraction methods can predict soil N supply consistently (Ros, in press). As organic C is often closely linked with N, distinct extractable organic matter (EOM) fractions have been used to assess the soil's capacity to supply N. However, none of these SOM fractions has been universally accepted as an indicator of the soils' capacity to supply N nor is their role in the N cycle certain. This is unfortunate since accurate estimation of the N mineralized from SOM would enable farmers to estimate the rate of N fertilizer applications to optimize crop yield and reduce N losses to the environment, thereby improving the sustainability of agriculture (Ros *et al.*, 2011).

One of the principle advantages of community fingerprinting using PCR-DGGE combined with CLPP (using BIOLOG microplates) was that with both procedures, multiple soil subsamples from the different land uses and treatments, could be screened simultaneously in a relatively short time, and reproducible results obtained. Another benefit of these fingerprinting methods was that they revealed links between

the agricultural crops, the soil physicochemical properties and the structure and function of the microbial communities from soils under the various land uses and management practices.

In the present study, as universal primers were used for PCR-DGGE, information on microbial community structure was obtained but not on the functional aspects of these communities. This shortcoming was overcome by CLPP using BIOLOG plates, which served to show the catabolic (functional) diversity of the microbial communities, and thus complemented the structural diversity analysis.

8.3.1 Evaluation of the soil sampling procedures

Sampling of the soils from both sites took place at the end of the dry winter periods of 2004 and 2008. This was successful in the case of the Baynesfield soils, as high concentrations of total genomic DNA were obtained in both studies. However, at Mount Edgecombe, while reasonable genomic DNA yields were obtained from all the land management soils in the initial study, in the follow-up work, even after two separate soil sampling attempts, total DNA concentrations were too low to be visualised in the agarose gels. Had the sampling taken place after the first spring rains, it is likely that higher DNA yields would have been obtained, as the microbial biomass is generally larger and more active during warmer, wet weather. Notwithstanding this, it is also possible that chemical inhibition prevented DNA isolation from the Mount Edgecombe soils, as BIOLOG analysis of the same soils clearly demonstrated the presence of bacteria and fungi, again illustrating the advantage of a dual approach.

The experimental design was suitably rigorous for the Mount Edgecombe soils, as site BT/139 is replicated in a randomized split plot design, which allowed inferential statistics to be applied to the data. However, since Baynesfield Estate is an established commercial venture, it was not possible to replicate the various land use treatments in a randomized, statistically correct experimental design.

8.3.2 Evaluation of the PCR-DGGE profiling method

The PCR-DGGE approach was successful in separating the soil microbial communities under the different land uses and sugarcane trash managements, revealing the genetic diversity of the bacterial and fungal populations. PCR-DGGE was particularly useful for the bacterial structural diversity study, as the G + C content of these organisms covers a wider range than that of the fungi, resulting in a better spread of bands in the bacterial DGGE gels.

Since the objective of this study was to show the response of entire soil bacterial and fungal communities to different land uses and treatments, without any deliberate selection by specific primers, universal primers were used. This undoubtedly left some of the community members undetected.

While the initial fungal DGGE studies had underestimated fungal community richness and diversity, the redesigned follow-up study largely addressed these concerns. The strategy applied here, of whole community DNA extraction followed by PCR-DGGE, gel band excision and sequencing, provided a better insight into the structure of soil fungal communities as affected by the various land uses and treatments, than had been possible initially. However, temporal effects could not be discounted as the DNA used in the two studies was isolated at different times.

Because the resolving power of DGGE is limited for fragments that are greater than 500 bp in length, the separation efficiency of large fragments in the gels was poor. Conversely, bands from longer PCR products (such as those produced in the initial study) provide better identification when sequenced as they contain more genetic information. The follow-up study proved therefore, that primer pair FR1GC/FF390 was well-suited to DGGE, albeit not to specific sequencing studies. As indicated above, this was appropriate given the broad-based nature of the current study.

8.3.3 Evaluation of the BIOLOG method for physiological profiling

In the CLPP analyses of both sites, the BIOLOG EcoPlates successfully differentiated the catabolic capabilities of the various bacterial communities, and the SF-N2 plates those of the corresponding soil fungal communities. EcoPlates are a more recent development and an improvement on the older GN microplates used in an earlier study at these sites. The former have the advantage of being specifically designed for environmental studies, and contain substrates known to occur in the rhizosphere, unlike the latter, which were originally intended for use in clinical analyses. The SF-N2 plates were well-suited to the soil fungal analyses, as they lacked the tetrazolium dye (present in the bacterial microplates) which is highly toxic to many fungi.

A shortcoming of the BIOLOG method is that in both the bacterial and the fungal analyses, the communities selected for were probably fast-growing microbes that could survive under the microplate conditions. Despite this, the advantages of this method outweighed the disadvantages, as all samples for both sites were screened in a relatively short time, thereby reducing the risk of contamination and avoiding the undesirable effects of sample 'aging'.

8.4 IMPLICATIONS OF THIS RESEARCH FOR AGRICULTURE

This study has revealed the impact of different agricultural land uses and management practices, on soil microbial diversity at the two long-term sites. As a diverse microbial community is generally regarded as an early indicator of soil health, those agricultural practices that increase microbial diversity and soil fertility are preferable. Unfortunately the importance of soil health is often not considered. In order to maintain productivity and profitability, a compromise is usually needed between operating efficiency, cost saving, and agricultural practices that promote soil health and fertility.

8.4.1 Baynesfield Estate

The benefits of residue retention for arable crops such as maize and sugarcane were shown in the present study. At this site, the low soil microbial structural diversity and the low soil organic C content under maize (CT with tops raked off), could be improved by reducing tillage of these soils, and retaining crop residues on the soil surface. Crop rotation with ley crops or legumes would help increase soil fertility and microbial diversity. Under sugarcane, a change from burnt cane to green cane harvesting with trash retention, could increase the soil organic matter content. In addition the cessation of burning would prevent the volatilization and subsequent loss, of large quantities of nutrients such as C, N and S, present in the plant residues. Fallow periods between replanting with cover crops (such as leys or legumes) would also be advantageous. However, the author recognises that these practices may not have been adopted at this commercial site, because of possible financial implications.

While the kikuyu pasture monoculture had the highest soil organic C content compared to the other land uses, the microbial structural richness and diversity was lower than that under native grassland. Crop rotation would be beneficial in increasing microbial diversity in the former soils, since it would increase the variety of plant inputs and root exudates to the soil, and hence the variety of substrates for microbial degradation.

The present study showed that under exotic forestry plantations, the microbial communities had become highly specialized in response to the prevailing conditions. For the long-term success of plantation management, it is important to maintain and enhance the productivity of these soils. The pH of the wattle plantation soil had dropped from 5.2 in 2004, to 4.6 in 2008. The acidification of soils under wattle plantations over time, poses problems if such land is subsequently used for the production of food crops. Liming is a possible strategy to improve crop production in these soils, as raising the soil pH allows for the establishment of a more diverse microbial population, especially among the bacteria, which have very rapid metabolic rates. However, the large quantities of lime required to overcome Al and Mn toxicity, make this an expensive option. Problems of low N levels may also occur under wattle

plantations. While wattle trees are significant N-fixers, wattle litter may immobilise N. In addition, only a low release of N from the litter may occur, as a result of N complexation with the reactive polyphenols that are present in high concentrations and are very toxic to other plants.

Under pine, the soils were characterised by high exchangeable acidity and the lowest pH of all the land uses. Soil infertility due to acidity is a major limiting factor in crop production. As with the wattle soils, liming is an option for raising the soil pH, thereby preventing the precipitation of phytotoxic Al, and improving conditions for a more diverse microbial community. A method for assessing soil quality under pines was suggested by Kelting *et al.* (1999), using a soil quality index (SQI) model. This monitors the impacts of different management practices on soils. Under this model, five key soil attributes are measured; (i) root growth promotion; (ii) the storage, supply and cycling of nutrients; (iii) the ability to accept, hold and supply water; (iv) the promotion of gas exchange; and (v) the promotion of biological activity. This approach could prove beneficial at the Baynesfield site.

8.4.2 Mount Edgecombe

At this site, those treatments retaining crop residues were preferable to burnt cane harvesting with tops raked off, as they increased the soil organic C content in the surface soils. This encouraged heterotrophic microbial activity which resulted in an improved cycling of nutrients. As previously noted, burning resulted in nutrient loss from the soils, which adversely affected soil fertility, and enhanced soil degradation. Applications of inorganic fertilizers increased organic matter returns to the soil in the form of roots and trash, thereby enhancing the organic C yields. However, fertilization also lowered the soil pH, and increased levels of extractable P and exchangeable K, compared to unfertilized treatments. In the long-term, this resulted in increased Al availability and problems of Al toxicity and a lower soil pH. As recommended for the Baynesfield sugarcane site, greencane harvesting with retention of crop residues is preferable to burnt cane harvesting with trash removal, and crop rotation with legumes and leys would increase soil fertility and microbial diversity.

This would help reduce the soil degradation that occurs under long-term sugarcane production.

8.5 RECOMMENDATIONS FOR FUTURE WORK

As traditional agricultural land use and management practices have been found to lead to the observed global decline in soil quality and fertility, it is vital that future work should concentrate on strategies to overcome this. It is to be hoped that this study will encourage those agriculturalists involved in all aspects of crop production, to acknowledge the importance of microorganisms in soil fertility, structure and health and to adopt practices to promote their wellbeing. Understanding the interactions of soil-inhabiting microbes and their environment, will enable predictions to be made of the impact on the soil, of various agricultural practices, both positive and negative, leading to better management of soil structure and functions. Unless greater care is taken of the soil microbial communities, sustainable agriculture will not be possible in the long term. For these reasons, constant monitoring of the soils' living components is essential to good farming practice.

While PCR-DGGE remains the mainstay among soil ecologists for determining microbial community structural diversity, it is only semi quantitative. The traditional end-point PCR used in this study has several limitations (section 2.4.2.2.2), making real-time PCR a possible alternative in future, as it has been shown to be more precise. DGGE is also limited, as communities constituting fewer than ~1% of the total population are not usually represented and, as shown in this study, many bands of similar sequence may run together in the gels. Thus, accurate determination of actual microbial species richness may not be possible. Alternative methods to estimate soil microbial diversity, together with their advantages and shortcomings, for example, T-RFLP analysis of SSU rRNA genes from environmental samples which may be used for the rapid comparison of microbial DNA sequences from PCR amplicons, are reviewed in section 2.4.

For future studies, the present work could be expanded using newly-developed methods, such as high-throughput genomic technologies, that make the identification

of differentially expressed genes possible. By profiling untargeted transcriptomes using *in silico* and microarray approaches, a more accurate and qualitative analysis of the effects of various factors on soil microbial diversity might be possible.

8.6 CONCLUDING REMARKS

This study has helped improve our understanding of the effects of land use and management practices, on the structural and catabolic diversity of soil microbial communities, however, considerable scope exists for further work. Serious limiting factors to realising the objective of a more quantitative and qualitative analysis of soil microbial diversity, are the availability of appropriate equipment and the high costs associated with many of the experimental procedures. Nonetheless, it is hoped that this work will form the basis of future studies, as this field has hitherto largely been neglected in South Africa, and many of the secrets of the ‘black box’ are yet to be revealed.

Until such time as a more holistic approach to soil fertility and health, involving conservation farming practices such as no-till/minimum till, biological control and organic matter retention is adopted, which recognises the importance of the soil microorganisms, intensive agricultural production cannot be sustainable in the long term.

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APPENDIX A

BAYNESFIELD ESTATE

TABLE A1 ANOVA of bacterial community species evenness and land use means at Baynesfield

| Source of variation | d.f. | s.s. | m.s. | f-ratio | p-value |
|---------------------|------|-------|-------|---------|---------|
| Land use | 5 | 0.154 | 0.030 | 0.91 | 0.509 |
| Residual | 12 | 0.408 | 0.034 | | |
| Total | 17 | 0.562 | | | |

| | | | | | | |
|------------|-------|--|--|--|--|--|
| Grand mean | 0.863 | | | | | |
|------------|-------|--|--|--|--|--|

| Land use | SC | M | Kik | Nat | PF | W |
|----------|------|------|------|------|------|------|
| | 0.87 | 0.88 | 0.88 | 0.94 | 0.67 | 0.95 |

($p < 0.05$)

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

MOUNT EDGECOMBE

TABLE A2 ANOVA and land treatment means (\pm sd) of the effects of sugarcane trash management on soil bacterial species richness, evenness and diversity at Mount Edgecombe

| Treatment | Species Richness (S) | Species Evenness (J) | Shannon-Weaver Diversity index (H') |
|------------------|----------------------|----------------------|-------------------------------------|
| Trash Management | | | |
| TF | 27 ns (2.08) | 0.88 ns (0.071) | 2.86 ns (0.295) |
| TFo | 28 ns (2.65) | 0.89 ns (0.033) | 2.97 ns (0.174) |
| BtoF | 26 ns (0.00) | 0.86 ns (0.015) | 2.81 ns (0.050) |
| BtoFo | 30 ns (2.65) | 0.87 ns (0.049) | 2.96 ns (0.130) |

Means followed by ns are not significantly different ($p < 0.05$).

Key: T = green cane harvested with trash retention; Bto = burnt cane with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

APPENDIX B

BAYNESFIELD ESTATE

TABLE B1 ANOVA of fungal community species evenness and land use means at Baynesfield

| Source of variation | d.f. | s.s. | m.s. | f-ratio | p-value |
|---------------------|------|-------|-------|---------|---------|
| Land use | 5 | 0.299 | 0.059 | 1.15 | 0.389 |
| Residual | 12 | 0.627 | 0.052 | | |
| Total | 17 | 0.927 | | | |

| | | | | | | |
|------------|-------|--|--|--|--|--|
| Grand mean | 0.825 | | | | | |
|------------|-------|--|--|--|--|--|

| Land use | SC | M | Kik | Nat | PF | W |
|----------|-------|-------|-------|-------|-------|-------|
| | 0.942 | 0.901 | 0.639 | 0.881 | 0.937 | 0.652 |

($p < 0.05$)

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

TABLE B2 ANOVA of soil fungal community species diversity and land use means at Baynesfield using the Shannon Weaver Diversity Index (H')

| Source of variation | d.f. | s.s. | m.s. | f-ratio | p-value |
|---------------------|------|-------|-------|---------|---------|
| Land use | 5 | 2.190 | 0.438 | 2.16 | 0.127 |
| Residual | 12 | 2.431 | 0.202 | | |
| Total | 17 | 4.621 | | | |

| | | | | | | |
|------------|------|--|--|--|--|--|
| Grand mean | 1.57 | | | | | |
|------------|------|--|--|--|--|--|

| Land use | SC | M | Kik | Nat | PF | W |
|----------|------|------|------|------|------|------|
| | 1.96 | 1.71 | 0.94 | 1.79 | 1.73 | 1.27 |

($p < 0.05$)

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

APPENDIX C

BAYNESFIELD ESTATE

TABLE C1 Summary of the results of a Monte Carlo permutation test of CCA data on the effects of land use on the soil fungal communities at Baynesfield

| Axes | 1 | 2 | 3 | 4 | Total inertia |
|---|------------|-------|-------|-------|---------------|
| Eigenvalue | 0.386 | 0.267 | 0.131 | 0.097 | 2.209 |
| Species-environment correlations | 0.966 | 0.917 | 0.869 | 0.747 | |
| Cumulative percentage variance of species data | 17.5 | 29.6 | 35.5 | 39.9 | |
| Cumulative percentage variance of species environment relation | 43.8 | 74.2 | 89.0 | 100.0 | |
| Summary of Monte Carlo Test | | | | | |
| | eigenvalue | Trace | F | P | |
| First canonical axis | 0.386 | | 2.754 | 0.002 | |
| All canonical axes | | 0.881 | 2.155 | 0.002 | |

MOUNT EDGECOMBE

TABLE C2 Summary of the results of a Monte Carlo permutation test of CCA data on the effects of land management at Mount Edgecombe

| Axes | 1 | 2 | 3 | 4 | Total inertia |
|---|------------|-------|-------|-------|---------------|
| Eigenvalue | 0.293 | 0.176 | 0.127 | 0.290 | 1.582 |
| Species-environment correlations | 0.940 | 0.971 | 0.805 | 0.000 | |
| Cumulative percentage variance of species data | 18.5 | 29.7 | 37.7 | 56.0 | |
| Cumulative percentage variance of species environment relation | 49.1 | 78.7 | 100.0 | 0.0 | |
| Summary of Monte Carlo Test | | | | | |
| | eigenvalue | Trace | F | P | |
| First canonical axis | 0.293 | | 1.819 | 0.010 | |
| All canonical axes | | 0.597 | 1.616 | 0.008 | |

APPENDIX D

TABLE D1 The substrates in the wells of a BIOLOG EcoPlate™ replicated three times across the plate (BIOLOG, 2008)

| | | | | | | | | | | | |
|------------------------------------|--|---|----------------------------------|------------------------------------|--|---|----------------------------------|------------------------------------|--|---|----------------------------------|
| A1 Water | A2 B-methyl-D- Glucoside | A3 D-Galactonic Acid γ -Lactose | A4 L-Arginine | A1 Water | A2 B-methyl-D- Glucoside | A3 D-Galactonic Acid γ -Lactose | A4 L-Arginine | A1 Water | A2 B-methyl-D- Glucoside | A3 D-Galactonic Acid γ -Lactose | A4 L-Arginine |
| B1 Pyruvic acid Methyl Ester | B2 D-Xylose | B3 D-Galacturonic Acid | B4 L-Asparigine | B1 Pyruvic acid Methyl Ester | B2 D-Xylose | B3 D-Galacturonic Acid | B4 L-Asparigine | B1 Pyruvic acid Methyl Ester | B2 D-Xylose | B3 D-Galacturonic Acid | B4 L-Asparigine |
| C1 Tween 40 | C2 i-Erythritol | C3 2-Hydroxy Benzoic Acid | C4 L- Phenylalanine | C1 Tween 40 | C2 i-Erythritol | C3 2-Hydroxy Benzoic Acid | C4 L- Phenylalanine | C1 Tween 40 | C2 i-Erythritol | C3 2-Hydroxy Benzoic Acid | C4 L- Phenylalanine |
| D1 Tween 80 | D2 D- Mannitol | D3 4-Hydroxy Benzoic Acid | D4 L-Serine | D1 Tween 80 | D2 D- Mannitol | D3 4-Hydroxy Benzoic Acid | D4 L-Serine | D1 Tween 80 | D2 D- Mannitol | D3 4-Hydroxy Benzoic Acid | D4 L-Serine |
| E1 α Cyclodextrin | E2 N-acetyl-D- Glucosamine | E3 γ Hydroxybutyri c Acid | E4 L-Threonine | E1 α Cyclodextrin | E2 N-acetyl-D- Glucosamine | E3 γ Hydroxybutyri c Acid | E4 L-Threonine | E1 α Cyclodextrin | E2 N-acetyl-D- Glucosamine | E3 γ Hydroxybutyri c Acid | E4 L-Threonine |
| F1 Glycogen | F2 D- Glucosaminic Acid | F3 Itaconic Acid | F4 Glycil-L- Glutamic Acid | F1 Glycogen | F2 D- Glucosaminic Acid | F3 Itaconic Acid | F4 Glycil-L- Glutamic Acid | F1 Glycogen | F2 D- Glucosaminic Acid | F3 Itaconic Acid | F4 Glycil-L- Glutamic Acid |
| G1 D-Cellobiose | G2 Glucose-1- Phosphate | G3 α -Ketobutyric Acid | G4 Phenylethyl- amine | G1 D-Cellobiose | G2 Glucose-1- Phosphate | G3 α -Ketobutyric Acid | G4 Phenylethyl- amine | G1 D-Cellobiose | G2 Glucose-1- Phosphate | G3 α -Ketobutyric Acid | G4 Phenylethyl- amine |
| H1 α -D-Lactose | H2 D,L- α - Glycerol Phosphate | H3 D-Malic Acid | H4 Putrescine | H1 α -D-Lactose | H2 D,L- α - Glycerol Phosphate | H3 D-Malic Acid | H4 Putrescine | H1 α -D-Lactose | H2 D,L- α - Glycerol Phosphate | H3 D-Malic Acid | H4 Putrescine |

BAYNESFIELD ESTATE

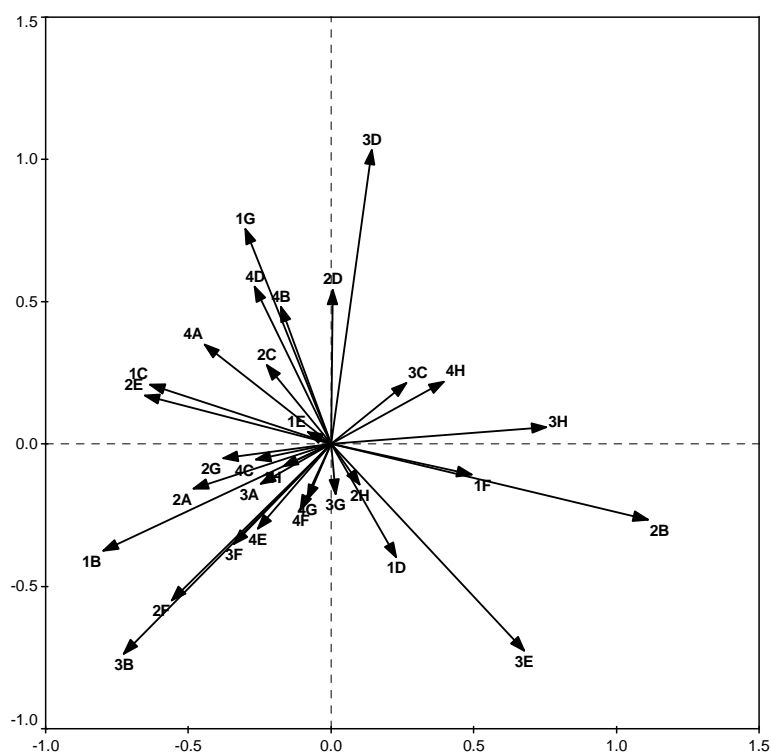


FIGURE D1 A PCA plot of all carbon sources along gradients of bacterial functional diversity at Baynesfield (Figure 6.2).

TABLE D2 MRPP of log-transformed data from Baynesfield bacterial functional diversity PCA (Figure 6.2)

| Land use | Av. distance | T | p-value |
|-------------|--------------|--------|---------|
| | | -2.762 | 0.005 |
| KIK 1, 2, 3 | 0.219 | | |
| SC 1, 2, 3 | 0.349 | | |
| PF 1, 2, 3 | 0.371 | | |
| W1, 2, 3 | 0.441 | | |
| M 1, 2, 3 | 0.578 | | |
| NAT 1, 2, 3 | 0.642 | | |

Distance measure: Sørensen (Bray-Curtis)

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

TABLE D3 Non-parametric one-way analysis (Kruskal-Wallis test) of catabolic richness (S) of soil bacterial communities (number of substrates utilized) under different land uses at Baynesfield Estate

| Species richness (S) | | non-significant (p = 0.1331) | | | | |
|---|-----------------|-------------------------------------|-----------------|----------------|-----------------|---------------|
| Multiple comparisons p values (2-tailed); S (BIOLOG) Baynesfield bacteria | | | | | | |
| Independent (grouping) variable: LAND USE | | | | | | |
| Kruskal-Wallis test: H (5, N = 18) = 8.449669 p = 0.1331 | | | | | | |
| Depend: S | KIK R:15.667 | M R: 4.0000 | NAT R:7.6667 | PF R:9.3333 | SC R:12.0000 | W R:8.3333 |
| KIK | | 0.111588 | 0.996861 | 1.000000 | 1.000000 | 1.000000 |
| M | 0.111588 | | 1.000000 | 1.000000 | 0.996861 | 1.000000 |
| NAT | 0.996861 | 1.000000 | | 1.000000 | 1.000000 | 1.000000 |
| PF | 1.000000 | 1.000000 | 1.000000 | | 1.000000 | 1.000000 |
| SC | 1.000000 | 0.996861 | 1.000000 | 1.000000 | | 1.000000 |
| W | 1.000000 | 1.000000 | 1.000000 | 1.000000 | 1.000000 | |

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

TABLE D4 Non-parametric one-way analysis (Kruskal-Wallis test) of catabolic evenness (J) (colour distribution) of soil bacterial communities under different land uses at Baynesfield Estate

| Species evenness (J) | | non-significant (p = 0.0706) | | | | |
|---|-----------------|-------------------------------------|-----------------|----------------|----------------|---------------|
| Multiple comparisons p values (2-tailed); J (BIOLOG) Baynesfield bacteria | | | | | | |
| Independent (grouping) variable: LAND USE | | | | | | |
| Kruskal-Wallis test: H (5, N = 18) = 10.16959 p = 0.0706 | | | | | | |
| Depend: J | KIK R:15.667 | M R:5.3333 | NAT R:6.0000 | PF R:8.0000 | SC R14.333: | W R:7.6667 |
| KIK | | 0.266368 | 0.398644 | 1.000000 | 1.000000 | 0.996861 |
| M | 0.266368 | | 1.000000 | 1.000000 | 0.584212 | 1.000000 |
| NAT | 0.398644 | 1.000000 | | 1.000000 | 0.838532 | 1.000000 |
| PF | 1.000000 | 1.000000 | 1.000000 | | 1.000000 | 1.000000 |
| SC | 1.000000 | 0.584212 | 0.838532 | 1.000000 | | 1.000000 |
| W | 0.996861 | 1.000000 | 1.000000 | 1.000000 | 1.000000 | |

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

TABLE D5 Non-parametric one-way analysis (Kruskal-Wallis test) of catabolic functional diversity (H') of soil bacterial communities (a composite of substrate richness and evenness) under different land uses at Baynesfield Estate

| Shannon index (H') non-significant ($p = 0.1129$) | | | | | | |
|--|----------|----------|----------|----------|----------|----------|
| Multiple comparisons p values (2-tailed); H (BIOLOG) Baynesfield bacteria | | | | | | |
| Independent (grouping) variable: LAND USE | | | | | | |
| Kruskal-Wallis test: H (5, N = 18) = 8.906433 p = 0.1129 | | | | | | |
| Depend: | KIK | M | NAT | PF | SC | W |
| H | R:16.000 | R:5.0000 | R:6.3333 | R:8.6667 | R12.667 | R:8.3333 |
| KIK | | 0.174253 | 0.398644 | 1.000000 | 1.000000 | 1.000000 |
| M | 0.174253 | | 1.000000 | 1.000000 | 1.000000 | 1.000000 |
| NAT | 0.398644 | 1.000000 | | 1.000000 | 0.838532 | 1.000000 |
| PF | 1.000000 | 1.000000 | 1.000000 | | 1.000000 | 1.000000 |
| SC | 1.000000 | 1.000000 | 1.000000 | 1.000000 | | 1.000000 |
| W | 1.000000 | 1.000000 | 1.000000 | 1.000000 | 1.000000 | |

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

TABLE D6 Summary of the results of the Monte Carlo permutation test of RDA data on the effects of land use on the bacterial communities at Baynesfield

| Axes | 1 | 2 | 3 | 4 | Total Variance |
|--|------------|-------|---------------|--------|----------------|
| Eigenvalue | 0.146 | 0.087 | 0.070 | 0.014 | 1.000 |
| Species-environment correlations | 0.910 | 0.865 | 0.868 | 0.550 | |
| Cumulative percentage variance of species data | 14.6 | 23.3 | 30.3 | 31.7 | |
| Cumulative percentage variance of species environment relation | 46.0 | 73.5 | 95.5 | 100.0 | |
| Sum of all eigenvalues | | | | | 1.000 |
| Sum of all canonical eigenvalues | | | | | 0.317 |
| Summary of Monte Carlo Test | | | | | |
| | Eigenvalue | Trace | F | P | |
| First canonical axis | 0.146 | | 2.219 | 0.022* | |
| All canonical axes | | 0.317 | 1.510 | 0.012* | |
| (499 permutations under reduced model) | | | * significant | | |

MOUNT EDGECOMBE

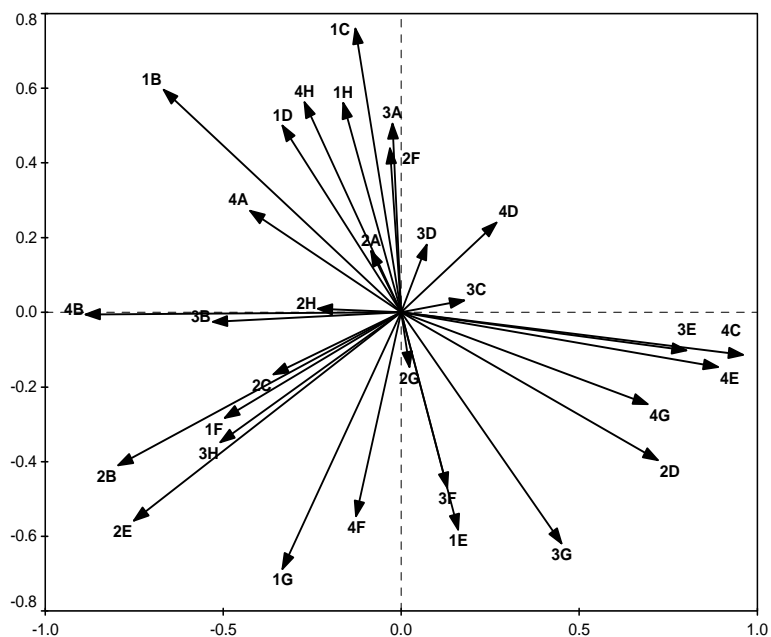


FIGURE D2 A PCA plot of all carbon sources along gradients of bacterial functional diversity at Mt Edgecombe (Figure 6.7).

TABLE D7 MRPP of log-transformed data from Mount Edgecombe bacterial functional diversity PCA (Figure 6.7)

| Land management | Av. distance | T | p-value |
|-----------------|--------------|--------|---------|
| | | -0.288 | 0.361 |
| TFo 1, 2, 3 | 0.271 | | |
| TF 1, 2, 3 | 0.316 | | |
| BtoFo 1, 2, 3 | 0.490 | | |
| BtoF 1, 2, 3 | 0.514 | | |

Distance measure: Sørensen (Bray-Curtis)

Key: T = green cane harvested with trash retention; Bto = burnt cane with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

TABLE D8 Non-parametric one-way analysis (Kruskal-Wallis test) of catabolic evenness (J) (colour distribution) of soil bacterial communities under different land treatments at Mount Edgecombe

| Species evenness (J) | | Non-significant (p = 0.7395) | | |
|---|---------------|-------------------------------------|-----------------|------------------|
| Multiple comparisons p values (2-tailed); J (BIOLOG) Mount Edgecombe bacteria | | | | |
| Independent (grouping) variable: TREATMENT | | | | |
| Kruskal-Wallis test: H (3, N = 12) = 1.2564 p = 0.7395 | | | | |
| Depend: J | TF R:6.333 | TFo R:7.333 | BtoF R:4.667 | BtoFo R:7.667 |
| TF | | 1.000000 | 1.000000 | 1.000000 |
| TFo | 1.000000 | | 1.000000 | 1.000000 |
| BtoF | 1.000000 | 1.000000 | | 1.000000 |
| BtoFo | 1.000000 | 1.000000 | 1.000000 | |

Key: T = green cane harvested with trash retention; Bto = burnt cane with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

TABLE D9 Non-parametric one-way analysis (Kruskal-Wallis test) of catabolic diversity (H') (a composite of substrate richness and evenness) of soil bacterial communities under different land treatments at Mount Edgecombe

| Species diversity (H') | | Non-significant (p = 0.1349) | | |
|---|---------------|-------------------------------------|-----------------|------------------|
| Multiple comparisons p values (2-tailed); H (BIOLOG) Mount Edgecombe bacteria | | | | |
| Independent (grouping) variable: TREATMENT | | | | |
| Kruskal-Wallis test: H (3, N = 12) = 5.564103 p = 0.1349 | | | | |
| Depend: H | TF R:6.667 | TFo R:10.333 | BtoF R:3.667 | BtoFo R:5.333 |
| TF | | 1.000000 | 1.000000 | 1.000000 |
| TFo | 1.000000 | | 1.141214 | 0.536576 |
| BtoF | 1.000000 | 1.141214 | | 1.000000 |
| BtoFo | 1.000000 | 0.536576 | 1.000000 | |

Key: T = green cane harvested with trash retention; Bto = burnt cane with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

TABLE D10 Summary of the results of the Monte Carlo permutation test of RDA data on the effects of land management on the bacterial communities at Mount Edgecombe

| Axes | 1 | 2 | 3 | 4 | Total Variance |
|--|------------|-------|-------|-------|----------------|
| Eigenvalue | 0.125 | 0.107 | 0.058 | 0.244 | 1.000 |
| Species-environment correlations | 0.896 | 0.750 | 0.656 | 0.000 | |
| Cumulative percentage variance of species data | 12.5 | 23.1 | 28.9 | 53.3 | |
| Cumulative percentage variance of species environment relation | 43.1 | 80.0 | 100.0 | 0.0 | |
| Sum of all eigenvalues | | | | | 1.000 |
| Sum of all canonical eigenvalues | | | | | 0.289 |
| Summary of Monte Carlo Test | | | | | |
| | Eigenvalue | Trace | F | P | |
| First canonical axis | 0.125 | | 1.138 | 0.708 | |
| All canonical axes | | 0.289 | 1.084 | 0.338 | |

(499 permutations under reduced model)

APPENDIX E

TABLE E1 The substrates in the wells of a BIOLOG SF-N2™ MicroPlate (BIOLOG, 2008)

| | | | | | | | | | | | |
|--|--|---|--|---|----------------------------------|---------------------------------------|-------------------------------------|--------------------------------|---|---|---|
| A1 Water | A2 α -Cyclodextrin | A3 Dextrin | A4 Glycogen | A5 Tween 40 | A6 Tween 80 | A7 N-Acetyl-D-Galactosamine | A8 N-Acetyl-D-Glucosamine | A9 Adonitol | A10 L-Arabinose | A11 D-Arabitol | A12 D-Cellulose |
| B1 i-Erythritol | B2 D-Fructose | B3 L-Fucose | B4 D-Galactose | B5 Gentiobiose | B6 α -D-Glucose | B7 m-Inositol | B8 α -D-Lactose | B9 Lactulose | B10 Maltose | B11 D-Mannitol | B12 D-Mannose |
| C1 D-Melibiose | C2 β -Methyl-D-Glucoside | C3 D- Psicose | C4 D-Raffinose | C5 L-Rhamnose | C6 D-Sorbitol | C7 Sucrose | C8 D-Trehalose | C9 Turannose | C10 Xylitol | C11 Pyruvic Acid Methyl Ester | C12 Succinic Acid Mono-Methyl Ester |
| D1 Acetic Acid | D2 Cis-Aconitic Acid | D3 Citric Acid | D4 Formic Acid | D5 D-Galactonic Acid Lactone | D6 D-Galacturonic Acid | D7 D-Gluconic Acid | D8 D-Glucosaminic Acid | D9 D-Glucuronic Acid | D10 α -Hydroxybutyric Acid | D11 β -Hydroxybutyric Acid | D12 γ -Hydroxybutyric Acid |
| E1 p-Hydroxy-phenylacetic Acid | E2 Itaconic Acid | E3 α -Ketobutyric Acid | E4 α -Ketoglutaric Acid | E5 α -Ketovaleric Acid | E6 D,L-Lactic Acid | E7 Malonic Acid | E8 Propionic Acid | E9 Quinic Acid | E10 D-Saccharic Acid | E11 Sebacic Acid | E12 Succinic Acid |
| F1 Bromosuccinic Acid | F2 Succinamic Acid | F3 Glucuronamide | F4 L-Alaninamide | F5 D-Alanine | F6 L-Alanine | F7 L-Alanyl-Glycine | F8 L-Asparagine | F9 L-Aspartic Acid | F10 L-Glutamic Acid | F11 Glycyl-L-Aspartic Acid | F12 Glycyl-L-Glutamic Acid |
| G1 L-Histidine | G2 Hydroxy-L-Proline | G3 L-Leucine | G4 L-Ornithine | G5 L-Phenylalanine | G6 L-Proline | G7 L-Pyroglutamic Acid | G8 D-Serine | G9 L-Serine | G10 L-Threonine | G11 D,L-Carnitine | G12 γ -Aminobutyric Acid |
| H1 Urocanic Acid | H2 Inosine | H3 Uridine | H4 Thymidine | H5 Phenylethyl-amine | H6 Putrescine | H7 2-Aminoethanol | H8 2,3-Butanediol | H9 Glycerol | H10 D,L, α -Glycerol Phosphate | H11 α -D-Glucose-1-Phosphate | H12 D-Glucose-6-Phosphate |

BAYNESFIELD ESTATE

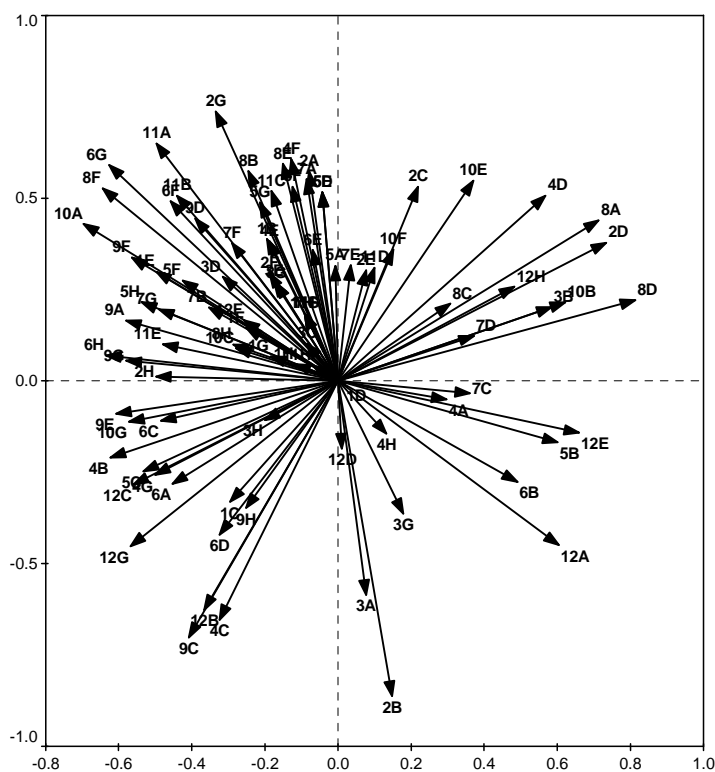


FIGURE E1 A PCA plot of all C sources along gradients of fungal functional diversity at Baynesfield (Figure 7.2).

TABLE E2 MRPP of log-transformed data from Baynesfield fungal functional diversity PCA (Figure 7.2)

| Land use | Av. distance | T | p-value |
|-------------|--------------|--------|---------|
| | | -2.996 | 0.004 |
| SC 1, 2, 3 | 0.123 | | |
| PF 1, 2, 3 | 0.204 | | |
| NAT 1, 2, 3 | 0.359 | | |
| M 1, 2, 3 | 0.407 | | |
| W1, 2, 3 | 0.442 | | |
| KIK 1, 2, 3 | 0.531 | | |

Distance measure: Sørensen (Bray-Curtis)

Key: SC = sugarcane; M = maize; KIK = kikuyu pasture; NAT = native grassland; PF = pine plantation; W = wattle plantation.

TABLE E3 Non-parametric one-way analysis (Kruskal-Wallis test) of catabolic richness (S) of soil fungal communities (number of substrates utilized) under different land uses at Baynesfield Estate

| Species richness (S) | | non-significant (p = 0.0740) | | | | |
|--|----------|-------------------------------------|-----------|----------|----------|----------|
| Multiple comparisons p values (2-tailed); S (BIOLOG) Baynesfield fungi | | | | | | |
| Independent (grouping) variable: LAND USE | | | | | | |
| Kruskal-Wallis test: H (5, N = 18) = 10.04279 p = 0.0740 | | | | | | |
| Depend: | KIK | M | NAT | PF | SC | W |
| S | R:3.3333 | R: 9.5000 | R: 9.3333 | R:15.667 | R:12.667 | R:6.5000 |
| KIK | | 1.000000 | 1.000000 | 0.069940 | 0.483852 | 1.000000 |
| M | 1.000000 | | 1.000000 | 1.000000 | 1.000000 | 1.000000 |
| NAT | 1.000000 | 1.000000 | | 1.000000 | 1.000000 | 1.000000 |
| PF | 0.069940 | 1.000000 | 1.000000 | | 1.000000 | 0.532016 |
| SC | 0.483852 | 0.996861 | 1.000000 | 1.000000 | | 1.000000 |
| W | 1.000000 | 1.000000 | 1.000000 | 0.532016 | 1.000000 | |

Key: SC = sugarcane (burnt cane harvested); M = maize (conventional tillage); KIK = permanent kikuyu pasture; NAT = native, undisturbed grassland; PF = pine plantation; W = wattle plantation.

TABLE E4 Summary of the results of the Monte Carlo permutation test of RDA data on the effects of land use on soil fungal communities at Baynesfield

| Axes | 1 | 2 | 3 | 4 | Total Variance |
|--|-------|-------|-------|-------|----------------|
| Eigenvalue | 0.153 | 0.086 | 0.062 | 0.039 | 1.000 |
| Species-environment correlations | 0.891 | 0.929 | 0.740 | 0.815 | |
| Cumulative percentage variance of species data | 15.3 | 23.9 | 30.1 | 34.0 | |
| Cumulative percentage variance of species environment relation | 44.9 | 70.3 | 88.5 | 100.0 | |
| Sum of all eigenvalues | | | | | 1.000 |
| Sum of all canonical eigenvalues | | | | | 0.340 |

Summary of Monte Carlo Test

| | eigenvalue | Trace | F | P |
|--|------------|-------|-------|---------------|
| First canonical axis | 0.153 | | 2.343 | 0.008* |
| All canonical axes | | 0.340 | 1.678 | 0.002* |
| (499 permutations under reduced model) | | | | * significant |

MOUNT EDGECOMBE

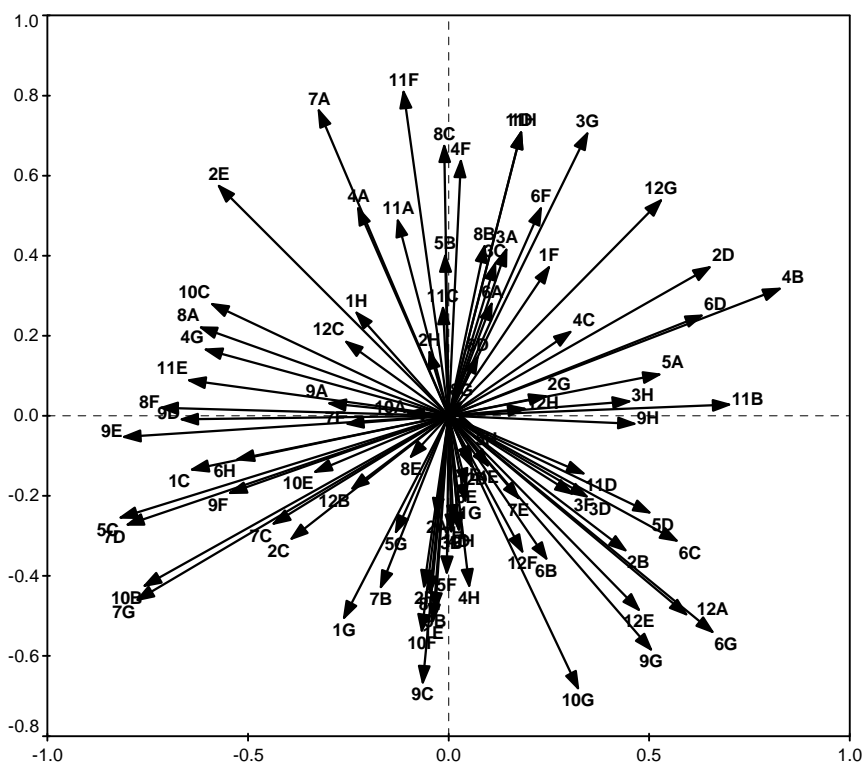


FIGURE E2 A PCA plot of all C sources along gradients of fungal functional diversity at Mount Edgecombe (Figure 7.7).

TABLE E5 MRPP of log-transformed data from Mount Edgecombe fungal functional diversity PCA (Figure 7.7)

| Land management | Av. distance | T | p-value |
|-----------------|--------------|--------|---------|
| | | -1.895 | 0.038 |
| TF 1, 2, 3 | 0.293 | | |
| TFo 1, 2, 3 | 0.332 | | |
| BtoF 1, 2, 3 | 0.377 | | |
| BtoFo 1, 2, 3 | 0.388 | | |

Distance measure: Sørensen (Bray-Curtis)

Key: T = green cane harvested with trash retention; Bto = burnt cane with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

TABLE E6 Non-parametric one-way analysis (Kruskal-Wallis test) of fungal catabolic richness under different treatments at Mount Edgecombe

| Species richness (S) | | Non-significant (p = 0.7069) | | |
|---|----------|-------------------------------------|----------|----------|
| Multiple comparisons p values (2-tailed); S (BIOLOG) Mt Edgecombe fungi | | | | |
| Independent (grouping) variable: TREATMENT | | | | |
| Kruskal-Wallis test: H (3, N = 12) = 1.394366 p = 0.7069 | | | | |
| Depend: | TF | TFo | BtoF | BtoFo |
| S | R:7.8333 | R:5.8333 | R:4.8333 | R:7.5000 |
| TF | | 1.000000 | 1.000000 | 1.000000 |
| TFo | 1.000000 | | 1.000000 | 1.000000 |
| BtoF | 1.000000 | 1.000000 | | 1.000000 |
| BtoFo | 1.000000 | 1.000000 | 1.000000 | |

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

TABLE E7 Non-parametric one-way analysis (Kruskal-Wallis test) of fungal catabolic evenness under different treatments at Mount Edgecombe

| Species evenness (J) | | Non-significant (p = 0.5360) | | |
|---|----------|-------------------------------------|----------|----------|
| Multiple comparisons p values (2-tailed); J (BIOLOG) Mt Edgecombe fungi | | | | |
| Independent (grouping) variable: TREATMENT | | | | |
| Kruskal-Wallis test: H (3, N = 12) = 2.179487 p = 0.5360 | | | | |
| Depend: | TF | TFo | BtoF | BtoFo |
| J | R:8.3333 | R:7.6667 | R:4.6667 | R:5.3333 |
| TF | | 1.000000 | 1.000000 | 1.000000 |
| TFo | 1.000000 | | 1.000000 | 1.000000 |
| BtoF | 1.000000 | 1.000000 | | 1.000000 |
| BtoFo | 1.000000 | 1.000000 | 1.000000 | |

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

TABLE E8 Non-parametric one-way analysis (Kruskal-Wallis test) of fungal catabolic diversity under different treatments at Mount Edgecombe

| Shannon Diversity Index (H') | | | | |
|---|----------|----------|----------|----------|
| Non-significant ($p = 0.8260$) | | | | |
| Multiple comparisons p values (2-tailed); H(BIOLOG) Mt Edgecombe fungal diversity | | | | |
| Independent (grouping) variable: TREATMENT | | | | |
| Kruskal-Wallis test: $H(3, N = 12) = 0.8974359$ $p = 0.8260$ | | | | |
| Depend: | TF | TFo | BtoF | BtoFo |
| H | R:7.6667 | R:6.3333 | R:5.0000 | R:7.0000 |
| TF | | 1.000000 | 1.000000 | 1.000000 |
| TFo | 1.000000 | | 1.000000 | 1.000000 |
| BtoF | 1.000000 | 1.000000 | | 1.000000 |
| BtoFo | 1.000000 | 1.000000 | 1.000000 | |

Key: T = green cane harvested with trash retention; Bto = burnt with harvest residues removed; F = fertilized annually with N, P and K; Fo = unfertilized.

TABLE E9 Summary of the results of the Monte Carlo permutation test of RDA data on the effects of land management on soil fungal communities at Mount Edgecombe

| Axes | 1 | 2 | 3 | 4 | Total Variance |
|--|-------|-------|-------|-------|----------------|
| Eigenvalue | 0.153 | 0.117 | 0.056 | 0.143 | 1.000 |
| Species-environment correlations | 0.909 | 0.931 | 0.929 | 0.000 | |
| Cumulative percentage variance of species data | 15.3 | 27.0 | 32.6 | 46.9 | |
| Cumulative percentage variance of species environment relation | 47.1 | 82.9 | 100.0 | 0.0 | |
| Sum of all eigenvalues | | | | | 1.000 |
| Sum of all canonical eigenvalues | | | | | 0.326 |

Summary of Monte Carlo Test

| | eigenvalue | Trace | F | P | |
|----------------------|------------|-------|-------|--------|-----------|
| First canonical axis | 0.153 | | 1.448 | 0.0620 | Near sig. |
| All canonical axes | | 0.326 | 1.288 | 0.0600 | Near sig. |

(499 permutations under reduced model)