
**MEASURING REHABILITATION SUCCESS OF COAL
MINING DISTURBED AREAS**

**A SPATIAL AND TEMPORAL INVESTIGATION INTO THE USE
OF SOIL MICROBIAL PROPERTIES AS ASSESSMENT CRITERIA**

Sarina Claassens (M.Env.Sci)

Thesis submitted for the degree Philosophiae Doctor in Environmental Sciences at the
Potchefstroom Campus of the North-West University

Promoter: Prof. L. van Rensburg
Co-promoter: Dr. M.S. Maboeta

May 2007
Potchefstroom

To my parents, whose loving support never fails their children

"You are never dedicated to something you have complete confidence in. No one is fanatically shouting that the sun is going to rise tomorrow. They know it is going to rise tomorrow. When people are fanatically dedicated to political or religious faiths or any kinds of dogmas or goals, it's always because these dogmas or goals are in doubt."

— Robert M. Pirsig, *Zen and the Art of Motorcycle Maintenance*

TABLE OF CONTENTS

Acknowledgements -----	i
Preface -----	ii
Summary -----	iii
Key terms -----	iii
Opsomming -----	iv
Sleuteltermen -----	iv
List of Figures -----	v
List of Tables -----	vi
List of Abbreviations -----	vii

CHAPTER 1: INTRODUCTION

1. BACKGROUND-----	2
1.1. The Importance of Microorganisms in the Maintenance of Soil Quality-----	2
1.2. The Impact of Environmental Disturbance on Soil Ecosystems-----	5
1.3. Assessing Soil Quality-----	8
1.4. The Space-for-Time Hypothesis-----	18
2. PERSPECTIVE, AIM, AND OUTLINE OF THESIS-----	20
REFERENCES-----	23

CHAPTER 2: GENERAL MATERIALS AND METHODS

1. SITE DESCRIPTION-----	30
2. SAMPLING PROCEDURE-----	33
3. ESTIMATION OF VEGETATION COVER-----	34
4. PHYSICAL AND CHEMICAL SOIL ANALYSIS-----	34
5. ASSAYS OF ENZYMATIC ACTIVITIES-----	35
5.1. Dehydrogenase Activity-----	35
5.2. B-glucosidase and Phosphomonoesterase Activities-----	36
5.3. Urease Activity-----	37
6. LIPID EXTRACTION, FRACTIONATION, AND ANALYSIS-----	38
7. STATISTICAL ANALYSIS-----	39
REFERENCES-----	41

CHAPTER 3: SOIL MICROBIAL COMMUNITY FUNCTION IN A POST-MINING CHRONOSEQUENCE

1. INTRODUCTION-----	44
2. MATERIALS AND METHODS-----	45
2.1. Site Description and Sampling-----	45

2.2. Estimation of Vegetation Cover-----	46
2.3. Physical and Chemical Soil Analysis-----	46
2.4. Assays of Enzymatic Activities -----	46
2.5. Statistical Analysis -----	46
3. RESULTS AND DISCUSSION-----	47
3.1. Physical and Chemical Soil Properties -----	47
3.2. Enzymatic Activities-----	51
4. CONCLUSIONS-----	53
REFERENCES-----	55

CHAPTER 4: SOIL MICROBIAL COMMUNITY STRUCTURE IN A POST-MINING CHRONOSEQUENCE

1. INTRODUCTION-----	58
2. MATERIALS AND METHODS-----	58
2.1. Site Description and Sampling -----	58
2.2. Estimation of Vegetation Cover-----	59
2.3. Physical and Chemical Soil Analysis-----	59
2.4. Lipid Extraction, Fractionation, and Analysis -----	59
2.5. Statistical Analysis -----	60
3. RESULTS AND DISCUSSION-----	60
3.1. Physical and Chemical Soil Properties -----	60
3.2. Phospholipid Fatty Acid Analysis-----	60
4. CONCLUSIONS-----	66
REFERENCES-----	67

CHAPTER 5: MICROBIAL MEASURES OF REHABILITATION SUCCESS OF COAL DISCARD IN TWO POST-MINING CHRONOSEQUENCES

1. INTRODUCTION-----	70
2. MATERIALS AND METHODS-----	71
2.1. Site Description and Sampling -----	71
2.2. Estimation of Vegetation Cover-----	72
2.3. Physical and Chemical Soil Analysis-----	72
2.4. Assays of Enzymatic Activities -----	72
2.5. Lipid Extraction, Fractionation, and Analysis -----	73
2.6. Statistical Analysis -----	73
3. RESULTS AND DISCUSSION-----	74
4. CONCLUSIONS-----	88
REFERENCES-----	90

CHAPTER 6: GENERAL DISCUSSION AND CONCLUDING REMARKS

1. GENERAL DISCUSSION OF RESULTS -----	93
--	----

1.1. Microbial Community Function and Structure in a Post-Mining Chronosequence	93
1.2. The Assessment of Reference Sites	96
1.3. The Comparison of Two Chronosequences	97
2. CONCLUDING OBSERVATIONS	99
3. CLOSING REMARKS AND RECOMMENDATIONS	101
REFERENCES	103
APPENDIX A	104

ACKNOWLEDGEMENTS

Like most achievements in life, this one would not have been possible on my own. Before I render thanks to all those who have contributed to the successful completion of this study, I want to give all the honour to my Heavenly Father. I am so thankful for the talents He gave me and for the opportunity to do something as fulfilling as this.

My promoters were invaluable in helping me put everything to paper. Leon – thank you for your constructive criticism of the manuscript and for always having an open door. Mark – I really appreciate you coming on board this project with me. Thank you for your many helpful suggestions on the manuscript.

I am deeply indebted to Peet Jansen van Rensburg, my co-investigator on the greater NRF Thuthuka project of which this thesis forms a part. Peet – without you, I would not have come this far and this thesis would never have seen the light of day. You were the one I constantly bombarded with questions (always urgent!) and the one who often had to listen when things got tough. Your sound ideas on the project and clear comments on the manuscript contributed greatly to its realisation. Thank you for your patience, your kindness, and the many hours you have spent working with me. Thank you for your wisdom, your humour, and your good-hearted nature. You made this a wonderful experience for me and I am delighted to call you my friend.

My sincerest appreciation to Jaco Bezuidenhout for assisting (so patiently) with some of the statistical aspects of the study and to the technicians who helped throughout the project.

To my family and friends – for your patience and support that know no bounds, I can only be grateful. Thank you for your love and encouragement. Michelle, thank you for always being there, no matter how far we are apart. Daléne, you are a remarkable friend and if not for you, I might still be stuck – thank you for that little bit of inspiration when I needed it most. I am so grateful to have wonderful parents. Mom and Dad, thank you for your genuine interest in what I do, for believing in me, and for always doing your best for your children. The examples you set taught us the truly important things in life and gave us the platform from which to launch successful lives. I love you both.

I wish to acknowledge the mining companies involved in this project for their cooperation and for access to the rehabilitated sites.

This material is based upon work financially supported by the National Research Foundation (NRF), South Africa.

PREFACE

The experimental work discussed in this thesis was conducted during the period of February 2004 to May 2007 in the School of Environmental Sciences and Development, North-West University, Potchefstroom Campus, Potchefstroom, South Africa.

The research conducted and presented in this thesis represents original work undertaken by the author and has not been previously submitted for degree purposes to any university. Where use has been made of the work of other researchers, it is duly acknowledged in the text.

The reference style used in this thesis is according to the specifications given by the Council of Biology Editors (CBE) Scientific Style using the name-year system (<http://writing.colostate.edu/references/sources/cbe/index.cfm>).

Any opinion, findings, and conclusions or recommendations expressed in this material are those of the author and therefore the NRF does not accept any liability in regard thereto.

SUMMARY

The rehabilitation of degraded soils, such as those associated with post-mining sites, requires knowledge of the soil ecosystem and its physical, chemical, and biological composition in order for rehabilitation efforts to fulfil the long-term goal of reconstructing a stable ecosystem for rehabilitated mine soil. This study addresses the need for appropriate assessment criteria to determine the progress of rehabilitation and subsequently the success of management practices.

Significant contributions made by this investigation included the establishment of minimum and maximum values for microbial community measurements from two case studies of rehabilitated coal discard sites. Furthermore, it was shown that there was no relationship between changes in microbial community function and structure and the rehabilitation age of the sites. Following this, the considerable impact of management practices on microbial communities was illustrated.

The first part of the study investigated the temporal changes in microbial community function and structure in a chronosequence of rehabilitated coal discard sites aged 1 to 11 years. The most important observation made during the investigation of the microbial communities in the different aged soil covers of the rehabilitated coal discard sites, was that there was no relationship between rehabilitation age and microbial activity or abundance of certain microbial groups. What was responsible for a clear differentiation between sites and a shift in microbial community attributes was the management practices applied.

A comparison of two chronosequences of rehabilitated coal discard sites was achieved by an application of the 'space-for-time' hypothesis. Sites of different ages and at separate locations ('space') were identified to obtain a chronosequence of ages ('time'). The two chronosequences included sites aged 1 to 11 years (chronosequence A) and 6 to 17 years (chronosequence B), respectively. Sites in the same chronosequence were managed identically, while there was a distinct difference in management practices applied to each chronosequence. The long-term effect of the different management regimes on the soil microbial community function and structure was investigated. Again, there was no relationship between rehabilitation age and microbial community measurements. Fluctuations of selected microbial properties occurred in both chronosequences and similar temporal trends existed over the rehabilitation periods. However, the less intensively managed chronosequence (B) seemed more stable (less fluctuation occurred) over the rehabilitation period than the more intensively managed chronosequence (A). It was therefore concluded that the microbial communities in the less managed sites maintained their functional and structural integrity within bounds in the absence of management inputs or disturbance. While there was similarity in the trends over time for individual microbial community measurements, the seemingly more stable conditions in chronosequence B are important in terms of the goal of rehabilitation.

Key terms: chronosequence; coal discard; enzymatic activity; management; microbial community; phospholipid fatty acid; rehabilitation.

OPSOMMING

Die rehabilitasie van gedegradeerde grond, soos die grond wat geassosieer word met terreine waar voorheen mynbedrywighede plaasgevind het, vereis 'n grondige kennis van die grondekosisteam en die fisiese, chemiese, en biologiese samestelling daarvan. Indien hierdie kennis afwesig is, mag rehabilitasie praktyke dalk nie die lang termyn doel van 'n stabiele en volhoubare ekosisteam vir gerehabiliteerde myngrond bereik nie. Hierdie studie spreek die behoefte vir toepaslike assesseringskriteria om die progressie van rehabilitasie en gevolglik die sukses van bestuurspraktyke te bepaal aan.

Belangrike bydraes wat deur hierdie ondersoek gelewer is, sluit in die vasstelling van minimum en maksimum waardes vir mikrobiiese gemeenskapsmetings afkomstig van twee gevallestudies van gerehabiliteerde steenkoolafvalterreine. Verder, is daar getoon dat geen verhouding bestaan tussen veranderinge in mikrobiiese gemeenskapsfunksie en –struktuur en die rehabilitasie ouderdom van die terreine nie. Gevolglik is die beduidende impak van bestuurspraktyke op mikrobiiese gemeenskappe geïllustreer.

Die eerste gedeelte van die studie het die temporale veranderinge in mikrobiiese gemeenskapsfunksie en –struktuur in 'n chronovolgorde van gerehabiliteerde steenkoolafvalterreine van ouderdomme 1 tot 11 jaar ondersoek. Die belangrikste waarneming wat tydens hierdie ondersoek gemaak is, is dat daar geen verhouding was tussen rehabilitasie-ouderdom en mikrobiiese aktiwiteit of die voorkoms van sekere groepe mikroörganismes nie. Daar kon wel 'n duidelike onderskeid getref word tussen die mikrobiologiese eienskappe van die terreine op grond van die bestuurspraktyke wat toegepas is.

'n Vergelyking tussen twee chronovolgordes van gerehabiliteerde steenkoolafvalterreine is gemaak deur 'n toepassing van die 'ruimte-vir-tyd' hipotese. Terreine van verskillende ouderdomme en met verskillende liggings ('ruimte') is geïdentifiseer om 'n chronovolgorde van ouderdomme ('tyd') te verkry. Die twee chronovolgordes het terreine ingesluit met ouderdomme van 1 tot 11 jaar (chronovolgorde A) en 6 tot 17 jaar (chronovolgorde B), respektiewelik. Terreine in dieselfde chronovolgorde is identies bestuur, terwyl daar 'n pertinente verskil was in die bestuurspraktyke wat op elke chronovolgorde toegepas is. Die lang termyn effek van die verskillende bestuurswyses op die grondmikrobiiese gemeenskapsfunksie en –struktuur is ondersoek. Weereens, was daar geen verband tussen rehabilitasie-ouderdom en mikrobiiese gemeenskapsmetings nie. Fluktuasies in geselekteerde mikrobiiese eienskappe het in beide chronovolgordes voorgekom en soortgelyke temporale tendense is oor die rehabilitasieperiodes waargeneem. Nogtans, het die chronovolgorde onder minder intensiewe bestuur (chronovolgorde B), meer stabiel voorgekom (minder fluktuasie het plaasgevind) oor die rehabilitasieperiode as chronovolgorde A wat meer intensief bestuur is. Om hierdie rede is daar tot die gevolgtrekking gekom dat mikrobiiese gemeenskappe op die terreine van chronovolgorde B (minder intensiewe bestuur) hulle funksionele en strukturele integriteit binne perke kon handhaaf in die afwesigheid van bestuursinsette of versteuring. Terwyl daar ooreenkomstigheid was in die tendense oor tyd vir individuele mikrobiiese gemeenskapsmetings, is die klaarblyklik meer stabiele toestand in chronovolgorde B van belang in terme van die doel van rehabilitasie.

Sleuteltermes: bestuur; chronovolgorde; ensiematiese aktiwiteit; fosfolipied-vetsuur; mikrobiiese gemeenskap; rehabilitasie; steenkoolafval.

LIST OF FIGURES

Figure 1.1. Possible temporal trends in dynamic soil quality assessments (Karlen <i>et al.</i> , 2003) -----	3
Figure 1.2. Graphical representation of trajectory of resistance and resilience in perturbed systems. Broken line shows time course of response variable in unperturbed (control) systems, solid line shows response following perturbation. Resistance is measured as the degree of impairment of response relative to control; resilience as the rate and extent of recovery. Recovery may be incomplete within the measured timescale (Ritz <i>et al.</i> , 2003) -----	4
Figure 1.3. Classification of phospholipid fatty acids (PLFAs) (Kaur <i>et al.</i> , 2005) -----	14
Figure 3.1. Principal components analysis (PCA) ordination diagram illustrating the relationship between coal discard sites based on physical and chemical soil properties. Each site is indicated according to the name of the site, followed by the time of sampling and the rehabilitation age in brackets, e.g. Site 1 sampled in 2002 was 1 year old: 1_2002 (1) -----	48
Figure 3.2. Enzymatic activities of topsoil covers from the coal discard sites (Mine A) for 2002, 2004, and 2005: (a) dehydrogenase, (b) β -glucosidase, (c) acid phosphatase, (d) alkaline phosphatase, and (e) urease -----	52
Figure 4.1. Ratios of fungal to bacterial PLFA (Frostegård and Bååth, 1996) in the coal discard sites (Mine A) for 2002 (a), 2004 (c), and 2005 (e), and Gram positive PLFA markers to total PLFA for 2002 (b), 2004 (d), and 2005 (f). Gram positive bacteria were i10me16:0, i15:0, a15:0, i16:0, and 17:0 (McKinley <i>et al.</i> , 2005) -----	64
Figure 5.1. Changes in dehydrogenase activity during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence -----	79
Figure 5.2. Changes in β -glucosidase activity during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence -----	80
Figure 5.3. Changes in alkaline phosphatase activity during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence -----	81
Figure 5.4. Changes in acid phosphatase activity during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence -----	82
Figure 5.5. Changes in urease activity during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence -----	83
Figure 5.6. Changes in microbial biomass during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence -----	84
Figure 5.7. Changes in the fungal to bacterial ratio during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence -----	85
Figure 5.8. Canonical correspondence analysis (CCA) ordination diagram illustrating the relationship between the coal discard sites of chronosequences A and B based on enzymatic activities and phospholipid fatty acid (PLFA) profiles. Each site is indicated according to the chronosequence to which it belongs (A or B) followed by the rehabilitation age of the site -----	87
Figure 6.1. Canonical correspondence analysis (CCA) ordination diagram illustrating the relationship between the coal discard sites of chronosequence A based on enzymatic activities and phospholipid fatty acid (PLFA) profiles. Each site is indicated according to the name of the site, followed by the time of sampling and the rehabilitation age in brackets, e.g. Site 1 sampled in 2002 was 1 year old: 1_2002 (1) -----	95

LIST OF TABLES

Table 1.1. Major phospholipid fatty acid (PLFA) groups associated with the membranes of various microorganisms (Guckert <i>et al.</i> , 1985; Olsson, 1999; Ponder and Tadros, 2002; Peacock, 2005) -----	16
Table 2.1. Coal discard sites sampled to derive the two chronosequences of rehabilitation ages ranging from 1 to 11 years and 6 to 17 years, respectively -----	32
Table 2.2. Locations of coal discard sites sampled to derive the two chronosequences of rehabilitation ages -----	33
Table 3.1. Physico-chemical properties and vegetation cover of topsoil covers obtained from the coal discard sites managed by Mining Company A -----	49
Table 4.1. Phospholipid fatty acid (PLFA) composition and ratios of topsoil covers obtained from the coal discard sites managed by Mining Company A -----	62
Table 5.1. Physico-chemical properties and vegetation cover of topsoil covers obtained from the coal discard sites managed by Mining Company B -----	76
Table 5.2. Phospholipid fatty acid (PLFA) composition and ratios of topsoil covers obtained from the coal discard sites managed by Mining Company B -----	77
Table 6.1. Enzymatic activities, phospholipid fatty acid (PLFA) composition, and PLFA ratios of soil samples obtained from reference sites in 2002 -----	96
Table 6.2. Minimum and maximum values for enzymatic activities, phospholipid fatty acid (PLFA) composition, and PLFA ratios of obtained from individual sites of chronosequences A and B over the study period (2002 – 2005) -----	98

LIST OF ABBREVIATIONS

AcdP	acid phosphatase
AlkP	alkaline phosphatase
ANOVA	analysis of variance
ATP	adenosine triphosphate
BRANC	branched chain fatty acid
C	carbon
Ca	calcium
CCA	canonical correspondence analysis
CEC	cation exchange capacity
CFE	chloroform fumigation extraction
CFI	chloroform fumigation incubation
Cl	chloride
DNA	deoxyribonucleic acid
EC	electrical conductivity
EL	ester-linked
FAME	fatty acid methyl ester
F:B	fungal to bacterial
HYFA	hydroxyl substituted fatty acid
INF	iodonitrotetrazolium violet-formazan
INT	iodonitrotetrazolium chloride
K	potassium
LPS	lipopolysaccharide
Mg	magnesium
MUFA	monounsaturated fatty acid
N	nitrogen
Na	sodium
NEL	non-ester linked
NH ₄	ammonia
NO ₃	nitrate
P	phosphorus
PCA	principal components analysis
PLFA	phospholipid fatty acid
PUFA	polyunsaturated fatty acid
S	sulphur
SATFA	saturated fatty acid
SFT	space-for-time
SO ₄	sulphate
SS	sum-of-squares
STRA	straight chain fatty acid
THAM	Tris (hydroxymethyl)-aminomethane
UNFA	unsubstituted fatty acid

CHAPTER 1

INTRODUCTION

*"Vapor from the sea;
rain, snow, and ice on the summits;
glaciers and rivers — these form a wheel that grinds the mountains thin and
sharp,
sculptures deeply the flanks,
and furrows them into ridge and canyon, and crushes
the rocks into soils
on which the forests and the meadows and gardens and fruitful
vine and tree and grain are growing"*

– John Muir, John of the Mountains: The Unpublished Journals of John Muir

1. BACKGROUND

1.1. The Importance of Microorganisms in the Maintenance of Soil Quality

Soil is a vital natural resource that is non-renewable on a human time-scale (Tate, 2000) and should therefore be preserved and if possible, its quality and productive capacity must be improved (Izquierdo *et al.*, 2005). It is a key component of the biosphere and soil quality is intrinsically linked to overall environmental quality and ultimately, sustainability (Marcote *et al.*, 2001). In recent years, the interconnected and mutually dependent relationship of soil quality to water and atmospheric quality has been recognised and research has focussed increasingly on sustaining and/or improving the quality of soils (Karlen *et al.*, 2003). The capacity of soil to function in a manner that upholds vital soil processes depends on the health or quality of that soil. According to Harris and Bezdicek (1994), the terms ‘soil quality’ and ‘soil health’ are often used in the same context in literature with scientists generally giving preference to soil quality and producers to soil health. The two terms are also sometimes used without qualification. Use of the term soil health depicts soil as a living, dynamic organism as opposed to soil quality, which rather gives a description of the physical, chemical, and biological characteristics (Doran and Safley, 1997). Although the literature provides a profusion of explanations for ‘soil quality’, a widely accepted definition is that of Doran and Safley (1997): “*Soil quality is the capacity of a soil to function within ecosystem and land-use boundaries, to sustain biological productivity, maintain environmental quality and promote plant, animal, and human health*”. Soil functions to produce plant biomass; maintain animal and human health; recycle nutrients; store carbon; partition rainfall; buffer anthropogenic acidity; remediate added animal and human wastes; and regulate energy transformations (Doran and Safley, 1997; Pascual *et al.*, 2000; Ruf *et al.*, 2003). Soil quality is influenced by a suite of physical, chemical, and biological properties that affect each other and the overall state of quality in the soil ecosystem (Karlen *et al.*, 2003).

Soil quality assessments indicate three possible temporal trends, namely aggrading, sustaining, or degrading (Figure 1.1) (Karlen *et al.*, 2003). It is important to note that the soil ecosystem is still dynamic and functioning, irrespective of which of these states it occupies. However, the level at which soil processes are maintained may be significantly compromised. In a degrading state, soil function would be impaired and may become increasingly compromised, while soil in an aggrading state tends to show enhanced function. A stable ecosystem would be one capable of sustaining its homeostatic state

within bounds – a function that could be attributed to the degree of resistance and/or resilience of that system (Beedlow *et al.*, 1988; Orwin and Wardle, 2004).

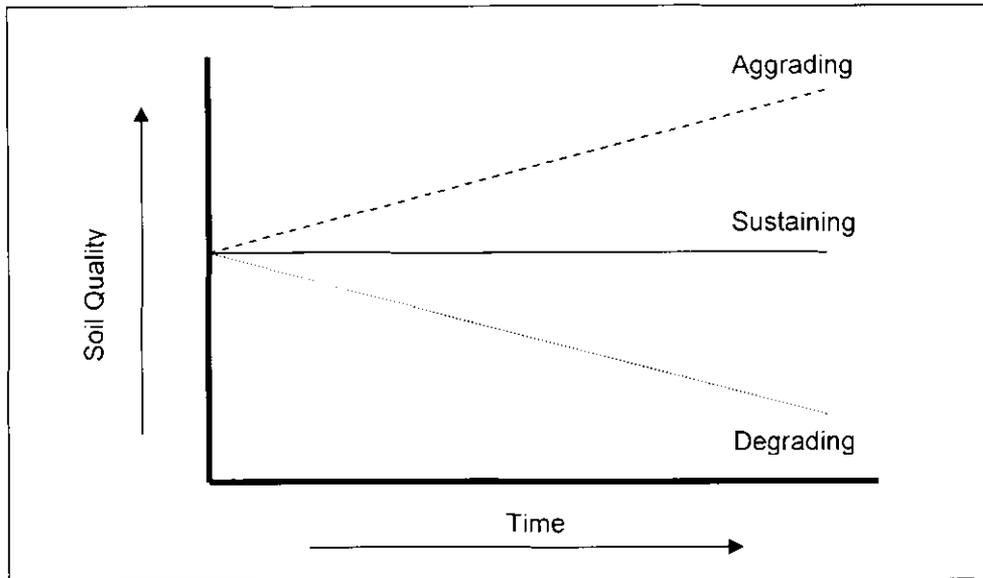


Figure 1.1. Possible temporal trends in dynamic soil quality assessments (Karlen *et al.*, 2003).

Resilience refers to the ability of the system to return to a state of equilibrium after it has been disturbed, in other words, to regain functional and structural characteristics that may have been subjected to stress or disturbance and return to a pre-disturbance level. On the other hand, resistance describes the amount of change caused by a disturbance and the ability of the system to maintain functional and structural equilibrium under conditions of stress (Figure 1.2) (SER, 2004; Ritz *et al.*, 2003; Orwin and Wardle, 2004). Considering the frequency of disturbance to the soil environment – whether natural or anthropogenic, it is clear that resilience and/or resistance in soil ecosystems are essential in order for these systems to maintain a certain degree of normal functionality. The relationship between ecosystem stability and microbial function is complex and the focal point of much contemporary research. What is obvious, is that microbial populations are of great significance in the maintenance of several fundamental soil processes and overall soil quality (Doran and Zeiss, 2000; Ritz *et al.*, 2003).

Microorganisms are interlaced into all the systems that support life on earth (Hawksworth, 1996) and microbial communities fulfil unique functional roles that are vital to the upholding of fundamental soil processes. Together with exocellular enzymes and other soil biota, they conduct all known metabolic reactions in the soil they inhabit. Soil microorganisms that produce trace gases (such as methane), can be applied as biocontrol

tools and are part of the food chains and food webs on which all macroorganisms depend (Richards, 1994; Ashman and Puri, 2002).

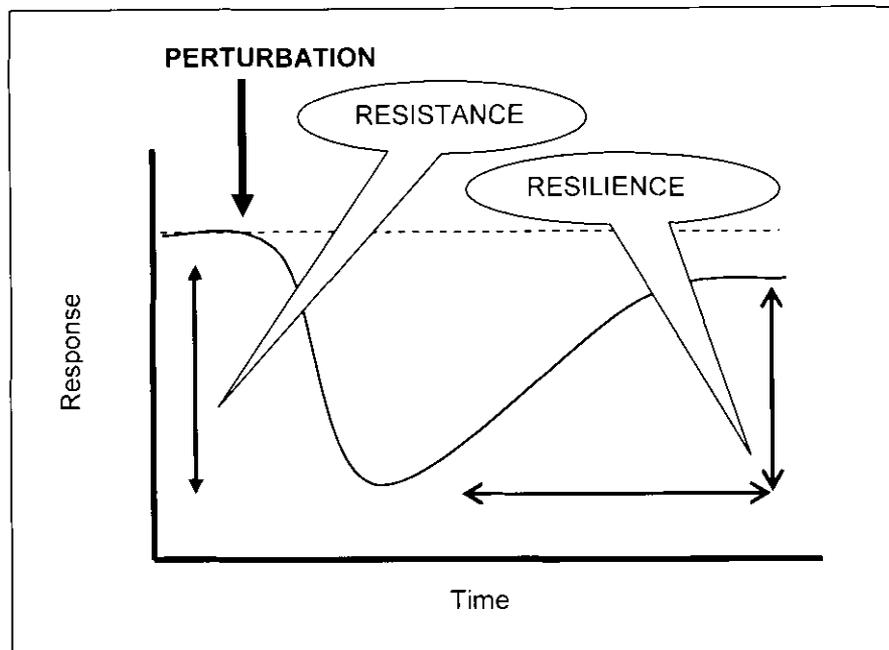


Figure 1.2. Graphical representation of trajectory of resistance and resilience in perturbed systems. Broken line shows time course of response variable in unperturbed (control) systems, solid line shows response following perturbation. Resistance is measured as the degree of impairment of response relative to control; resilience as the rate and extent of recovery. Recovery may be incomplete within the measured timescale (Ritz *et al.*, 2003).

Soil microbial communities are also central to processes of nutrient cycling; maintenance of soil structure; degradation of pollutants; and aspects pertaining to human, plant, and animal health (Doran and Zeiss, 2000; Ritz *et al.*, 2003). They play crucial roles in the biogeochemical cycling of C, N, P, and S (Bandick and Dick, 1999; Masciandaro and Ceccanti, 1999; Aon and Colaneri, 2001). Physical and chemical soil properties, such as pH, cation exchange capacity, salinity, solubility of soil mineral components, and aggregate stability are constantly being altered by the activities of soil microorganisms (Tate, 2000). It is understood that at least some minimum number of species are essential for ecosystem functioning under steady conditions and that high species diversity is probably essential for maintaining ecosystem stability (Nannipieri *et al.*, 2003). Consequently, the loss of functional groups of microorganisms performing essential ecological roles will lead to ecosystem modification (Hawksworth, 1996). It therefore follows that soil microbial communities are of great importance in restoring fertility in degraded soils (Harris and Birch, 1989) and the value of a diverse, resilient soil microbial

community in the development of sustainable soil ecosystems has been recognised for some time (Tate and Rogers, 2002).

1.2. The Impact of Environmental Disturbance on Soil Ecosystems

Land degradation has become a problem of global concern. Soil has been recognised for its value as a natural resource and accordingly, serious concerns exist regarding anthropogenic activities, including civil engineering programmes (such as opencast coal mining) and intensive agriculture that causes environmental degradation (Harris, 2003). The past half a century has seen considerable losses in terms of healthy soil ecosystems worldwide. Of the 8.7 billion hectares of agricultural land, permanent pastures, forests, and woodlands, around 2 billion hectares have been degraded (Arshad and Martin, 2002). In 1999, the National State of the Environment Report for South Africa indicated the average soil loss as 2.5 tonnes per hectare per annum. At that stage, the rate of soil loss in South Africa was estimated at eight times that of the rate of soil formation – a clearly unsustainable situation (DEAT, 2007). Although more recent estimates are unavailable, it can only be expected, with regard to land-use practices and the difficulties associated with restoration of damaged environments, that the situation has at best stayed unchanged.

Mining in South Africa provides a vast contribution to the economy, both in terms of the actual materials that are mined and in the creation of literally hundreds of thousands of jobs, with benefits to many aspects of society (Mining Review Africa, 2003). However, mining activities inherently hold extensive adverse effects for the biophysical, social, and economic environment and results in severe disturbance of large land areas (Milton, 2001; Mummey *et al.*, 2002a). The natural grassland biome of South Africa is poorly conserved and its fragmentation by an abundance of mine tailings and discard sites leads to degradation of environmental quality and eventually affects human living standards (Van Wyk, 2002). Tailings material is processed at a rate of millions of tonnes per year (Rösner *et al.*, 2001) and massive tailings dams originating from coal, gold, and base metal mining litters the South African landscape. In 1996, the mining industry was responsible for the production of 377 million tonnes of tailings (Van Wyk, 2002) and is still the principal contributor to the solid waste stream (72.3%) (DEAT, 2007). Cyanide compounds, heavy metals, radionuclides, and asbestos are all possible components of mine waste. If mine waste is not managed properly, it represents a potential hazard for surrounding ecosystems and public health in nearby communities (Hoskin, 2003). Other impacts of mining include

destruction of land and vegetation, pollution, and changes in surface drainage. As a result, environments are prone to increased soil erosion, compaction, changes in topsoil characteristics, and a reduced capacity to support vegetation growth (Arendse and Wilkinson, 2002). Although mines are expected to provide for and apply rehabilitative measures before closure is granted (Milton, 2001), it is much more complicated than simply restoring the disturbed area. The consequence of mining activities is a soil environment typified by poor physical characteristics, such as poor textural material properties, combined with the effect of the slopes of the discard sites (Van Wyk, 2002); low levels of plant nutrients and organic matter; pH extremes; and the presence of heavy metals (Mining Review Africa, 2003). The processing of mine tailings and discard material usually results in an elevated topography which means that these discard sites are particularly exposed to the adverse effects of wind and water erosion (Van Wyk, 2002). These aspects, often accompanied by harsh climatic conditions characteristic to the arid and semi-arid areas of southern Africa, deter the establishment of permanent self-sustaining vegetation cover (revegetation) on mine discard and tailings (Milton, 2001; Mining Review Africa, 2003). Often in mining projects, topsoil has to be stripped from mining sites and stored (stockpiled), with subsequent adverse changes in the structure, physical and chemical characteristics, and biology of soils (Harris and Birch, 1989). The heaps of spoil material produced during open-cast coal mining operations typically contains low amounts of organic matter and display low soil biological activity (Frouz *et al.*, 2001; Frouz and Nováková, 2005). This can have serious consequences for soil quality and the rehabilitation of land disturbed by opencast mining.

Changes in plant diversity not only affects aboveground ecosystem functioning, but also have implications for belowground communities (Bartelt-Ryser *et al.*, 2005). The quantity and quality of plant inputs, such as litter, root turnover, root exudation, and plant productivity are coupled to soil microbial community function and structure (Grayston *et al.*, 2001; Rutigliano *et al.*, 2004). Due to the effect of belowground communities on decomposition of organic material and the mineralisation of nutrients, feedback effects may be caused on the plant community (Bardgett *et al.*, 2005). It has been indicated that such feedback mechanisms between plants and microbial communities can last for a year or even longer. Hence, the importance of microbial communities with regard to ecosystem development might be a more important factor than previously assumed (Bartelt-Ryser *et al.*, 2005). Vegetation cover decline has also been linked with changes in the nitrogen cycle, lower urease and protease activity (Garcia *et al.*, 2002), and considerable soil losses

by wind and water erosion (Castillo and Joergensen, 2001). Virtually 91% of South Africa is situated within the United Nations definition of ‘affected drylands’ (UNCCD, 1994). These terrestrial ecosystems are extremely dry areas where rainfall is low and potential evaporation is high. Dryland systems are fragile and need to be managed carefully. The loss of vegetation cover from such areas poses an increased risk of erosion, the outcome being soil with a reduced capacity to support vegetation (DEAT, 2007).

The depletion of natural resources that result in the transformation and fragmentation of natural habitats leads to changes in the number and type of species that occur there and inevitably, to impaired ecosystem functioning (Hawksworth, 1996). Soil microbial communities are critical to the ecological functioning of terrestrial ecosystems (Bandick and Dick, 1999; Aon and Colaneri, 2001) and exhibit great sensitivity to changes in their physical and chemical environment (Ibekwe *et al.*, 2002). Therefore, disturbance of the soil ecosystem that disrupts normal microbial community function and structure, is potentially detrimental to short- and long-term ecological stability (Mummey *et al.*, 2002a,b) and places enormous strain on the resilience of soil and natural processes to maintain global balances of energy and organic matter (Doran and Safley, 1997).

The Society for Ecological Restoration (SER) defines ecological restoration as “*the process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed*” (SER, 2004). Rehabilitation and restoration share a fundamental focus on pre-existing ecosystems as references, but the two activities encompass different goals and strategies. While the goals of restoration include the re-establishment of pre-existing biotic integrity in terms of species composition and community structure, rehabilitation emphasises the reparation of damaged ecosystem functions, with the primary goal of raising ecosystem productivity for the benefit of society (SER, 2004). Soil is a natural resource essential to life, making its preservation and the restoration of already damaged environments critical in achieving sustainable development and feeding the growing world population (Arshad and Martin, 2002). It is fundamental in the functioning of ecosystems, as an economic resource, and as a platform for infrastructural development (Rapport *et al.*, 1997). The ultimate objective of all rehabilitation decisions should be to minimise environmental degradation and to establish stability in disturbed ecosystems. According to Harris and Birch (1989), a key feature of successful rehabilitation is the interaction between improvement in soil structure and recovery of soil microbial communities. Furthermore, for rehabilitation attempts to be successful it is necessary to realise that soil is a dynamic resource with both inherent qualities and characteristics resulting from

disturbance which should be monitored and managed. Even when ecological rehabilitation have been adequately defined, the central question facing the land manager attempting to remediate or restore degraded land is how to measure the success or failure of rehabilitation efforts on a particular site or landscape (Harris, 2003). Vegetative stabilisation appears to be the answer to achieving rehabilitation success on mine discard sites (Carroll *et al.*, 2000) and traditionally attention was focused on vegetation development on discard dumps. However, re-establishment of ecosystem function in post-mining landscapes calls for a reconstruction of soils – a process to which soil biota are key. It therefore follows that since soil is the growth medium for all vegetation, the rehabilitation of mining discard sites is unlikely in the absence of soil organic matter accumulation and microbial activity (Frouz *et al.*, 2001; Frouz and Nováková, 2005). Accordingly, it is of the essence to find suitable methods to assess and improve the quality of this growth medium. Research should thus be focused on an integrated approach that takes physical, chemical, and biological properties and their interactions into account. The need for timely indicators of trends in ecosystem recovery is an important component of this (Harris, 2003).

1.3. Assessing Soil Quality

Soil properties are changed by anthropogenic influences and this alters the ability of the soil to sustain equilibrium in the environment. It is therefore vital that these properties should be measured and the measurements understood in order for discussions concerning effective management or environmental issues to be founded on exact information (Rowell, 1994).

“As the complexity of the issues involving management and stewardship of soil systems continue to increase, the need to assess the status and function of soil microbial communities is becoming more acute. Whether the need is to evaluate the quality of agricultural soils, the impact of anthropogenic intrusion (e.g., chemical pollution), the status of nutrient cycling in native systems, or the results of reclamation management, accurate and reproducible indicators of soil microbial community sustainability or resilience are essential for achievement of project goals and development of appropriate (sound) soil stewardship plans” (Tate and Rogers, 2002).

Considering the multitude of physical, chemical, and biological properties that influence soil ecosystem stability, it is clear that soil quality cannot be measured directly.

Instead, assessment should be focused on the use of key components or processes as indicators that would provide a simplified mechanism to signify the degree to which an ecosystem is performing. In the case of soil quality, an indicator should be a measurable soil attribute that reflects soil functionality (Schoenholtz *et al.*, 2000), or indicates whether a specific management strategy has a positive or negative influence on soil quality. In other words, it should be able to guide management efforts. The selection of suitable indicators is complicated for a number of reasons. It is important to realise that results obtained from the individual measurements of soil ecosystem components represent the summed response of the whole system (Elliot, 1997). In order to achieve a comprehensive assessment of soil quality it is necessary to take into account both the numerous dimensions of soil function, such as productivity and environmental fitness, and the assortment of physical, chemical, and biological factors which control biogeochemical processes; and their variation in intensity over time and space (Doran and Safley, 1997).

Traditionally, criteria for judging the success of rehabilitation have focused on visually distinguishable aboveground indicators, such as soil erosion, vegetation cover, and diversity of vegetation. However, these criteria fail to account for the composition of the soil microbiota, which are the basis of terrestrial ecosystems (Mummey *et al.*, 2002a). Other properties applied in the characterisation of soil include physical and chemical soil analyses and for a long time, these properties formed the foundation for the majority of management decisions. The occurrence of certain morphological phenomena, such as loss of organic matter, water and wind erosion, salinisation, acidification, poor drainage, and structural deterioration are important signs of degradation in soil quality (Doran and Safley, 1997). However, the response of physical and chemical parameters can only be measured effectively over an extensive period (Pascual *et al.*, 2000). Furthermore, soil is a very complex ecosystem and physical and chemical characterisation does not allow insight into the biological structures and functions within the soils (White *et al.*, 1996; Widmer *et al.*, 2001).

The significance of microbial communities in sustainable soil ecosystems has been acknowledged for some time and an essential element for evaluating impacts of management regimes is the accurate assessment of microbial community function and structure (Tate and Rogers, 2002). Accordingly, soil microbial properties have often been proposed as timely and sensitive indicators of soil ecological stress or restoration processes (Bandick and Dick, 1999; Badiane *et al.*, 2001; Ibekwe *et al.*, 2002). According to Harris

(2003), analysis of the soil microbial community meets all five criteria against which the potential of a particular ecosystem metric could be judged. These include:

- It should be relevant to the ecosystem under study and to the objectives of the assessment programmes.
- It should be sensitive to anthropogenic changes.
- It should provide a response that can be differentiated from natural variation.
- It should be environmentally benign.
- It should be cost effective to measure.

(Andreasen *et al.*, 2001)

Some of the methods used to investigate soil microorganisms include cultivation-dependent techniques and cultivation-independent community profiling methods. The latter can be divided into biochemical, physiological, and molecular approaches (Leckie, 2005). However, assessing the status of microbial community function and structure has been problematic and indices in this regard are still lacking. This is especially true for post-mining landscapes and is complicated by the myriad of different and often unique environments land managers and researchers deal with.

The classification and identification of microorganisms based on morphological traits is complicated because microorganisms are small and lack conspicuous external features (Muyzer, 1999). Until recently, the analysis of soil microbial communities relied extensively on cultivation-dependent techniques using a variety of enriched culture media and direct viable counts (Kirk *et al.*, 2004). These techniques are fast and inexpensive; however, they are insensitive and provide little insight into the nutritional and/or environmental status *in situ* (Hill *et al.*, 2000). Two main reasons for the insensitivity of these techniques can be identified. First, it is difficult to extract microorganisms from soil. Even after multi-stage extractions using chemical and physical dispersion treatments, large proportions of microorganisms remain associated with soil particles. Second, the isolated microorganisms are restricted to those that can grow on the medium of choice (Peacock *et al.*, 2001; Taylor *et al.*, 2002). According to Hill *et al.* (2000), it has been estimated that less than 0.1% of the microorganisms found in typical soil environments are cultivable using modern culture media formulations. This can be attributed to the lack of knowledge concerning culture conditions under which microorganisms thrive in their natural environment (Muyzer, 1999). Cultivable microorganisms recovered from environmental samples therefore represent only a fraction of the extant microbiota (White *et al.*, 1996). Thus, bias exists towards those organisms that can be grown successfully in the laboratory

(Leckie, 2005) and towards fast growing individuals and fungal species that produce large quantities of spores (Kirk *et al.*, 2004).

Other traditional analyses include the measurement of non-viable microbial biomass by the chloroform fumigation-extraction (CFE) or chloroform fumigation-incubation (CFI) methods (Bailey *et al.*, 2002a). The use of CFE or CFI provides a direct measurement of total soil biomass (Wang *et al.*, 2003) and has the advantage of not requiring direct counts and size conversions (Elliot, 1997). These methods give an indication of the function of microbial life as a pool, but provide no information on community structure (Alef and Nannipieri, 1995; Peacock *et al.*, 2001). Other negative aspects include different extraction efficiencies for different soils and difficulties in separating root from microbial biomass (Elliot, 1997).

Soil enzyme activity may be associated with various biotic and abiotic components, including proliferating cells, latent cells, cell debris, clay minerals, humic colloids, and the soil aqueous phase (Burns, 1982). The assay of a variety of soil enzymes gives an indication of the diversity of functions that can be assumed by the microbial community (Brohon *et al.*, 2001). Several studies have suggested the use of soil enzyme assays to investigate biochemical soil processes and to reflect the status of biological activity (Dick, 1994; Bandick and Dick, 1999; Aon *et al.*, 2001; Knight and Dick, 2004). Enzymatic activities, such as that of dehydrogenase, β -glucosidase, urease, and phosphatase show significant correlation with total organic carbon, total nitrogen, water-filled pore space, and heterotrophic bacterial and fungal biomass (Aon and Colaneri, 2001). Numerous studies indicate the sensitivity of these enzymes to management practices and disturbance (Dick *et al.*, 1996; Dick, 1997; Aon *et al.*, 2001; Badiane *et al.*, 2001) and they are frequently used to estimate changes in soil quality (Bandick and Dick, 1999; Masciandaro and Ceccanti, 1999; Gil-Sotres *et al.*, 2005).

Dehydrogenase is present in all microorganisms (Dick, 1997) and dehydrogenase activity is regarded as an accurate measure of the microbial oxidative capacity of soil and therefore of viable microorganisms (Dick, 1994; Taylor *et al.*, 2002). According to Smith and Pugh (1979), the dehydrogenase assay can provide a valid indication of soil microbial activity and could be valuable in ecological investigations. Measures of dehydrogenase activity have been applied to estimate the degree of recovery of degraded soils (Gil-Sotres *et al.*, 2005).

B-Glucosidase (EC 3.2.1.21, β -D-glucoside glucohydrolase) activity is very useful in the monitoring of soil ecosystems for several reasons. It shows low seasonal variability

(Knight and Dick, 2004), plays a central role in the cycling of organic matter, is the most abundant of the three enzymes involved in cellulose degradation, and is rarely substrate limited (Turner *et al.*, 2002). Soil management effects on β -glucosidase can be detected within relatively short time periods (1-3 years) and it is possible to perform the assay on air-dried soil, making this assay more accessible for routine soil quality testing (Bandick and Dick, 1999). Several studies have found β -glucosidase to be sensitive to soil management and it has been proposed as an indicator for soil quality (Bandick and Dick, 1999; Ndiaye *et al.*, 2000; Pascual *et al.*, 2000; Vepsäläinen *et al.*, 2001). A study by Hayano and Katami (1977) found that fungi were the primary source for β -glucosidase assayed in their study. In contrast, Waldrop *et al.* (2000), found correlations between β -glucosidase activity and signature lipid biomarkers for Gram-positive and Gram-negative bacteria in soil.

Phosphomonoesterases are involved in organic phosphorus transformations in soil (Nannipieri *et al.*, 2002). They have low substrate specificity and are the predominant phosphatases in most soils. They are classified as acid phosphatases (E.C. 3.1.3.2, orthophosphoric-monoester phosphohydrolase) and alkaline phosphatases (E.C. 3.1.3.1, orthophosphoric-monoester phosphohydrolase), depending on the pH-optima of activity. Soil microorganisms produce alkaline phosphatases, while acid phosphatases are mainly attributed to plant roots (Criquet *et al.*, 2004). Phosphatase activity measured in soil has been used to evaluate the quality of soil or to describe the functioning of the ecosystem (Aon and Colaneri, 2001; Brohon *et al.*, 2001). It is influenced by management practices (Aon *et al.*, 2001), biotic and abiotic factors (Criquet *et al.*, 2004) and the soil microclimate (Krämer and Green, 2000). The sensitivity of these enzymes to soil pH was utilised by Dick *et al.* (2000) to determine the optimum soil pH for crop production. The study showed the potential of using the ratios of alkaline phosphatase to acid phosphatase (AlkP/AcdP) instead of chemical methods to assess effective soil pH. A study by Lim *et al.* (1996), found that osmotic stress resulted in increased alkaline phosphatase activity in bacteria and it has been suggested that an increase in alkaline phosphatase activity may reflect a stress response to unfavourable environmental conditions (Criquet *et al.*, 2004). Acid phosphatase activity is also useful as an indicator for recovery of degraded soils due to its association with organic matter content (Gil-Sotres *et al.*, 2005).

The hydrolysis of urea is catalysed by the enzyme urease (EC 3.5.1.5, urea amidohydrolase). Although urease occurs in bacteria, fungi, algae, and higher plants (Samborska *et al.*, 2004), urease activity in soil has been correlated with microbial

biomass. This suggests the microbial origin of urease in soil (Klose and Tabatabai, 2000). Urease activity is often measured due to its importance in the nitrogen cycle and it has been widely used to evaluate changes in soil quality due to soil management (Gil-Sotres *et al.*, 2005).

According to Knight and Dick (2004), a variety of environmental factors and soil characteristics affect the activity of microbial communities or uptake of nutrients by plants. Furthermore, the presence of abiotic enzymes also moderates the reflection of microbial community dynamics. As a result, enzyme assays often do not correlate with microbial activity nor do they predict nutrient availability to plants. Data interpretation can be difficult due to mixtures of active and inactive populations and the accumulation of stabilised enzymes, which may or may not contribute to ecosystem function (Tate and Rogers, 2002). Results for enzymatic activities are highly variable and thresholds for interpreting enzyme assays as soil quality indicators are unavailable. However, enzymatic assays may provide valuable information of microbial community function if applied for the monitoring of trends over time (Bandick and Dick, 1999; Aon and Colaneri, 2001; Hinojosa *et al.*, 2004; Gil-Sotres *et al.*, 2005).

A comprehensive assessment of soil microbial community characteristics is one way in which to address the incomplete picture of soil status that current methods provide (Harris, 2003). Phospholipid fatty acid (PLFA) analysis allows phenotypic fingerprinting of soil microbial communities (Leckie, 2005) and has been found to be a reliable tool for distinguishing ecosystem types and for assessing management effects on soil microbial community structure (Tate and Rogers, 2002). The use of a cultivation-independent method, such as PLFA analysis is a powerful means to examine *in situ* microbial community structure. This technique, also known as signature lipid biomarker analysis, circumvents many of the problems frequently associated with conventional cultivation-based techniques (Pinkart *et al.*, 1998; Waldrop *et al.*, 2000). The analyses provide a quantitative description of the microbial community in the particular environment sampled at a given time. Fatty acids are extracted directly from soil samples with organic solvents – a total or representative extraction. The microbial lipid extract is then fractionated into neutral lipids, glycolipids, and phospholipids by silicic acid chromatography. The phospholipid fraction is subjected to mild alkaline methanolysis to produce fatty acid methyl esters (FAMES) for quantitative analysis by capillary gas chromatography and gas chromatography-mass spectrometry (Guckert *et al.*, 1985; White and Ringelberg, 1998). A sample profile represents the abundance of each of the extracted PLFAs. Previous studies

have indicated correlations of PLFA analyses with enzymatic activities (Waldrop *et al.*, 2000), acridine orange direct counts, ATP content (Balkwill *et al.*, 1998), microbial community metabolic profiles (Biolog™), and DNA analyses (Widmer *et al.*, 2001).

Phospholipid fatty acids can be classified into ester-linked (esterified) PLFAs (EL-PLFAs) and non-ester linked (non-esterified) PLFAs (NEL-PLFAs). The EL-PLFAs comprise 60-90% and the NEL-PLFAs 10-40% of the total PLFAs. A schematic representation of the classification of PLFAs is provided in Figure 1.3. Ester-linked PLFAs are divided into ester-linked unsubstituted fatty acids (EL-UNFAs) and hydroxyl substituted fatty acids (EL-HYFAs). The EL-UNFAs are further subdivided into saturated (EL-SATFA), monounsaturated (EL-MUFA), and polyunsaturated (EL-PUFA) fatty acids. Two subgroups, branched chain fatty acids (BRANCs) and straight chain fatty acids (STRAs) constitute the EL-SATFAs. Non-ester linked PLFAs include the unsubstituted (NEL-UNFA) and hydroxy substituted (NEL-HYFA) fatty acids. Hydroxy substituted fatty acids that are localised in the lipopolysaccharide portion of the cell wall in Gram-negative bacteria are designated as LPS-HYFA (Zelles, 1999; Kaur *et al.*, 2005).

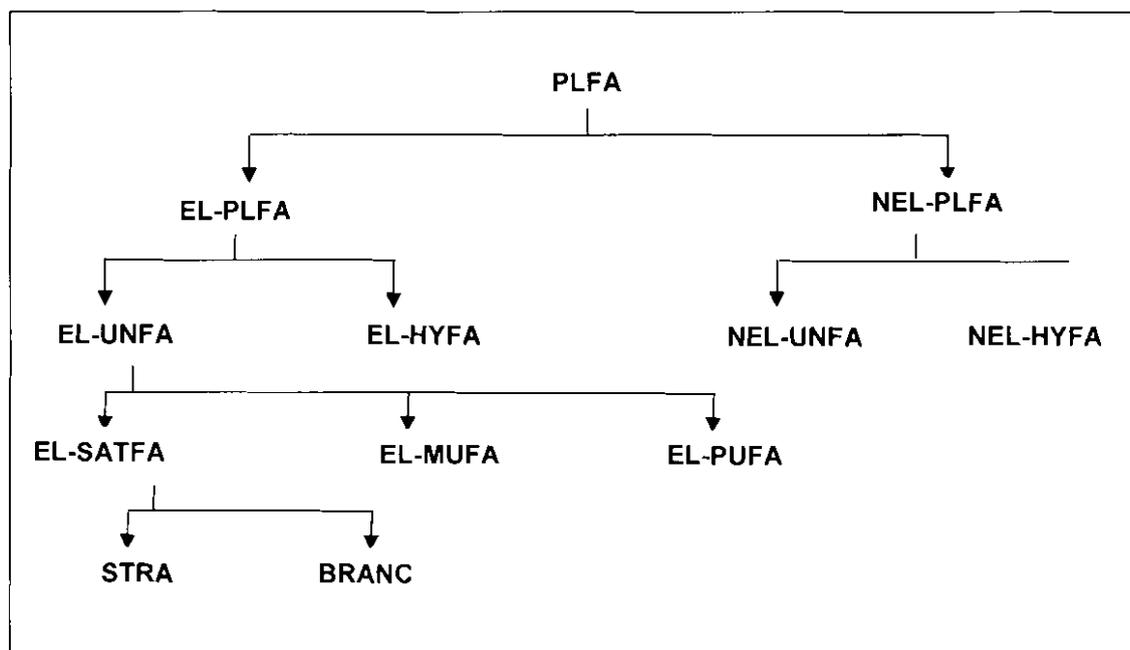


Figure 1.3. Classification of phospholipid fatty acids (PLFAs) (Kaur *et al.*, 2005).

Signature lipid biomarker analysis is based on the variability of fatty acids present in the cell membranes of different organisms. The composition of PLFA profiles in microorganisms is determined by fatty acids of varying chain length, saturation, and

branching and can therefore be used as ‘fingerprints’ of the soil community (Steer and Harris, 2000; Leckie, 2005). It is also affected by the metabolic state of the organism, environmental factors, and exposure to toxic substances (Frostegård *et al.*, 1997). Accordingly, when bacteria are cultured under standardised conditions, they maintain a constant fatty acid composition unique to specific groups of microorganisms (Keweloh and Heipieper, 1996). PLFAs are easily extracted from soil and the technique is optimised for phospholipid molecules, so that other free fatty acids are not detected. Therefore, it provides insight into a greater portion of the whole community composition than cultivation-based practices would (Hill *et al.*, 2000; Peacock *et al.*, 2001).

The cell membranes of all living microorganisms contain PLFAs, which function to maintain cell fluidity, enable transport of nutrients into the cell, and eliminate metabolic products (Ponder and Tadros, 2002). Since PLFAs are not associated with storage functions, they represent a constant portion of the cell mass. Following cell death, PLFAs are rapidly degraded and therefore does not exist extracellularly (Zelles *et al.*, 1992). This makes them valuable as signature molecules and indicators of viable microbial biomass (Calderón, *et al.*, 2000; Rütters *et al.*, 2002). In addition, the physiological status and community structure of microbial populations can be inferred from lipid profiles (Steenwerth *et al.*, 2003) since certain fatty acids respond to environmental disturbances and are unique to specific groups of organisms (White *et al.*, 1996). Subsequently, PLFA profiles can signify changes in the bacterial and/or fungal composition of a soil (Ibekwe and Kennedy, 1998; Hill *et al.*, 2000). The major PLFA groups associated with the membranes of various microorganisms are indicated in Table 1.1.

The use of PLFA analysis to differentiate between bacterial and fungal biomass, is very useful since other techniques, such as substrate induced respiration and direct microscopy, is time-consuming and sometimes imprecise (Bååth and Anderson, 2003). Lipids unique to fungi and bacteria, respectively, can be summed as indices of each of these groups of soil microorganisms, creating a fungal to bacterial (F:B) ratio of living soil microbial biomass (Bardgett and McAlister, 1999). The quantity of 18:2 ω 6c is used as an indicator of fungal biomass, since it is mainly of fungal origin (Federle, 1986; Merilä *et al.*, 2002). As an index of bacterial biomass, Frostegård and Bååth (1996) suggested the use of the sum of the following PLFAs considered to be predominantly of bacterial origin: i15:0, a15:0, 15:0, i16:0, 16:1 ω 9, 16:1 ω 7t, i17:0, a17:0, 17:0, cy17:0, 18:1 ω 7, and cy19:0. According to Bailey *et al.* (2002b), the F:B biomass ratios obtained from PLFA analysis can be compared to those obtained by selective inhibition of substrate-induced respiration

to assess the relative dominance of fungi over bacteria in a set of soil samples. A F:B activity ratio (as determined by substrate induced respiration) of 1.0 indicates an equal contribution of fungi and bacteria to the microbiological activity in the soil sample. When using PLFA analysis to determine F:B ratios, the ratio is usually less than 1.0 since the saturated fatty acids included in the prokaryotic lipids are ubiquitous and found in most organisms (Bailey, *et al.*, 2002b).

Table 1.1. Major phospholipid fatty acid (PLFA) groups associated with the membranes of various microorganisms (Guckert *et al.*, 1985; Olsson, 1999; Ponder and Tadros, 2002; Peacock, 2005).

PLFA Structure Group	Fatty Acids	General Classification
Normal saturated	14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, 24:0	A general microbial biomarker found in both the prokaryotic and eukaryotic (polyenoic fatty acids) kingdoms; a relative increase has been shown to correlate with decreased diversity.
Monounsaturated	14:1 ω 5c, 15:1, 16:1 ω 9c, 16:1 ω 7c, 16:1 ω 7t, 16:1 ω 5c, 17:1 ω 8, cy17:0, 17:1, 18:3 ω 6, 18:3 ω 3, 18:1 ω 9c, 18:1 ω 7c, 18:1 ω 7t, 18:1 ω 5c, 19:1 ω 12c, 19:1 ω 12, cy19:0, 20:1 ω 9c, 20:1 ω 9t, 22:1 ω 9c, 22:1 ω 9t	Indicative of predominantly Gram-negative bacteria, which is fast-growing, utilise many carbon sources and adapt quickly to a variety of environments; may also be found in the cell membranes of obligate anaerobes such as sulphate or iron-reducing bacteria; an increase in the amount and type of carbon sources has been shown to increase this marker.
Terminally-branched saturated	i14:0, i15:0, a15:0, i16:0, i17:0, a17:0, i18:0	Common to Gram-positive bacteria, including <i>Arthrobacter</i> and <i>Bacillus</i> spp. Many of these types of bacteria can be spore formers and can exist in environments that are lower in overall organic carbon content.
Mid-chain branched saturated	i10Me15:0, a10Me15:0, br15:0a, 10Me15:0, br16:0a, br16:0b, br16:0c, 10Me16:0, 11Me16:0, 12Me16:0, br17:0, 2Me17:0, 10Me17:0, 12Me18:0	Primarily indicative of Actinomycete type bacteria in surface soils. It has been hypothesised that since these bacteria grow hyphae they are able to better survive in harsh environments due to their ability to span interstitial spaces to collect water and nutrient sources.
Polyunsaturated	18:2 ω 6, 18:3 ω 3, 20:2 ω 6, 20:5 ω 3	Representative of fungi and other microeukaryotic organisms; this marker too shows significant differences due to land-use.

Ratios of F:B biomass derived from PLFA analysis have been used to measure recovery of soil. In a study conducted by Bardgett and McAlister (1999), F:B ratios were found to be indicative of ecosystem self-regulation. Results from the study suggest that the ratio of F:B

PLFAs is higher in soils that are unimproved, in contrast to soils that have been fertilised and show a lower ratio of F:B PLFAs. Similarly, native soil systems show a tendency to be characterised by high ratios of fungi to bacteria compared to managed systems (Bardgett and McAlister, 1999). The ratio of F:B biomass was also positively correlated to soil pH (Bååth and Anderson, 2003).

According to Kieft *et al.* (1994), bacteria alter their membrane fatty acid components in response to environmental stress, thereby generating characteristic PLFA stress signatures. In this regard, increased ratios of saturated to unsaturated fatty acids, increased ratios of *trans*- to *cis*- monoenoic fatty acids, and increased ratios of cyclopropyl fatty acids to their monoenoic precursors are known as stress signatures. The growth rate, medium composition, and environmental factors under which microorganisms grow, influence the relative amounts of *trans* fatty acids present in the cells. Accordingly, the measurement of the *trans/cis* ratio of 16:1 fatty acids and 18:1 fatty acids are applied as a general measure of stress or starvation to determine the physiological status of the microbial population (Kieft *et al.*, 1994; Keweloh and Heipieper, 1996). The concentration of *trans* monoenoic fatty acids usually increases during nutrient deprivation, while the concentration of *cis* monoenoic fatty acids decline (Guckert *et al.*, 1986). *Trans/cis* ratios greater than 0.1 are considered indicative of starvation or exposure to toxins (Guckert *et al.*, 1986; Keweloh and Heipieper, 1996). In contrast, non-stressed microbial communities are generally considered to have ratios of 0.05 or less (White *et al.*, 1996). Stress on microbial populations can also result in physiological changes that show an increased concentration of cyclopropyl fatty acids (Guckert *et al.*, 1991). Such changes may be stimulated by starvation, high temperatures, high magnesium ion concentrations, and low pH (Guckert *et al.*, 1986). Therefore, increases in cy17:0 and cy19:0 relative to their respective metabolic precursors, 16:1 ω 7c and 18:1 ω 7c, may rather indicate physiological stress in microbial communities than a change in the community composition (Leckie, 2005). The cyclopropane/monoenoic PLFA ratio usually falls within the range of 0.05 (for log phase) to 2.5 or greater (stationary phase) (Guckert *et al.*, 1985; Guckert *et al.*, 1986). According to Smith *et al.* (2000), a ratio of greater than 0.1 is indicative of nutritional stress, while cells in the exponential growth phase have ratios of less than 0.05.

In spite of the value of PLFA analysis, some restrictions of this technique should be taken into consideration. In a number of cases, a specific fatty acid present in a soil sample cannot be linked with a specific microorganism or group of microorganisms, because appropriate signature molecules are not known for all organisms (Hill *et al.*, 2000).

Different microbial species can share various fatty acids, therefore PLFA profiles cannot be used to identify species within a community (Hill *et al.*, 2000; Ibekwe *et al.*, 2002).

1.4. The Space-for-Time Hypothesis

It is widely accepted that the most reliable manner to measure change in an ecosystem and to gain an understanding of the basic structure and function of that ecosystem, is by long-term study employing appropriate spatial and temporal scales. In terms of obtaining a realistic ecological assessment of a restoration project, this implies monitoring the same site through time (Michener, 1997; Sparling *et al.*, 2003). However, with respect to ecological function and structure, “long-term” often represents decades to centuries (Michener, 1997). Soil recovery (in terms of soil formation) takes thousands of years (Eijsackers, 2004), topsoil components such as organic matter may take hundreds of years to reach equilibrium (Sparling *et al.*, 2003), and even the repopulation of soil by microflora can take up to a decade (Eijsackers, 2004). It is thus clear, that “long-term” with respect to ecological function and structure implies timescales that are beyond the scope of typical investigations. Studies of biological soil communities are frequently restricted to seasonal or other types of short-term investigations or even sampling at only a single point in time (Mummey *et al.*, 2002b; Taylor *et al.*, 2003; Hinojosa *et al.*, 2004; Zhang *et al.*, 2006). This is especially true for rehabilitation projects where time and expense must be kept to a minimum. Rehabilitation projects in the South African mining environment are implemented and managed by mining companies in order to restore a disturbed area according to a mine closure plan. Often these projects have been in progress for a number of years before scientists become involved and an opportunity arises to evaluate biological soil properties. Even if biological properties are evaluated at the start of a rehabilitation project, it is improbable that such monitoring will be conducted consistently over a long-term period due to difficulties associated with funding and collaboration with mining companies. In addition, there are other research constraints linked to restoration studies. These include uncontrollable events that cannot be replicated or studied using traditional experimental approaches and statistical analyses (Michener, 1997). Complications such as these and a general difficulty in monitoring soils over long periods have necessitated the use of alternative investigative approaches to monitoring through time in order to quantitatively evaluate the success of an ecological restoration activity and to advance

restoration ecology as a scientific discipline. One such approach is a substitution of ‘space’ for ‘time’ (Michener, 1997; Sparling *et al.*, 2003).

According to Pickett (1989), one of the most commonly encountered techniques in ecology is inferring a temporal trend from a study of different aged sites. This technique is called space-for-time (SFT) substitution, and results in a chronosequence. The SFT approach assumes that sites in different locations were initially similar, and that simultaneous sampling of different sites of increasing age is equivalent to resampling the same site through time. Therefore, when applying this approach, sites of different ages and stages of development at separate locations (‘space’) are identified to obtain a chronosequence of ages (‘time’) (Sparling *et al.*, 2003). Due to the extensive periods associated with recovery of ecosystems from disturbance, extrapolation of temporal trends from chronosequences (sites or samples of different ages) that are obtained by sampling certain environmental variables may be a practical alternative to long-term studies, especially where general or qualitative trends are sought (Pickett, 1989; Michener, 1997).

Space-for-time substitution has mostly been applied to vegetation studies (Pickett, 1989; Molnár and Botta-Dukát, 1998; Le Duc *et al.*, 2000), including studies of primary and secondary succession. In this context, SFT substitution has been found useful for illustrating general trends and regional variability in composition and community structure in some systems; and to expose patterns of functional characteristics through succession (Pickett, 1989). Other applications of SFT substitution include the following investigations: comparative plant successional studies on topsoiled mining-spoils (Martínez-Ruiz and Fernández-Santos, 2005); changes in soil microbial community structure in a tallgrass prairie chronosequence (Allison *et al.*, 2005); vegetation response to longer-term climate shifts (Scanlon *et al.*, 2005); as a surrogate for a long-term study on ant succession (Dauber and Wolters, 2005); and as a reliable approach to assessing topsoil recovery after landslip erosion in New Zealand (Sparling *et al.*, 2003). Studies on the biological impact of acid deposition, recovery of stripmines, dynamics of debris dams, and disturbance in landscapes have also employed SFT substitution (Pickett, 1989).

From a review by Pickett (1989), it is clear that the applications of SFT substitution cover a wide range of systems, “*from aquatic to terrestrial, from those having fast dynamics to those having slow dynamics, and from rich to poor implications of the past*”. Furthermore, it is apparent that there are different strategies for studying the past of an ecological system. Different approaches to ecological studies (e.g. long-term study and SFT substitution) expose different levels of detail and address different characteristics of

systems. Therefore, the manner in which SFT substitution is applied relies on the objectives of the relevant study and the workings of the system to which it is applied (Pickett, 1989).

2. PERSPECTIVE, AIMS, AND OUTLINE OF THESIS

Although mining companies are expected to provide for and apply rehabilitative measures before closure permits are granted, the rehabilitation of areas disturbed by mining activities is a complicated and multifaceted process. Past rehabilitative management strategies that relied extensively on the rehabilitation of disturbed areas by application of agronomic approaches, proved unsuccessful in the semi-arid and arid areas of southern Africa (Milton, 2001). Furthermore, the rehabilitation process is complicated due to the lack of suitable assessment criteria for restoration success. It seems that vegetative stabilisation is the most successful answer to achieving sustainable rehabilitation of mine discard sites (Carroll *et al.*, 2000) and since soil is the growth medium for all vegetation, it is important to find suitable methods to assess and improve the quality of this growth medium. Soil quality and the associated success of rehabilitation efforts are difficult to measure because soil and its functions are an ecologically complex phenomenon that cannot be readily assessed by any single soil parameter. Research should thus be focused on a more integrated approach that takes the physical, chemical, and biological properties and their interactions into account.

A preliminary investigation to the current study was conducted to ascertain whether microbial enumeration techniques, assays of enzymatic activities, and analysis of signature lipid biomarkers (PLFA analysis) could be applied to differentiate between the soil cover layers of coal discard sites of varying rehabilitation ages that were managed in an identical manner. The most significant findings of this study will be briefly discussed here, while the article containing the results of the investigation (Claassens *et al.*, 2006) is included in Appendix A.

The investigation was conducted on seven revegetated coal discard dumps in South Africa varying from 1 to 8 years in rehabilitation age at the time of the investigation. In this preliminary investigation as well as in the current study, the term ‘rehabilitation age’ refers to the time since the first application of a soil cover layer and subsequent amelioration and revegetation of a specific coal discard dump.

Statistical analysis of the microbial counts indicated no significant differences ($p = 0.05$, Tukey’s test) between soil covers of discard sites based on microbial enumeration

using a variety of culture media. Significant differences ($p = 0.05$, Tukey's test) between sites of varying rehabilitation ages could be observed based on assays of enzymatic activities (dehydrogenase, β -glucosidase, acid and alkaline phosphatases, and urease) as well as analyses of PLFA profiles. The relationship between soil physico-chemical properties, vegetation cover, and microbial properties was investigated using multivariate statistical techniques. These included ordinations of principal components analysis (PCA) and canonical correspondence analysis (CCA). Discard dumps with relatively higher vegetation cover and organic C content had a positive association with enzymatic activities and microbial biomass. Organic C content correlated significantly with β -glucosidase ($r = 0.80$, $p < 0.05$); urease ($r = 0.96$, $p < 0.05$); acid phosphatase ($r = 0.76$, $p < 0.05$); dehydrogenase ($r = 0.69$, $p < 0.01$); and microbial biomass ($r = 0.73$, $p < 0.01$). Results obtained from the multifactorial analysis of microbial properties illustrated their interconnectedness with physico-chemical soil properties and vegetation cover. It also seemed that there was a relationship between the progress of rehabilitation and r- and K-strategic microorganisms, with r-strategists favouring younger dumps or dumps under fertiliser treatment and K-strategists favouring more stable environments.

The conclusion drawn from this preliminary investigation, was that microbial community function (enzymatic activities) and structure (PLFA analyses) was sufficiently sensitive to differentiate between coal discard dumps of varying rehabilitation ages and could be applied in a more comprehensive spatial and temporal investigation to assess the progress of rehabilitation on coal discard sites.

The aim of the current investigation was to apply aspects of microbial community function and structure in a spatial and temporal assessment of the status of the soil covers of seven coal discard sites of different rehabilitation ages (managed by Mining Company A). Specific objectives included ascertaining the relationship between rehabilitation age and microbial community function and structure; and determining whether information obtained from the assessment of these microbial properties could be used in soil monitoring programmes for the rehabilitation of coal discard sites.

Another objective was to apply 'space-for-time' (SFT) substitution as a tool to facilitate the comparison of long-term effects of management practices on rehabilitated coal discard sites. It was hypothesised that the application of SFT substitution would be a successful approach to obtain two chronosequences of rehabilitation ages and to compare long-term microbial community dynamics that would otherwise not have been possible. The two chronosequences of coal discard sites were managed by different mining

companies (Mining Company A and Mining Company B, respectively). The coal discard sites constituting chronosequence A, were aged 1 to 11 years and those constituting chronosequence B, 6 to 17 years. The specific objectives of the application of SFT substitution was to determine whether distinctive management practices had different long-term effects on the microbial communities in the soil covers of the discard sites; and to establish minimum and maximum values for microbial activity and abundance measurements in these rehabilitated ecosystems.

Multivariate statistical analysis was performed on data from all coal discard sites to investigate the relationship between the respective sites, soil physical and chemical characteristics, vegetation cover, and microbial community function and structure.

Chapter 2 contains a description of the sites (at Mine A and Mine B, respectively); sampling procedures; materials and methods used; and statistical analyses performed throughout the study.

Chapter 3 describes the spatial and temporal investigation into the functional diversity of the microbial communities of the seven coal discard sites sampled at Mine A. Functional diversity was assessed by assaying the activities of the major enzymes representative of the main steps of soil biogeochemical nutrient cycles, i.e. C (B-glucosidase), N (urease), and P (acid and alkaline phosphatases). Dehydrogenase activity was assayed as an indicator of overall microbial activity.

Chapter 4 describes the spatial and temporal investigation into the structural diversity of the microbial communities at the seven coal discard sites sampled at Mine A. Soil microbial community structure was determined by means of analysis and quantification of specific signature lipid biomarkers (PLFA analysis).

Chapter 5 describes an application of the SFT hypothesis. Two chronosequences of rehabilitation ages from coal discard dumps under different management regimes (Mine A and Mine B) were compared in terms of microbial community function and structure to assess the long-term effects of different management practices.

Finally, the results of Chapters 3 to 5 are summarised and discussed in Chapter 6.

REFERENCES

- Alef, K., Nannipieri, P. 1995. *Methods in applied soil microbiology and biochemistry*. Boston: Academic Press. 576p.
- Allison, V.J., Miller, R.M., Jastrow, J.D., Matamala, R., Zak, D.R. 2005. Changes in soil microbial community structure in a tallgrass prairie chronosequence. *Soil Sci Soc Am J* 69:1412-1421.
- Andreasen, J.K., O'Neill, R.V., Noss, R., Slosser, N.C. 2001. Considerations for the development of a terrestrial index of ecosystem integrity. *Ecol Indic* 1:21-35.
- Aon, M.A., Colaneri, A.C. 2001. Temporal and spatial evolution of enzymatic activities and physico-chemical properties in an agricultural soil. *Appl Soil Ecol* 18:255-270.
- Aon, M.A., Sarena, D.E., Burgos, D.E., Cortassa, S. 2001. (Micro)biological, chemical, and physical properties of soils subjected to conventional or no-till management: an assessment of their quality status. *Soil Tillage Res* 60:173-186.
- Arendse, L., Wilkinson, M. 2002. National environmental indicators programme, Specialist Report 3, Vol 4: Land Use. Department of Environmental Affairs and Tourism, South Africa. 44p.
- Arshad, M.A., Martin, S. 2002. Identifying critical limits for soil quality indicators in agro-ecosystems. *Agric Ecosyst Environ* 88:153-160.
- Ashman, M.R., Puri, G. 2002. *Essential soil science: a clear and concise introduction to soil science*. Blackwell Science Ltd., Oxford, 198p.
- Bååth, E., Anderson, T.-H. 2003. Comparison of soil fungal/bacterial ratios in a pH gradient using physiological and PLFA-based techniques. *Soil Biol Biochem* 35:955-965.
- Badiane, N.N.Y., Chotte, J.L., Pate, E., Masse, D., Rouland, C. 2001. Use of soil enzyme activities to monitor soil quality in natural and improved fallows in semi-arid tropical regions. *Appl Soil Ecol* 18:229-238.
- Bailey, V.L., Peacock, A.D., Smith, J.L., Bolton Jr. H. 2002a. Relationship between soil microbial biomass determined by chloroform fumigation-extraction, substrate-induced respiration, and phospholipid fatty acid analysis. *Soil Biol Biochem* 34:1385-1389.
- Bailey, V.L., Smith, J.L., Bolton Jr. H. 2002b. Fungal-to-bacterial ratios in soil investigated for enhanced C sequestration. *Soil Biol Biochem* 34:997-1007.
- Balkwill, D.L., Leachs, F.R., Wilson, J.T., McNabb, J.F., White, D.C. 1998. Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate and direct counts in sub-surface aquifer sediments. *Microb Ecol* 16:73-84.
- Bandick, A.K., Dick, R.P. 1999. Field management effects on soil enzyme activities. *Soil Biol Biochem* 31:1471-1479.
- Bardgett, R.D., McAlister, E. 1999. The measurement of soil fungal:bacterial biomass ratios as an indicator of ecosystem self-regulation in temperate meadow grasslands. *Biol Fertil Soils* 29:282-290.
- Bardgett, R.D., Bowman, W.D., Kaufmann, R., Schmidt, S.K. 2005. A temporal approach to linking aboveground and belowground ecology. *Trends Ecol Evol* 20:634-641.
- Bartelt-Ryser, J., Joshi, J., Schmid, B., Brandl, H., Balsler, T. 2005. Soil feedbacks of plant diversity on soil microbial communities and subsequent plant growth. *Perspect Plant Ecol Evol Syst* 7:27-49.
- Beedlow, P.A., Van Voris, P., Rogers, L.E. 1988. Theoretical perspective on ecosystem disturbance and recovery. In: Rickard, W.H., editor. *Shrub-steppe: balance and change in a semi-arid terrestrial ecosystem*, New York: Elsevier. pp 257-269.
- Brohon, B., Delolme, C., Goudon, R. 2001. Complementarity of bioassays and microbial activity measurements for the evaluation of hydrocarbon-contaminated soils quality. *Soil Biol Biochem* 33:883-891.
- Burns, R.G. 1982. Enzyme activity in soil: location and a possible role in microbial ecology. *Soil Biol Biochem* 14:423-427.

- Calderón, F.J., Jackson, L.E., Scow, K.M., Rolston, D.E. 2000. Microbial responses to simulated tillage in cultivated and uncultivated soils. *Soil Biol Biochem* 32:1547-1559.
- Carroll, C., Merton, L., Burger, P. 2000. Impact of vegetative cover and slope runoff, erosion, and water quality for field plots on a range of soil and spoil materials on central Queensland coal mines. *Aust J Soil Res* 38:313-327.
- Castillo, X., Joergensen, R.G. 2001. Impact of ecological and conventional arable management systems on chemical and biological soil quality indices in Nicaragua. *Soil Biol Biochem* 33:1591-1597.
- Claassens, S., Riedel, K.J., Van Rensburg, L., Bezuidenhout, J.J., Jansen van Rensburg, P.J. 2006. Microbial community function and structure on coal mine discard under rehabilitation. *S Afr J Plant Soil* 23:105-112.
- Criquet, S., Ferre, E., Farnet, A.M., Le Petit, J. 2004. Annual dynamics of phosphatase activities in an evergreen oak litter: influence of biotic and abiotic factors. *Soil Biol Biochem* 36:1111 – 1118.
- Dauber, J., Wolters, V. 2005. Colonization of temperate grassland by ants. *Basic Appl Ecol* 6:83-91.
- DEAT. 2007. The Department of Environmental Affairs and Tourism. The National State of the Environment Report for South Africa. [cited February 2007]; Available from <http://www.environment.gov.za/soesa/nsoer/issues/land.htm>
- Dick, R.P. 1994. Soil enzyme activities as indicators of soil quality. In: Doran, J.W., Coleman, D.C., Bezdicek, D.F., Stewart, B.A., editors. *Defining soil quality for a sustainable environment*, SSSA Special Publication 35, Madison: SSSA. pp 107-124.
- Dick, R.P. 1997. Soil enzyme activities as integrative indicators. In: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R., editors. *Biological indicators of soil health*. New York: CAB International. pp 121-156.
- Dick, R.P., Breakwell, D.P., Turco, R.F. 1996. Soil enzyme activities and biodiversity measurements as integrative microbiological indicators. In: Doran, J.W., Jones, A.J., editors. *Methods for assessing soil quality*, SSSA Special Publication 49, Madison: SSSA. pp 247-271.
- Dick, W.A., Cheng, L., Wang, P. 2000. Soil acid and alkaline phosphatase activity as pH adjustment indicators. *Soil Biol Biochem* 32:1915-1919.
- Doran, J.W., Safley, M. 1997. Defining and assessing soil health and sustainable productivity. In: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R., editors. *Biological indicators of soil health*. New York: CAB International. pp 1-28.
- Doran, J.W., Zeiss, M.R. 2000. Soil health and sustainability: managing the biotic component of soil quality. *Appl Soil Ecol* 15:3-11.
- Eijsackers, H. 2004. Leading concepts towards vital soil. In: Doelman, P., Eijsackers, H., editors. *Vital Soil: function, value and properties*. Developments in soil science vol 29. Amsterdam: Elsevier. pp 1-20.
- Elliot, E.T. 1997. Rationale for developing bioindicators of soil health. In: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R., editors. *Biological indicators of soil health*. New York: CAB International. pp 49-78.
- Federle, T.W. 1986. Microbial distribution in soil – new techniques. In: Megusar, F., Gantar, M., editors. *Perspectives in microbial ecology*. Ljubljana: Slovene Society of Microbiology. pp 493-498.
- Frostegård, A., Bååth, E. 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol Fertil Soils* 22:59-65.
- Frostegård, A., Petersen, S.O., Bååth, A., Nielsen, T.H. 1997. Dynamics of a microbial community associated with manure hot spots as revealed by phospholipid fatty acid analysis. *Appl Environ Microbiol* 63:2224-2231.
- Frouz, J., Keplin, B., Pižl, V., Tajovský, K., Starý, J., Lukešová, A., Nováková, A., Balík, V., Háněl, L., Materna, J., Düker, C., Chalupský, J., Rusek, J., Heinkele, T. 2001. Soil biota and upper soil layer development in two contrasting post-mining chronosequences. *Ecol Eng* 17:275-284.
- Frouz, J., Nováková, A. 2005. Development of soil microbial properties in topsoil layer during spontaneous succession in heaps after brown coal mining in relation to humus microstructure development. *Geoderma* 129:54-64.
- Garcia, C., Hernandez, T., Roldan, A., Martin, A. 2002. Effect of plant cover decline on chemical and microbiological parameters under Mediterranean climate. *Soil Biol Biochem* 34:635-642.

- Gil-Sotres, F., Trasar-Cepeda, C., Leirós, M.C., Seoane, S. 2005. Different approaches to evaluating soil quality using biochemical properties. *Soil Biol Biochem* 37:877-887.
- Grayston, S.J., Griffith, G.S., Mawdsley, J.L., Campbell, C.D., Bardgett, R.D. 2001. Accounting for variability in soil microbial communities of temperate upland grassland ecosystems. *Soil Biol Biochem* 33:533-551.
- Guckert, J.B., Antworth, C.P., Nichols, P.D., White, D.C. 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. *FEMS Microbiol Ecol* 31:147-158.
- Guckert, J.B., Hood, M.A., White, D.C. 1986. Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increases in the *trans/cis* ratio and proportions of cyclopropyl fatty acids. *Appl Environ Microbiol* 52:794-801.
- Guckert, J.B., Ringelberg, D.B., White, D.C., Hanson, R.S., Bratina, B.J. 1991. Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the Proteobacteria. *J Gen Microbiol* 137, 2631-2641.
- Harris, J.A. 2003. Measurements of the soil microbial community for estimating the success of restoration. *Eur J Soil Sci* 54:801-808.
- Harris, R.F., Bezdicsek, D.F. 1994. Descriptive aspects of soil quality/health. In: Doran, J.W., Coleman, D.C., Bezdicsek, D.F., Stewart, B.A., editors. *Defining soil quality for a sustainable environment*, SSSA Special Publication 35, Madison: SSSA. pp 23-35.
- Harris, J.A., Birch, P. 1989. Soil microbial activity in opencast coal mine restorations. *Soil Use Manage* 5:155-160.
- Hawksworth, D.L. 1996. Microorganisms: The neglected rivets in ecosystem maintenance. In: Di Castri, F., Younès, T., editors. *Biodiversity, science and development*. Oxon: CAB International. pp 130–138.
- Hayano, K., Katami, A. 1977. Extraction of β -glucosidase activity from pea field soil. *Soil Biol Biochem* 9:349-351.
- Hill, G.T., Mitkowski, N.A., Aldrich-Wolfe, L., Emele, L.R., Jurkonie, D.D., Ficke, A., Maldonado-Ramirez, S., Lynch, S.T., Nelson, E.B. 2000. Methods for assessing the composition and diversity of soil microbial communities. *Appl Soil Ecol* 15:25-36.
- Hinojosa, M.B., Carreira, J.A., García-Ruiz, R., Dick, R.P. 2004. Soil moisture pre-treatment effects on enzyme activities as indicators of heavy metal-contaminated and reclaimed soils. *Soil Biol Biochem* 36:1559-1568.
- Hoskin, W.M.A. 2003. Mine closure – The 21st century approach, avoiding future abandoned mines. CEPMLP Internet Journal Volume 12: Article 10. [cited March 2005]; Available from <http://www.dundee.ac.uk/cepmlp/journal/html/article12-10.html>
- Ibekwe, A.M., Kennedy, A.C. 1998. Phospholipid fatty acid profiles and carbon utilization patterns for analysis of microbial community structure under field and greenhouse conditions. *FEMS Microbiol Ecol* 26:151-163.
- Ibekwe, A.M., Kennedy, A.C., Frohne, P.S., Papiernik, S.K., Yang, C.H., Crowley, D.E. 2002. Microbial diversity along a transect of agronomic zones. *FEMS Microbiol Ecol* 39:183-191.
- Izquierdo, I., Caravaca, F., Alguacil, M.M., Hernández, G., Roldán, A. 2005. Use of microbiological indicators for evaluating success in soil restoration after revegetation of a mining area under subtropical conditions. *Appl Soil Ecol* 30:3-10.
- Karlen, D.L., Ditzler, C.A., Andrews, S.S. 2003. Soil quality: why and how? *Geoderma* 114:145-156.
- Kaur, A., Chaudhary, A., Kaur, A., Choudhary, R., Kaushik, R. 2005. Phospholipid fatty acid – A bioindicator of environment monitoring and assessment in soil ecosystem. *Curr Sci* 89:1103-1112.
- Keweloh, H., Heipieper, H.J. 1996. *Trans*-unsaturated fatty acids in bacteria. *Lipids* 31:129-135.
- Kieft, T.L., Ringelberg, D.B., White, D.C. 1994. Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and dessication in a porous medium. *Appl Environ Microbiol* 60:3292-3299.

- Kirk, J.L., Beaudette, L.A., Hart, M., Moutoglis, P., Klironomos, J.N., Lee, H., Trevors, J.T. 2004. Methods of studying soil microbial diversity. *J Microbiol Methods* 58:169-188.
- Klose, S., Tabatabai, M.A. 2000. Urease activity of microbial biomass in soils as affected by cropping systems. *Biol Fertil Soils* 31:191-199.
- Knight, T.R., Dick, R.P. 2004. Differentiating microbial and stabilized β -glucosidase activity relative to soil quality. *Soil Biol Biochem* 36:2089-2096.
- Krämer, S., Green, D.M. 2000. Acid and alkaline phosphatase dynamics and their relationship to soil microclimate in a semiarid woodland. *Soil Biol Biochem* 32:179-188.
- Leckie, S.E. 2005. Methods of microbial community profiling and their application to forest soils. *For Ecol Manage* 220:88-106.
- Le Duc, M.G., Pakeman, R.J., Marrs, R.H. 2000. Vegetation development on upland and marginal land treated with herbicide, for bracken (*Pteridium aquilinum*) control, in Great Britain. *J Environ Manage* 58:147-160.
- Lim, C.H., Ozkanca, R., Flint, K.P. 1996. The effects of osmotic stress on survival and alkaline phosphatase activity of *Aeromonas hydrophila*. *FEMS Microbiol Lett* 137:19-24.
- Marcote, I., Hernandez, T., Garcia, C., Polo, A. 2001. Influence of one or two successive annual applications of organic fertilisers on the enzyme activity of a soil under barley cultivation. *Bioresour Technol* 79:147-154.
- Martínez-Ruiz, C., Fernández-Santos, B. 2005. Natural revegetation on topsoiled mining-spoils according to the exposure. *Acta Oecol* 28:231-238.
- Masciandaro, G., Ceccanti, B. 1999. Assessing soil quality in different agro-ecosystems through biochemical and chemico-structural properties of humic substances. *Soil Tillage Res* 51:129-137.
- Merilä, P., Strömmer, R., Fritze, H. 2002. Soil microbial activity and community structure along a primary succession transect on the land-uplift coast in western Finland. *Soil Biol Biochem* 34:1647-1654.
- Michener, W.K. 1997. Quantitatively evaluating restoration experiments: research design, statistical analysis, and data management considerations. *Restor Ecol* 5:324-337.
- Milton, S.J. 2001. Rethinking ecological rehabilitation in arid and winter rainfall regions of southern Africa. *S Afr J Sci* 97:1-2.
- Mining Review Africa. 2003. The green mine. [cited April 2005]; Available from http://www.miningreview.com/archive/021/20_1.htm
- Molnár, Z., Botta-Dukát, Z. 1998. Improved space-for-time substitution for hypothesis generation: Secondary grasslands with documented site history in SE-Hungary. *Phytocoenologia* 28:1-29.
- Mummey, D.L., Stahl, P.D., Buyer, J.S. 2002a. Microbial biomarkers as an indicator of ecosystem recovery following surface mine reclamation. *Appl Soil Ecol* 21:251-259.
- Mummey, D.L., Stahl, P.D., Buyer, J.S. 2002b. Soil microbiological properties 20 years after surface mine reclamation: spatial analysis of reclaimed and undisturbed sites. *Soil Biol Biochem* 34:1717-1725.
- Muyzer, G. 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr Opin Microbiol* 2:317-322.
- Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G. 2003. Microbial diversity and soil functions. *Eur J Soil Sci* 54:655-670.
- Nannipieri, P., Kandeler, E., Ruggiero, P. 2002. Enzyme activities and microbiological and biochemical processes in soil. In: Burns, R.G., Dick, R.P., editors. *Enzymes in the environment - activity, ecology, and applications*, New York: Marcel Dekker Inc. pp 1-33.
- Ndiaye, E.L., Sandero, J.M., McGrath, D., Dick, R.P. 2000. Integrative biological indicators for detecting changes in soil quality. *Am J Altern Agric* 15:26 – 36.
- Olsson, P.A. 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. *FEMS Microbiol Ecol* 29:303-310.
- Orwin, K.H., Wardle, D.A. 2004. New indices for quantifying the resistance and resilience of soil biota to exogenous disturbances. *Soil Biol Biochem* 36:1907-1912.

- Pascual, J.A., Garcia, C., Hernandez, T., Moreno, J.L., Ros, M. 2000. Soil microbial activity as a biomarker of degradation and remediation processes. *Soil Biol Biochem* 32:1877-1883.
- Peacock, A.D. 2005. Center for Biomarker Analysis, University of Tennessee, Knoxville. Personal communication.
- Peacock, A.D., Mullen, M.D., Ringelberg, D.B., Tyler, D.D., Hedrick, D.B., Gale, P.M., White, D.C. 2001. Soil microbial community responses to dairy manure or ammonium nitrate applications. *Soil Biol Biochem* 33:1011-1019.
- Pickett, S.T.A. 1989. Space for time substitution as an alternative to long-term studies. In: Likens, G.E., editor. *Long-term studies in ecology*. New York: Springer. pp 110-135.
- Pinkart, H.C., Devereux, R., Chapman, P.J. 1998. Rapid separation of microbial lipids using solid phase extraction columns. *J Microbiol Methods* 34:9-15.
- Ponder, Jr. F., Tadros, M. 2002. Phospholipid fatty acids in forest soil four years after organic matter removal and soil compaction. *Appl Soil Ecol* 19:173-182.
- Rapport, D.J., McCullum, J., Miller, M.H. 1997. Soil health: its relationship to ecosystem health. In: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R., editors. *Biological indicators of soil health*. New York: CAB International. pp 29-47.
- Richards, B.N. 1994. *The microbiology of terrestrial ecosystems*. New York: Longman Scientific & Technical. 399p.
- Ritz, K., McHugh, M., Harris, J. 2003. Biological diversity and function in soils: contemporary perspectives and implications in relation to the formulation of effective indicators. OECD expert meeting on soil erosion and soil biodiversity indicators, March 2003. Rome. pp 1-11.
- Rösner, T., Boer, R., Reyneke, R., Aucamp, P., Vermaak, J. 2001. A preliminary assessment of pollution contained in the unsaturated and saturated zone beneath reclaimed gold-mine residue deposits. *Water Research Commission Report 797/1/01*. Pretoria. 210p.
- Rowell, D.L. 1994. *Soil science: methods and applications*. Essex: Addison Wesley Longman Ltd. 350p.
- Ruf, A., Beck, L., Dreher, P., Hund-Rinke, K., Römbke, J., Spelda, J. 2003. A biological classification concept for the assessment of soil quality: "biological soil classification scheme" (BBSK). *Agric Ecosyst Environ* 2077:1-9.
- Rutigliano, F.A., D'Ascoli, R., Virzo De Santo, A. 2004. Soil microbial metabolism and nutrient status in a Mediterranean area as affected by plant cover. *Soil Biol Biochem* 36:1719-1729.
- Rütters, H., Sass, H., Cypionka, H., Rullkötter, J. 2002. Phospholipid analysis as a tool to study complex microbial communities in marine sediments. *J Microbiol Methods* 48:149-160.
- Samborska, A., Stepniewska, Z., Stepniewski, W. 2004. Influence of different oxidation states of chromium (VI, III) on soil urease activity. *Geoderma* 122:317 – 322.
- Scanlon, T.M., Caylor, K.K., Manfreda, S., Levin, S.A., Rodriguez-Iturbe, I. 2005. Dynamic response of grass cover to rainfall variability: implications for the function and persistence of savanna ecosystems. *Adv Water Resour* 28:291-302.
- Schoenholtz, S.H., Van Miegroet, H., Burger, J.A. 2000. A review of chemical and physical properties as indicators of forest soil quality: challenges and opportunities. *For Ecol Manage* 138:335-356.
- SER. 2004. Society for Ecological Restoration International Science & Policy Working Group. *The SER primer on ecological restoration*. [cited June 2005]; Available from <http://www.ser.org/>
- Smith, C.A., Phiefer, C.B., Macnaughton, S.J., Peacock, A., Burkhalter, R.S., Kirkegaard, R., White, D.C. 2000. Quantitative lipid biomarker detection of unculturable microbes and chlorine exposure in water distribution system biofilms. *Water Res* 34: 2683-2688.
- Smith, S.N., Pugh, G.J.F. 1979. Evaluation of dehydrogenase as a suitable indicator of soil microflora activity. *Enzyme Microb Technol* 1:279-281.
- Sparling, G., Ross, D., Trustrum, N., Arnold, G., West, A., Speir, T., Schipper, L. 2003. Recovery of topsoil characteristics after landslip erosion in dry hill country of New Zealand, and a test of the space-for-time hypothesis. *Soil Biol Biochem* 35:1575-1586.

- Steer, J., Harris, J.A. 2000. Shifts in the microbial community in rhizosphere soils during the growth of *Agrostis stolonifera*. *Soil Biol Biochem* 32:869-878.
- Steenwerth, K.L., Jackson, L.E., Calderón, F.J., Stromberg, M.R., Scow, K.M. 2003. Soil microbial community composition and land use history in cultivated and grassland ecosystems of coastal California. *Soil Biol Biochem* 35:489-500.
- Tate, R.L. (III). 2000. *Soil microbiology*. 2nd Ed. New York: John Wiley & Sons, Inc. 508p.
- Tate, R.L. (III), Rogers, B.F. 2002. Soil ecosystem properties, microbial diversity, and ecosystem assessments. In: Violante, A., Huang, P.M., Bollag, J.-M., Gianfreda, L., editors. *Developments in soil science*, volume 28B, New York: Elsevier. pp 79-93.
- Taylor, J.P., Wilson, B., Mills, M.S., Burns, R.G. 2002. Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. *Soil Biol Biochem* 34:387-401.
- Turner, B.L., Hopkins, D.W., Haygarth, P.M., Ostle, N. 2002. β -Glucosidase activity in pasture soils. *Appl Soil Ecol* 20:157-162.
- UNCCD. 1994. The United Nations Convention to Combat Desertification. [cited February 2005]; Available from <http://www.unccd.int/convention/menu.php/>
- Van Wyk, S.J. 2002. An analytical investigation of the biophysical factors that inhibit successful ecological restoration of gold tailings dams (Dissertation – M.Env.Sci). Potchefstroom University for Christian Higher Education, Potchefstroom, South Africa. 154p.
- Vepsäläinen, M., Kukkonen, S., Vestberg, M., Sirviö, H., Niemi, R.M. 2001. Application of soil enzyme activity test kit in a field experiment. *Soil Biol Biochem* 33:1665-1672.
- Waldrop, M.P., Balsler, T.C., Firestone, M.K. 2000. Linking microbial community composition to function in a tropical soil. *Soil Biol Biochem* 32:1837-1846.
- Wang, W.J., Dalal, R.C., Moody, P.W., Smith, C.J. 2003. Relationships of soil respiration to microbial biomass, substrate availability and clay content. *Soil Biol Biochem* 35:273-284.
- White, D.C., Ringelberg, D.B. 1998. Signature lipid biomarker analysis. In: *Techniques in microbial ecology*. Burlage, R.S., Atlas, R., Stahl, D., Geesey, G., and Sayler, G., editors. New York: Oxford University Press. pp 255-272.
- White, D.C., Stair, J.O., Ringelberg, D.B. 1996. Quantitative comparisons of *in situ* microbial biodiversity by signature biomarker analysis. *J Ind Microbiol* 17:185-196.
- Widmer, F., Fließbach, A., Laczkó, E., Schulze-Aurich, J., Zeyer, J. 2001. Assessing soil biological characteristics: a comparison of bulk soil community DNA-, PLFA-, and BiologTM-analyses. *Soil Biol Biochem* 33: 1029-1036.
- Zelles, L. 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. *Biol Fertil Soils* 29: 111-129.
- Zelles, L., Bai, Q.Y., Beck, T., Beese, F. 1992. Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. *Soil Biol Biochem* 24:317-323.
- Zhang, C., Huang, L., Luan, T., Jin, J., Lan, C. 2006. Structure and function of microbial communities during the early stages of revegetation of barren soils in the vicinity of a Pb/Zn smelter. *Geoderma* 136:555-565.

CHAPTER 2

GENERAL MATERIALS AND METHODS

"As soils are depleted, human health, vitality and intelligence go with them."

– Louis Bromfield

1. SITE DESCRIPTION

The study was conducted on coal discard sites of varying rehabilitation ages located within the grassland biome of South Africa (average annual rainfall of 700 mm). Fourteen coal discard sites that were managed by two different mining companies (seven sites from each mine) were investigated. Both mining companies followed an ameliorative approach to revegetation. This approach relies on achieving optimum conditions for plant growth by the application of cover materials or by improving the physical and chemical nature of mine wastes using organic matter, fertilisers, and lime (Johnson *et al.*, 1994). However, each mining company had a distinct approach to the rehabilitation programmes applied to the respective coal discard sites under their management.

The first part of the study was conducted on seven revegetated coal discard sites under rehabilitation and managed by Mining Company A. At the start of the investigation (2002), discard sites sampled at Mine A varied in rehabilitation age from 1 to 8 years (Table 2.1). In this study, the term 'rehabilitation age' refers to the time since the first application of a soil cover layer and subsequent amelioration and revegetation of a specific coal discard site. Since the soil cover layers applied to discard material differed in thickness for sites managed by different mining companies, the soil cover thickness was considered part of the management practices for the purpose of this investigation. The same sites were resampled in 2004 and 2005 to obtain a chronosequence of rehabilitation ages ranging from 1 to 11 years (chronosequence A).

For the rehabilitation of coal discard sites, Mining Company A applied a soil cover layer of 10-15 cm to discard material before ameliorating the soil cover with lime, fertilisers (nitrate and super-phosphate), and organic material in the form of manure. Dumps were then revegetated with a grass seed mixture. The soil used as cover was excavated from adjacent borrow pits or stripped from the sites before mining (stockpiled). The grass seed mixture applied to the sites included the following commercially available grasses: annual teff [*Eragrostis tef* (Zuccagni) Trotter]; weeping lovegrass [*Eragrostis curvula* (Schrader) Nees]; Rhodesgrass (*Chloris gayana* Kunth); common fingergrass (*Digitaria eriantha*); Bermudagrass [*Cynodon dactylon* (L.) Pers. var. *dactylon*]; and kikuyu grass [*Pennisetum clandestinum* Chiov].

As part of the management regime, all sites were defoliated by means of cutting and baling at the end of each growing season until and including 2002. Sites were treated with variable amounts of lime, inorganic fertilisers, and well-cured manure so that conditions

would be optimal for vegetation growth after amelioration. From 2003, the cutting and baling practice was discontinued and grazing was applied to all sites. Subsequent amelioration of soil cover layers was conducted annually according to lime requirements, fertiliser recommendations, and organic carbon content, based on the results of soil physical and chemical analyses obtained by standard procedures. Macro-elements (Ca, Mg, and K) were always added as nitrates and phosphorus was supplemented using super-phosphate. In 2002, it was envisioned that new management practices would stabilise sites and that fertiliser application would be ceased after five years (2007). Therefore, application of fertilisers and organic material was reduced by the mining company over the study period.

Due to the nature and location of the study sites, the continuous use of reference sites to compare rehabilitated sites to was not possible during this study. The coal discard sites under investigation take up large areas of land (several hectares) and are surrounded by other anthropogenically disturbed areas (roads, farms, urban areas). Furthermore, these sites have a topography (slopes) unlike natural areas that influences vegetation growth and subsequently microbial communities. Reference sites sampled initially (2002), were located on farms adjacent to the study sites. Although these sites were representative of soil not directly impacted by mining activities, they could not be confidently applied as references to natural, undisturbed soils. After 2002, the same reference sites were no longer available due to expanded agricultural activities. The results obtained from the characterisation of the microbial community function and structure at these reference sites are discussed in Chapter 6.

During the second part of this investigation, soil samples were also obtained from coal discard sites (6 to 17 years old) from another coal mining site (managed by Mining Company B). As in the case of rehabilitation practices followed by Mining Company A, Mining Company B applied a soil cover layer to discard material before ameliorating the soil cover with lime, fertilisers (nitrate and super-phosphate), and organic material in the form of manure. However, the soil cover layer applied to the coal discard sites managed by Mining Company B was thicker (30-60 cm) than that applied by Mining Company A (10-15 cm). The soil used as cover was excavated from adjacent borrow pits or stripped from the sites before mining (stockpiled). Discard sites were revegetated with the same grass seed mixture applied at Mine A.

There were also differences in the management regimes applied by the two mining companies after the initial revegetation of the discard dumps. Mining Company A applied

more intensive management in the form of defoliation (cutting and baling, replaced by grazing in 2003) combined with the annual amelioration of soil cover layers according to lime requirements, fertiliser recommendations, and organic carbon content. In contrast, Mining Company B followed a less intensive management regime and applied no amelioration after initial revegetation. Sites were also grazed by livestock as for Mining Company A.

The two coal mines (Mine A and Mine B) chosen for this investigation offered an opportunity to apply space-for-time (SFT) substitution and in this way to compare the long-term effect of different rehabilitative management regimes on the microbial community function and structure of coal discard dumps. When applying this approach, sites of different ages and stages of development at separate locations ('space') are identified to obtain a chronosequences of ages ('time') (Sparling *et al.*, 2003). In this manner, a temporal trend is inferred from a study of different aged sites (refer to Chapter 1 for a discussion on the SFT hypothesis).

The site identities and rehabilitation ages of the coal discard sites sampled at the two mines to derive chronosequence A and chronosequence B, respectively, are presented in Table 2.1.

Table 2.1. Coal discard sites sampled to derive the two chronosequences of rehabilitation ages ranging from 1 to 11 years and 6 to 17 years, respectively.

	Rehabilitation Age																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
Mine A																		
Site 1	x		x	x														
Site 2			x		x	x												
Site 3				x		x	x											
Site 4				x		x	x											
Site 5				x		x	x											
Site 6				x		x	x											
Site 7								x		x	x							
Mine B																		
Site 1						x												
Site 2							x											
Site 3										x								
Site 4											x							
Site 5															x			
Site 6																	x	
Site 7																		x

At Mine A, seven discard sites that varied between 1 and 8 years old in 2002, were sampled in 2002, 2004, and 2005 to obtain a chronosequence of rehabilitation ages ranging from 1 to 11 years (chronosequence A). At Mine B, seven discard sites between the ages of 6 and 17 years old, were sampled in 2006 (chronosequence B). For those ages with data from several sites (replicates), mean values and standard errors were calculated (Martínez-Ruiz and Fernández-Santos, 2005).

Table 2.2. Locations of coal discard sites sampled to derive the two chronosequences of rehabilitation ages.

	Grid Reference
Mine A	
Site 1	26°10.80' S 29°21.20' E
Site 2	26°29.41' S 29°45.34' E
Site 3	26°20.40' S 29°53.19' E
Site 4	25°53.42' S 29°10.27' E
Site 5	25°54.35' S 29°11.21' E
Site 6	26°01.22' S 29°06.06' E
Site 7	27°51.14' S 29°57.23' E
Mine B	
Site 1	26°42.30' S 27°59.71' E
Site 2	26°41.65' S 27°59.32' E
Site 3	26°41.76' E 27°59.56' E
Site 4	26°41.87' S 27°59.32' E
Site 5	26°42.33' S 27°58.83' E
Site 6	26°41.33' S 27°58.83' E
Site 7	26°41.17' S 27°58.59' E

(Grid) Lat/Lon hddd°mm.mmm' (Datum) WGS 84.

2. SAMPLING PROCEDURE

A random sampling design was used to obtain three composite samples per site (five cores per composite sample) of the soil cover layers from all coal discard sites ($n = 21$ for Mine A and Mine B, respectively). All sites were sampled during summer, within one month after the first rainfall of the season. Samples were collected in the same quadrates used for the assessment of vegetation growth. A soil auger was used to obtain volume samples with a minimum of 1 kg of soil per sampling area. The top 0-10 cm of the soil cover layer was sampled since most of the discard dumps at Mine A had an effective soil cover thickness of only ≤ 15 cm. Although dumps from Mine B had thicker soil cover layers (at least 30 cm) than dumps from Mine A, only the top 0-10 cm of the soil cover layer was sampled to facilitate comparison between the two chronosequences.

Samples were obtained using aseptic techniques and subsamples (± 200 g) of each sample were kept at 4°C for enzymatic assays to preserve biological properties. For lipid analyses, separate subsamples (± 200 g) were sealed in plastic bags, frozen on site using dry ice and transported on dry ice to the laboratory, where it was stored at -80°C until lyophilisation. Composite (consolidated) samples were mixed thoroughly to contain equal weights of individual samples. Each consolidated sample was analysed for enzymatic activity within five days of sampling.

3. ESTIMATION OF VEGETATION COVER

The ground and crown vegetation cover of all the sites were estimated in three 1 m² quadrates randomly placed over a 50 m transect. All plants rooted in a 1 m² quadrate were included in the estimation per plot. The ground cover included all living and non-living organic material on the ground surface per area and the crown cover was regarded as the canopy cover spread of all grass species over a fixed area. A quantitative value (percentage) was attributed to every plot and both values (ground and crown cover) were expressed as a percentage per m² surface area (Van Rensburg *et al.*, 2004).

4. PHYSICAL AND CHEMICAL SOIL ANALYSIS

Physical and chemical analyses of soil samples were conducted by an independent laboratory according to standard procedures.

A 1:2 (v/v) water extraction procedure was conducted as described by Peech (1965) for the determination of the water-soluble basic cation fraction (Ca, Mg, K, and Na). Quantification was done by means of atomic absorption spectrometry with a Spectr. AA-250 (Varian, Australia) using acetylene-air for determining the basic cations (Ramirez-Munoz, 1968).

The exchangeable cation concentration was measured by replacement of the exchangeable cations with ammonium by adding excess ammonium acetate solution to the soil samples (Thomas, 1982) and analysed with a Spectr. AA-250 (Varian, Australia). The exchangeable-ion status of the soil samples was used to quantify the percentage base saturation, which expresses the content of exchangeable bases as a percentage of the cation exchange capacity (CEC), measured at pH 7.0 or 8.2. In equation form this becomes:

$$\text{Base saturation (\%)} = \frac{X_b}{\text{CEC}} \times 100$$

where: X_b = sum of exchangeable bases (Ca, Mg, K, and Na)

CEC = cation exchange capacity.

The anions (Cl, NO₃, and SO₄) were quantified by means of ion chromatography (Metrohm 761 Compact IC, Switzerland). Concentrations of NH₄ were quantified by means of the ammonia-selective electrode method (Banwart *et al.*, 1972). The pH value and electrical conductivity (EC) of the soil was determined in the 1:2 extract with a calibrated pH/conductivity meter (Radiometer PHM 80, Copenhagen) at 25°C after a 12 h equilibration period with intermittent stirring.

A P-Bray 1 analysis was also conducted to quantify the P concentration as described by Bray and Kurtz (1945) and organic carbon was determined according to the Walkley-Black procedure (Walkley and Black, 1934).

Quantification of the particle-size distribution of all soil samples was conducted according to the procedures advocated by the American Society for Testing and Materials (ASTM, 1961).

5. ASSAYS OF ENZYMATIC ACTIVITIES

Before analyses, consolidated soil samples were passed through a 2 mm sieve. For the determination of dehydrogenase activity, soil was kept at field water content, while air-dried samples were used for determination of β -glucosidase (β -D-glucoside glucohydrolase, EC 3.2.1.21), urease (urea amidohydrolase, EC 3.5.1.5), acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2, pH 6.5) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, pH 11.0) activities (Alef and Nannipieri, 1995; Dick *et al.*, 1996). Soil water content was determined gravimetrically after drying soil samples at 105°C overnight (Alef and Nannipieri, 1995). All analyses were carried out in triplicate.

5.1. Dehydrogenase Activity

Dehydrogenase activity was assayed according to the method of Von Mersi and Schinner (1991) described in Alef and Nannipieri (1995). The method is based on the incubation of

soil with the substrate iodonitrotetrazolium chloride (INT) at 40°C for 2 h followed by colorimetric estimation of the reaction product iodonitrotetrazolium violet-formazan (INF).

Field moist soil (1.0 g) was weighed into a 50 ml screw-cap Erlenmeyer flask and incubated in the dark for 2 h at 40°C with 1.5 ml Tris (hydroxymethyl)-aminomethane (THAM) buffer and 2 ml INT solution. Controls were performed with sterilised soil (1.0 g samples, autoclaved at 121°C for 20 min). The reaction was terminated by the addition of 10 ml *N,N*-dimethylformamide/ethanol (1:1 v/v) extraction solution and shaking at 20 min intervals for 1 h. The soil suspension was filtered through Whatman no. 2 filter paper and the absorbance of the filtrate was measured at 464 nm.

A calibration curve was prepared by pipetting 0, 1, 2, and 5 ml of INF solution into test tubes and adding 13.5 ml of extraction solution to each test tube. The calibration concentrations were: 0, 100, 200, and 500 µg INF per test.

The dehydrogenase activity is expressed as µg INF g⁻¹ dry weight 2h⁻¹ and calculated as follows:

$$INF (\mu\text{g g}^{-1} \text{dwt } 2\text{h}^{-1}) = \frac{S_1 - S_0}{\text{dwt}}$$

where: S_1 = INF (µg) of the test

S_0 = INF (µg) of the control

dwt = dry weight of 1 g moist soil.

5.2. B-glucosidase and Phosphomonoesterase Activities

B-glucosidase, acid phosphatase, and alkaline phosphatase activities were all based on *p*-nitrophenol release after cleavage of a synthetic substrate (*p*-nitrophenyl glucoside and *p*-nitrophenyl phosphate, respectively) (Alef and Nannipieri, 1995; Dick *et al.*, 1996). These procedures were first described by Eivazi and Tabatabai (1977), Tabatabai (1982), and Tabatabai and Bremner (1969).

For the β-glucosidase assay, 1.0 g soil (air dried) was placed in a 50 ml screw-cap Erlenmeyer flask and incubated for 1 h at 37°C with 0.25 ml toluene, 4 ml modified universal buffer (pH 6.0) and 1 ml *p*-nitrophenyl-β-D-glucoside. The reaction was terminated by the addition of 1 ml 0.5 M calcium chloride and 4 ml 0.1 M THAM buffer (pH 12.0). Controls were performed by adding substrate immediately after incubation, before the addition of calcium chloride and THAM buffer. The soil suspension was filtered

through Whatman no. 2 filter paper and the absorbance of the filtrate was measured at 410 nm.

A calibration curve was prepared with 1 ml of standard *p*-nitrophenol solution, diluted to 100 ml with distilled water in a volumetric flask. Aliquots (0, 1, 2, 3, 4, and 5 ml) of this diluted standard solution were pipetted into Erlenmeyer flasks (50 ml) and the volume adjusted to 5 ml by the addition of distilled water. The procedure for *p*-nitrophenol analysis of the incubated sample was then followed.

B-glucosidase activity was expressed as $\mu\text{g } p\text{-nitrophenol g}^{-1} \text{ dry weight h}^{-1}$ and calculated as follows:

$$p - \text{Nitrophenol } (\mu\text{g g}^{-1} \text{dwt h}^{-1}) = \frac{C \times v}{\text{dwt} \times SW \times t}$$

where: C = measured concentration of *p*-nitrophenol ($\mu\text{g ml}^{-1}$ filtrate)

dwt = dry weight of 1 g moist soil

v = total volume of the soil suspension in ml

SW = weight of soil sample used (1 g)

t = incubation time in hours.

Phosphomonoesterase assays differed from the above only in the choice of buffer. Modified universal buffers, pH 6.5 and pH 11.0, were used for acid and alkaline phosphomonoesterase, respectively. Phosphatase activity was expressed as $\mu\text{g } p\text{-nitrophenol g}^{-1} \text{ dry weight h}^{-1}$ and calculated as above.

5.3. Urease activity

Urease activity was assayed using the procedure of Kandeler and Gerber (1988) as described by Alef and Nannipieri (1995). The method is based on the colorimetric determination of released ammonia after the incubation of soil samples with urea solution for 2 h at 37°C.

Air-dried soil (5.0 g) was mixed with 2.5 ml urea solution and 20 ml borate buffer in an Erlenmeyer flask (100 ml) and incubated at 37°C for 2 h. After the incubation, 30 ml of 1.0 M potassium chloride solution was added and the flask was shaken for 30 min. After filtering the soil suspension through Whatman no. 2 filter paper, the filtrates were analysed for the ammonium content. Controls were prepared with 2.5 ml distilled water and the urea

solution was added at the end of the incubation, immediately before the addition of the potassium chloride solution.

For ammonium determination, 1 ml clear filtrate was added to an Erlenmeyer flask (50 ml) and mixed with 9 ml distilled water, 5 ml sodium salicylate/sodium hydroxide solution, and 2 ml sodium dichloroisocyanide solution. The mixture was left to stand at room temperature for 30 min prior to measuring the optical density at 690 nm.

A calibration curve was prepared as follows: ammonium standard solution I (0.0, 1.0, 1.5, 2.0, and 2.5 ml) was diluted with 100 ml potassium chloride solution to prepare ammonium standard solution II. Ammonium standard solution II (1 ml) was diluted with 9 ml distilled water and the ammonium determination performed as above. The ammonium concentrations were 0, 1, 1.5, 2, and 2.5 $\mu\text{g NH}_4\text{-N ml}^{-1}$.

Urease activity was expressed as $\mu\text{g NH}_4\text{-N g}^{-1}$ dry weight 2 h^{-1} and calculated as follows:

$$\mu\text{g NH}_4\text{-N g}^{-1} \text{dwt } 2\text{h}^{-1} = \frac{\mu\text{g NH}_4\text{-N ml}^{-1} \times V \times 10}{\text{dwt} \times 5}$$

where: *dwt* = dry weight of 1 g moist soil

V = total volume of the extract (52.5 ml)

10 = the dilution factor

5 = weight of soil used in the assay.

6. LIPID EXTRACTION, FRACTIONATION, AND ANALYSIS

Microbial biomass was estimated as the total extractable phospholipid fatty acids (PLFAs) (McKinley *et al.*, 2005) and microbial community composition was analysed on relative concentrations (mole percentages) of individual fatty acids.

Glassware for lipid analysis was freed of lipid contaminants by washing with non-phosphate containing soap and then heating in a muffle furnace at 450°C for 4 h.

Total lipids were extracted from 5 g lyophilised soil according to a modified Bligh and Dyer procedure (Peacock *et al.*, 2001) using a single-phase chloroform-methanol-aqueous buffer system in a ratio of 1:2:0.8 (v/v/v).

Silicic acid column chromatography (Guckert *et al.*, 1985) was used to fractionate the total lipid extract into neutral lipids, glycolipids, and polar lipids. The polar lipid fraction was transesterified to fatty acid methyl esters (FAMES) by mild alkaline

methanolysis (Guckert *et al.*, 1985).

FAMES were analysed by capillary gas chromatography with flame ionisation detection on an Agilent 6890 series II gas chromatograph, using a 60 m SPB-1 column (0.250 mm I.D., 0.250 μm film thickness) with the injector and detector maintained at 270°C and 290°C, respectively. Hydrogen was used as the carrier gas and sample injection was splitless. The column temperature was programmed to start at 60°C for 2 min, increased at a rate of 10°C min⁻¹ to 150°C, then increased at 3°C min⁻¹ to 312°C. Gas flow was at a constant pressure of 300kPa. Methyl nonadecanone (19:0) was used as a quantitative internal standard and definitive peak identification was made for representative samples by gas chromatography/mass spectrometry using an Agilent 6890 series II gas chromatograph interfaced with an Agilent 5973 mass selective detector under the same column and temperature programme described. Mass spectra were determined by electron impact at 70eV (McKinley *et al.*, 2005).

Fatty acid nomenclature is in the form of A:B ω C, where “A” is the number of carbon atoms in the chain, “B” is the number of double bonds, and “C” is the position of the double bond from the methyl end of the molecule; *cis* and *trans* geometries are indicated by the suffixes “c” and “t”, respectively. The prefixes “i”, “a”, and “me” refer to iso, anteiso, and midchain methyl branching, respectively, with “cy” indicating a cyclopropyl ring structure and “br” indicating a branched fatty acid with unknown branching configuration (Guckert *et al.*, 1985; Allison *et al.*, 2005).

The sum of the following PLFAs was used as a measure of the bacterial biomass: i15:0, a15:0, 15:0, i16:0, 16:1 ω 9, 16:1 ω 7t, i17:0, a17:0, 17:0, cy17:0, 18:1 ω 7, and cy19:0 (Frostegård and Bååth, 1996). The PLFA 18:2 ω 6 was used as a measure of fungal biomass (Federle, 1986; Frostegård and Bååth, 1996). The ratio of iso to anteiso branched PLFA was calculated as (i15:0 + i17:0/a15:0 + a17:0) and Gram positive PLFA markers were i10me16:0, i15:0, a15:0, i16:0, and 17:0 (McKinley *et al.*, 2005).

7. STATISTICAL ANALYSIS

Statistical analyses were performed and graphs generated using Statistica 7.1 (Statsoft Inc., Tulsa, Oklahoma, USA), SigmaPlot 10.0 (Systat Software Inc., San Jose, California, USA), GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, California, USA), and Canoco for Windows 4.5 (Biometris – Plant Research International, Wageningen, The Netherlands; Ter Braak and Šmilauer, 1998).

Data of physical and chemical characteristics, enzymatic activities, and PLFA composition of samples were subjected to analysis of variance (ANOVA) followed by Tukey's test for mean separation where significant differences ($p = 0.05$) were indicated.

Principal components analysis (PCA) and canonical correspondence analysis (CCA) (Canoco for Windows 4.5) were performed to investigate the relationships between sites, soil physical and chemical properties, and microbial community function and structure.

Chronosequence data for Mine A and B were analysed using the *Time Series Analysis* module in Statistica 7.1 (Statsoft Inc., Tulsa, Oklahoma, USA). Missing data (missing ages) embedded in either time series was replaced by using the *predicted values from linear trend regression* option in Statistica. With this option, Statistica fits a least-squares regression line to the time series. The missing data is then replaced by the values predicted by this regression line. This method implies that the strongest feature of the series is its linear trend across time and was chosen as the most reliable option to predict missing values in the time series even though the data was fitted with nonlinear regression. Other options predict missing data simply by connecting data points on either side of the missing value with a straight line (interpolation) or by replacing missing values with the overall mean of the series or by the mean/median of adjacent points. Linear regression may be viewed as just a simpler case of nonlinear regression and is not fundamentally different from nonlinear regression. Any nonlinear regression programme can be used to fit to a linear model and the results will be the same as if you had chosen linear regression (Motulsky and Christopoulos, 2005).

Curve fitting was performed in GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, California, USA) by using nonlinear regression. Due to the method chosen for predicting missing data points in the chronosequences, curve fits were also performed with linear regression. The p values obtained from both linear and nonlinear regressions after applying the F-test were then compared to determine whether there was consistency in the conclusions regarding the significant differences between the curve fits. Similarity or dissimilarity of fitted curves based on the F-test is expressed in terms of an F-ratio and corresponding p value. If $p < 0.05$, a statistically significant difference between the curves was depicted (Motulsky, 2007).

REFERENCES

- Alef, K., Nannipieri, P. 1995. *Methods in applied soil microbiology and biochemistry*. Boston: Academic Press. 576p.
- Allison, V.J., Miller, R.M., Jastrow, J.D., Matamala, R., Zak, D.R. 2005. Changes in soil microbial community structure in a tallgrass prairie chronosequence. *Soil Sci Soc Am J* 69:1412-1421.
- ASTM (American Society for Testing and Materials). (1961). Tentative method for grain-size analysis of soils. In: *The 1961 book of ASTM standards*. Philadelphia: ASTM.
- Banwart, W.L., Brember, J.M., Tabatabai, M.A. 1972. Determination of ammonium in soil extracts and water samples by an ammonia electrode. *Commun Soil Sci Plant Anal* 3:449.
- Bray, R.H., Kurtz, L.T. 1945. Determination of total, organic and available forms of phosphorus in soils. *Soil Sci* 59:39-45.
- Dick, R.P., Breakwell, D.P., Turco, R.F. 1996. Soil enzyme activities and biodiversity measurements as integrative microbiological indicators. In: Doran, J.W., Jones, A.J., editors. *Methods for assessing soil quality*, SSSA Special Publication 49. Madison: SSSA. pp 247-271.
- Eivazi, F., Tabatabai, M.A. 1977. Phosphatases in soils. *Soil Biol Biochem* 9:167-172.
- Federle, T.W. 1986. Microbial distribution in soil – new techniques. In: Megusar, F., Gantar, M., editors. *Perspectives in microbial ecology*. Ljubljana: Slovene Society of Microbiology. pp 493-498.
- Frostegård, A., Bååth, E. 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol Fertil Soil* 22:59-65.
- Guckert, J.B., Antworth, C.P., Nichols, P.D., White, D.C. 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. *FEMS Microbiol Ecol* 31:147-158.
- Johnson, M.S., Cooke, J.A., Stevenson, J.K.W. 1994. Revegetation of metalliferous wastes and land after metal mining. In: Hester, R.E., Harrison, R.M., editors. *Mining and its environmental impact*. Cambridge: Royal Society of Chemistry. pp 31-48.
- Kandeler, E., Gerber, H. 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. *Biol Fertil Soil* 6:68-72.
- Martínez-Ruiz, C., Fernández-Santos, B. 2005. Natural revegetation on topsoiled mining-spoils according to the exposure. *Acta Oecol* 28:231-238.
- McKinley, V.L., Peacock, A.D., White, D.C. 2005. Microbial community PLFA and PHB responses to ecosystem restoration in tallgrass prairie soils. *Soil Biol Biochem* 37:1946-1958.
- Motulsky, H. 2007. *Curvefit.com – A complete guide to nonlinear regression*. GraphPad Software Inc. [cited March 2007]; Available from http://www.graphpad.com/curvefit/1_model_2_datasets.htm
- Motulsky, H., Christopoulos, A. 2005. *Fitting models to biological data using linear and non-linear regression – a practical guide to curve fitting*. GraphPad Software Inc. 351p.
- Peech, M. 1965. Hydrogen-ion activity. In: Black, C.A., editor. *Methods of soil analysis, Part 2*. Agron Monogr 9. Madison: ASA. pp. 914-926.
- Peacock, A.D., Mullen, M.D., Ringelberg, D.B., Tyler, D.D., Hedrick, D.B., Gale, P.M., White, D.C. 2001. Soil microbial community responses to dairy manure or ammonium nitrate applications. *Soil Biol Biochem* 33:1011-1019.
- Ramiriz-Munoz, J. 1968. *Atomic absorption spectroscopy and analysis by atomic absorption flame photometry*. New York: Am Elsevier Publ. Co.
- Sparling, G., Ross, D., Trustrum, N., Arnold, G., West, A., Speir, T., Schipper, L. 2003. Recovery of topsoil characteristics after landslip erosion in dry hill country of New Zealand, and a test of the space-for-time hypothesis. *Soil Biol Biochem* 35:1575-1586.
- Tabatabai, M.A. 1982. Soil enzymes. In: Page, A.L., Miller, E.M., Keeney, D.R., editors. *Methods of soil analysis, Part 2 – chemical and microbiological properties*. Madison: ASA-SSSA. pp 903-947.

- Tabatabai, M.A., Bremner J.M. 1969. Use of *p*-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biol Biochem* 1:301-307.
- Ter Braak, C.F., Šmilauer, P. 1998. Canoco reference manual for user's guide to Canoco community ordination, ver. 4. New York: Microcomputer Power.
- Van Rensburg, L., Maboeta, M.S., Morgenthal, T.L. 2004. Rehabilitation of co-disposed diamond tailings: growth medium rectification procedures and indigenous grass establishment. *Water Air Soil Pollut* 154:101-113.
- Von Mersi, W., Schinner, F. 1991. An improved and accurate method for determining dehydrogenase activity of soils with iodinitrotetrazolium chloride. *Biol Fertil Soils* 11:216-220.
- Walkley, A., Black, I.A. 1934. An examination of the Degiareff method for determining SOM and a proposed modification of the chromic acid titration method. *Soil Sci* 37:29-38.

CHAPTER 3

SOIL MICROBIAL COMMUNITY FUNCTION IN A POST-MINING CHRONOSEQUENCE

"Population must increase rapidly, more rapidly than in former times, and ere long the most valuable of all arts will be the art of deriving a comfortable subsistence from the smallest area of soil."

– Abraham Lincoln

Part of this material has been published in *Applied Ecology and Environmental Research* 4:75-83 (2005) by the authors S. Claassens, K.J. Riedel, L. van Rensburg, T.L. Morgenthal, & P.J. Jansen van Rensburg.

Part of this material has been submitted for publication in *Water, Air, and Soil Pollution*.

1. INTRODUCTION

Soil has been recognised for its value as a natural resource and accordingly, serious concerns exist regarding anthropogenic disturbances such as mining activities that cause environmental degradation (Harris, 2003). The natural grassland biome of South Africa is fragmented by an abundance of mine tailings and discard sites. Revegetation (rehabilitation) of these areas are complicated by the poor physical and chemical soil characteristics associated with mining disturbed areas as well as difficult climatic conditions characteristic to the arid and semi-arid areas of southern Africa (Milton, 2001).

Traditionally, physical and chemical soil analyses and visually distinguishable aboveground indicators, such as vegetation, formed the foundation for the majority of management decisions in rehabilitation. However, these parameters fail to account for soil microbiota, which are the basis of terrestrial ecosystems (Pascual *et al.*, 2000; Mummey *et al.*, 2002). The significance of microbial communities in sustainable soil ecosystems has been acknowledged for some time and an essential element for evaluating impacts of management practices is the accurate assessment of microbial community function and structure (Tate and Rogers, 2002). In this chapter, the assessment of microbial community function based on assays of potential enzymatic activities is discussed. The assessment of microbial community structure is addressed in Chapter 4.

Microbial activity is fundamental in the processes that make energy and nutrients available for recycling in the ecosystem and soil microorganisms play crucial roles in the biogeochemical cycling of C, N, and P (Bandick and Dick, 1999). The assay of a variety of soil enzymes gives an indication of the diversity of functions that can be assumed by the microbial community (Brohon *et al.*, 2001; Hinojosa *et al.*, 2004). Enzymatic activities are also a direct expression of the soil microbial community to metabolic requirements and available nutrients. The capacity of soil microbial communities to maintain functional diversity of critical soil processes through disturbance, stress, or succession could ultimately be more important to ecosystem stability than taxonomic diversity (Caldwell, 2005). Several studies indicate the sensitivity of enzymatic activities to management practices, disturbance (Dick, 1997; Aon *et al.*, 2001; Badiane *et al.*, 2001), and soil quality (Gil-Sotres *et al.*, 2005). Enzyme assays are performed under optimised reaction conditions that give an indication of the potential (maximum) enzymatic activity in a soil sample (Tate, 2002). Rather than assaying actual enzymatic activity, the use of buffered and optimised methods have the advantage of standardising environmental factors, which

allows for comparison of soils from different geographical locations and environmental conditions (Schloter *et al.*, 2003).

During this investigation, the temporal changes in microbial community function in a post-mining chronosequence of coal discard sites (managed by Mining Company A) of varying rehabilitation ages (1 to 11 years) were studied. The objective was to determine whether temporal changes in microbial community function (enzymatic activities) were related to rehabilitation age and whether assays of enzymatic activity could indicate progress of rehabilitation. The enzymatic activities assayed included dehydrogenase, β -glucosidase, acid and alkaline phosphatases, and urease.

2. MATERIALS AND METHODS

Refer to Chapter 2 for a comprehensive site description and discussion of all materials and methods used in this investigation.

2.1. Site Description and Sampling

This investigation was conducted on seven revegetated coal discard sites under rehabilitation. At the start of the investigation, discard sites sampled at Mine A varied in rehabilitation age from 1 to 8 years. The same sites were resampled in 2004 and 2005 to obtain a chronosequence of rehabilitation ages ranging from 1 to 11 years (chronosequence A) (site identities and rehabilitation ages are presented in Table 2.1, Chapter 2).

During rehabilitation of coal discard sites, Mining Company A applied a soil cover layer that was ameliorated and revegetated with a mixture of commercially available grasses. As a management practice, all sites were defoliated by means of cutting and baling at the end of each growing season until and including 2002. From 2003, this practice was discontinued and grazing was applied to all sites. Amelioration of soil cover layers was conducted annually according to lime requirements, fertiliser recommendations, and organic carbon content, based on the results of soil physical and chemical analyses.

A random sampling design was used to obtain three composite samples per site (five cores per composite sample) of the soil cover layers from seven coal discard sites. The top 0-10 cm of the soil cover layer was sampled since most of the discard dumps had an effective soil cover thickness of only ≤ 15 cm. Samples were obtained using aseptic techniques and subsamples (± 200 g) were kept at 4°C (for enzymatic analyses) to preserve biological properties. Each sample was analysed for enzymatic activity within five days of

sampling.

2.2. Estimation of Vegetation Cover

The ground and crown vegetation cover of all the sites were estimated in three 1 m² quadrates randomly placed over a 50 m transect. The ground cover included all living and non-living organic material on the ground surface per area and the crown cover was regarded as the canopy cover spread of all grass species over a fixed area. Both values were expressed as a percentage per m² surface area (Van Rensburg *et al.*, 2004).

2.3. Physical and Chemical Soil Analysis

The extraction and analysis procedures for the physical and chemical characterisation of soil samples were conducted by an independent laboratory according to standard procedures (see Chapter 2).

2.4. Assays of Enzymatic Activities

Before analyses, consolidated soil samples were passed through a 2 mm sieve. For the determination of dehydrogenase activity, soil was kept at field water content, while air-dried samples were used for the determination of β -glucosidase, urease, and acid and alkaline phosphatase activities. Soil water content was determined gravimetrically after drying soil samples at 105°C overnight (Alef and Nannipieri, 1995).

Dehydrogenase and urease activities were assayed according to the procedures described in Alef and Nannipieri (1995). B-glucosidase as well as acid and alkaline phosphatase activities were based on *p*-nitrophenol release after cleavage of a synthetic substrate (*p*-nitrophenyl glucoside and *p*-nitrophenyl phosphate, respectively) (Alef and Nannipieri, 1995; Dick *et al.*, 1996). Modified universal buffers pH 6.5 and pH 11.0 were used for acid and alkaline phosphomonoesterase, respectively.

2.5. Statistical Analysis

Statistical analyses were performed and graphs generated using Statistica 7.1 (Statsoft Inc., Tulsa, Oklahoma, USA), SigmaPlot 10.0 (Systat Software Inc., San Jose, California, USA), and Canoco for Windows 4.5 (Biometris – Plant Research International, Wageningen, The Netherlands; Ter Braak and Šmilauer, 1998).

Data of physical and chemical characteristics, as well as enzymatic activities of samples were subjected to analysis of variance (ANOVA) followed by Tukey's test for mean separation where significant differences ($p = 0.05$) were indicated. A principal components analysis (PCA) (Canoco for Windows 4.5) was performed on the physical and chemical characteristics to determine whether there was differentiation between sites based on these variables.

3. RESULTS AND DISCUSSION

Throughout this discussion, coal discard sites are referred to in terms of their rehabilitation ages, with the site name and sampling year indicated in brackets (e.g. Site 1_2002). Refer to Table 2.1 (Chapter 2) for names and rehabilitation ages of all coal discard sites.

3.1. Physical and Chemical Soil Properties

Results from the physical and chemical characterisation of the soil cover layers showed few significant differences ($p < 0.05$) between sites of different rehabilitation ages (Table 3.1). A PCA (Figure 3.1) based on the physical and chemical properties of soil covers indicated only two outliers – one of the 4-year old sites (Site 1_2005) and the 11-year old site (Site 7_2005). The separate positions of these sites on the PCA could not be attributed to individual soil properties and may be due to the combined effect of the soil physical and chemical properties. No trends consistent with the rehabilitation ages of the respective discard sites were apparent from this investigation. The similarity in physical and chemical soil properties between sites of different rehabilitation ages is due to the homogeneity of the soil cover layers as well as the slow rate of soil evolution and therefore the time-scale of this investigation will not have a significant impact on these properties. This homogeneity of soil physical and chemical properties is important to facilitate the comparison of different aged sites in terms of microbial community function and structure and to assess the temporal effect of the applied management regime.

All sites showed an increase in P, organic C, and $\text{NO}_3\text{-N}$ from 2002 to 2005, with the exception of Site 3, which showed a decrease in $\text{NO}_3\text{-N}$ from 2002 to 2005 (Table 3.1). In addition, this site consistently had the highest P over the study period. These observations could be attributed to the variable applications of fertilisers and organic material (manure) to the sites. Application of fertilisers and organic material was performed by the mining company to ensure optimum vegetation growth. The soil pH varied between 5.51 and 7.81

and a general increase in pH was observed from 2002 to 2005 in sites of all rehabilitation ages (Table 3.1). However, differences in pH were not statistically significant ($p > 0.05$). Soil pH would not directly affect enzymatic activities assayed since the assays are performed using buffers at their optimal pH (Hinojosa *et al.*, 2004). However, the long-term effect of pH can cause shifts in the microbial community structure that would translate into a functional (enzymatic) response.

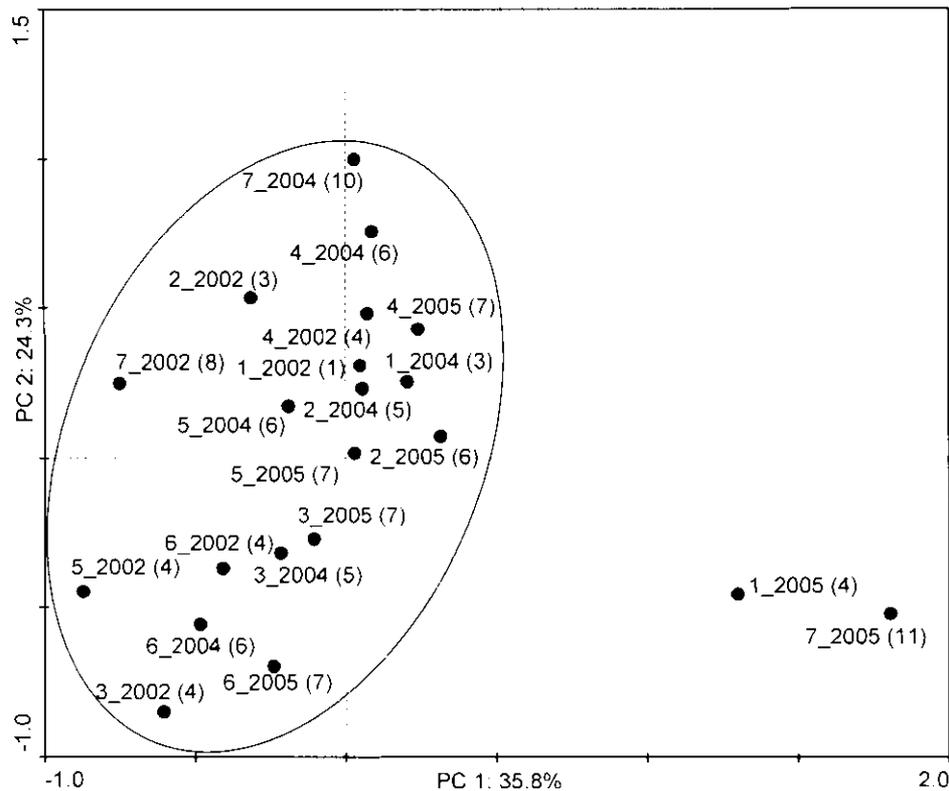


Figure 3.1. Principal components analysis (PCA) ordination diagram illustrating the relationship between coal discard sites based on physical and chemical soil properties. Each site is indicated according to the name of the site, followed by the time of sampling and the rehabilitation age in brackets, e.g. Site 1 sampled in 2002 was 1 year old: 1_2002 (1).

A general decrease in percentage ground and crown cover from 2002 to 2005 was observed. The only increases were observed in Site 1 (crown cover), Site 2 (crown cover), and Site 6 (ground cover) (Table 3.1). This could be attributed to a change in the management practices applied. Until 2002, sites were defoliated by means of cutting and baling of the grass; this was replaced by grazing of the sites by livestock. Initially (at the start of rehabilitation), fertilisers and organic material were applied at high rates to maximise vegetation growth but was optimised over the rehabilitation period to achieve stabilisation in vegetation growth.

Table 3.1. Physico-chemical properties and vegetation cover of soil cover layers obtained from the coal discard sites managed by **Mining Company A**.

	Site_1	Site_2	Site_3	Site_4	Site_5	Site_6	Site_7
2002							
Rehabilitation age (years)	1	3	4	4	4	4	8
Ca (mg kg ⁻¹)	25.10 (9.72)a	7.36 (0.27)a	3.73 (1.68)a	2.92 (0.53)a	3.13 (0.27)a	5.65 (1.29)a	49.30 (13.04)a
Mg (mg kg ⁻¹)	7.89 (4.41)a	2.08 (0.22)a	1.47 (0.64)a	0.92 (0.18)a	0.98 (0.16)a	1.22 (0.49)a	15.77 (3.40)a
K (mg kg ⁻¹)	5.90 (1.96)a	5.31 (0.78)a	25.08 (8.32)a	4.92 (1.26)a	12.59 (6.51)a	24.59 (9.31)a	6.29 (1.71)a
Na (mg kg ⁻¹)	1.56 (0.44)a	1.85 (0.12)a	1.21 (0.44)a	0.93 (0.12)a	0.87 (0.27)a	1.21 (0.17)a	2.08 (0.30)a
SO ₄ (mg kg ⁻¹)	122.04 (71.69)a	28.27 (2.17)a	13.77 (4.72)a	25.62 (12.21)a	26.34 (6.06)a	32.62 (15.90)a	239.00 (72.57)a
NO ₃ -N (mg kg ⁻¹)	6.38 (5.15)a	6.38 (1.30)a	16.19 (6.59)a	1.31 (0.16)a	6.05 (3.50)a	11.45 (7.31)a	2.45 (1.23)a
NH ₄ -N (mg kg ⁻¹)	0.30 (0.04)a	0.26 (0.07)a	0.73 (0.23)a	0.43 (0.09)a	0.64 (0.07)a	0.47 (0.21)a	0.39 (0.07)a
Cl (mg kg ⁻¹)	5.35 (0.71)a	12.75 (5.20)a	5.71 (1.96)a	1.69 (0.32)a	4.01 (1.35)a	3.03 (1.03)a	2.59 (0.18)a
P (P-Bray1) (mg kg ⁻¹)	18.23 (9.75)a	5.60 (0.84)a	146.43 (65.07)a	27.37 (7.27)a	38.13 (16.77)a	96.77 (17.27)a	18.97 (7.53)a
Organic carbon (%)	0.31 (0.16)a	0.52 (0.10)a	2.20 (0.74)a	0.32 (0.08)a	0.67 (0.01)a	2.72 (1.36)a	0.69 (0.14)a
pH	7.41 (0.44)a	6.20 (0.26)a	7.03 (0.03)a	7.15 (0.50)a	6.24 (0.41)a	6.90 (0.37)a	5.51 (0.27)a
EC (mS m ⁻¹)	37.00 (4.36)a	21.67 (4.10)a	29.00 (6.27)a	26.33 (2.03)a	18.67 (6.23)a	35.00 (8.02)a	117.33 (46.25)a
CEC (cmol(+) kg ⁻¹)	7.06 (1.77)a	17.55 (1.92)a	9.34 (1.56)a	4.19 (0.61)a	4.90 (1.01)a	9.92 (3.95)a	24.34 (1.60)a
Base saturation (%)	112.29 (17.54)a	70.13 (12.70)a	98.94 (15.35)a	96.49 (13.94)a	61.28 (13.34)a	6.90 (0.37)a	54.33 (3.07)a
Sand (%)	58.70 (1.00)a	39.80 (2.62)a	68.18 (2.53)a	61.23 (1.62)a	68.12 (2.61)a	67.46 (0.91)a	79.22 (5.53)a
Silt (%)	34.27 (1.54)a	42.66 (1.76)a	23.42 (2.84)a	26.27 (2.61)a	20.93 (2.92)a	25.25 (2.83)a	8.46 (1.96)a
Clay (%)	7.02 (1.63)a	17.55 (0.86)a	8.39 (0.51)a	12.51 (1.10)a	10.95 (1.75)a	7.29 (2.26)a	12.31 (3.81)a
Ground Cover (%)	41.57 (15.67)ab	63.21 (23.72)a	71.25 (19.10)cd	28.92 (12.30)ab	86.75 (16.59)d	60.91 (14.97)bcd	77.42 (17.71)abc
Crown Cover (%)	45.71 (15.97)a	38.75 (12.36)b	80.13 (12.95)b	42.50 (22.11)a	84.83 (14.27)b	65.91 (19.98)ab	56.29 (19.85)b
2004							
Rehabilitation age (years)	3	5	6	6	6	6	10
Ca (mg kg ⁻¹)	18.95 (2.91)a	25.00 (5.62)a	5.49 (1.22)a	13.51 (3.64)a	9.17 (6.96)a	7.56 (1.82)a	82.39 (19.82)ab
Mg (mg kg ⁻¹)	3.67 (0.57)a	7.52 (1.58)a	1.96 (0.51)a	1.83 (0.39)a	3.52 (2.95)a	2.69 (0.99)a	9.68 (3.13)a
K (mg kg ⁻¹)	8.25 (0.81)a	5.92 (2.41)a	18.98 (6.31)a	9.78 (2.61)a	12.34 (7.96)a	25.57 (13.55)a	12.47 (5.57)a
Na (mg kg ⁻¹)	0.81 (0.19)a	3.76 (2.69)a	1.68 (0.37)a	1.04 (0.43)a	1.39 (0.67)a	2.37 (0.93)a	5.27 (5.76)a
SO ₄ (mg kg ⁻¹)	42.29 (2.53)a	27.52 (11.54)a	10.75 (4.87)a	50.51 (11.47)a	45.31 (39.51)a	22.47 (10.97)a	28.17 (7.01)a
NO ₃ -N (mg kg ⁻¹)	1.72 (0.19)a	9.83 (8.70)a	10.28 (3.39)a	2.81 (1.44)a	6.30 (3.26)a	28.11 (11.35)a	10.14 (3.00)a
NH ₄ -N (mg kg ⁻¹)	1.13 (0.45)a	0.95 (0.83)a	1.02 (0.28)a	0.82 (0.33)a	0.57 (0.09)a	0.55 (0.13)a	3.04 (1.02)b
Cl (mg kg ⁻¹)	9.99 (0.93)a	17.3 (8.19)b	4.86 (1.64)a	4.91 (1.37)a	2.90 (0.96)a	2.59 (0.89)a	16.95 (9.19)b
P (P-Bray1) (mg kg ⁻¹)	15.37 (4.97)a	9.47 (1.06)a	149.34 (37.46)c	18.20 (5.06)a	53.61 (20.5)b	118.45 (15.12)c	30.07 (19.45)a
Organic carbon (%)	0.58 (0.05)a	0.67 (0.05)a	2.42 (0.24)a	0.78 (0.08)a	1.08 (0.27)a	2.33 (1.86)a	1.54 (0.36)a
pH	7.81 (0.15)a	7.32 (0.13)a	7.12 (0.31)a	6.57 (0.22)a	6.58 (0.36)a	6.86 (0.37)a	5.85 (0.28)a
EC (mS m ⁻¹)	47.33 (1.78)a	60.67 (25.26)a	36.33 (5.07)a	46.67 (6.68)a	43.67 (26.40)a	50.83 (18.00)a	60.00 (28.20)a

	Site_1	Site_2	Site_3	Site_4	Site_5	Site_6	Site_7
2004 (continued)							
CEC (cmol(+) kg ⁻¹)	5.06 (0.59)a	5.86 (1.04)a	9.55 (1.37)a	3.48 (1.07)a	4.86 (0.54)a	8.50 (2.94)a	9.90 (0.97)a
Base saturation (%)	99.18 (8.56)ab	97.04 (5.29)ab	99.21 (10.56)a	75.61 (3.19)ab	76.59 (10.70)a	50.86 (11.37)b	75.25 (5.38)ab
Sand (%)	73.20 (0.32)a	47.53 (2.74)a	74.23 (1.57)a	88.17 (1.10)a	74.69 (3.80)a	74.90 (2.81)a	56.03 (0.55)a
Silt (%)	8.27 (0.50)a	23.30 (3.20)a	16.68 (1.56)a	11.67 (1.04)a	14.33 (3.74)a	16.21 (2.96)a	11.43 (1.99)a
Clay (%)	18.53 (0.52)ab	29.17 (1.04)b	9.11 (0.40)a	10.17 (1.06)a	10.99 (1.63)a	8.91 (1.92)a	32.53 (1.55)b
Ground Cover (%)	19.99 (1.75)ab	27.54 (2.71)ab	39.28 (1.03)b	15.11 (3.57)a	24.06 (6.40)ab	39.50 (12.50)c	15.92 (2.34)a
Crown Cover (%)	61.36 (3.21)a	63.09 (3.07)a	61.82 (3.99)a	41.72 (6.97)a	62.28 (9.62)a	64.02 (3.50)a	49.34 (6.55)a
2005							
Rehabilitation age (years)	4	6	7	7	7	7	11
Ca (mg kg ⁻¹)	54.14 (18.70)ab	15.12 (6.53)a	7.62 (0.77)a	5.14 (0.57)a	15.22 (13.52)a	9.48 (2.35)a	12.23 (5.87)a
Mg (mg kg ⁻¹)	19.14 (7.01)ab	8.01 (4.65)a	2.45 (0.37)a	2.93 (0.47)a	6.05 (5.37)a	4.16 (1.49)a	14.17 (3.40)ab
K (mg kg ⁻¹)	40.42 (21.73)a	18.98 (10.19)a	12.88 (4.13)a	8.16 (1.94)a	12.10 (9.41)a	26.56 (17.80)a	35.11 (10.93)a
Na (mg kg ⁻¹)	11.68 (4.61)c	4.11 (1.84)a	2.14 (0.31)a	2.37 (0.19)a	2.19 (1.07)a	3.53 (1.69)a	10.01 (1.74)bc
SO ₄ (mg kg ⁻¹)	18.50 (6.26)a	33.31 (17.84)a	7.73 (5.03)a	34.28 (15.70)a	12.32 (6.04)a	14.66 (2.03)a	26.5 (7.31)a
NO ₃ -N (mg kg ⁻¹)	135.55 (23.60)c	13.57 (8.13)a	4.37 (0.19)a	15.29 (5.79)a	6.55 (3.03)a	44.77 (15.39)b	160.19 (93.33)c
NH ₄ -N (mg kg ⁻¹)	0.73 (0.09)ab	0.73 (0.06)ab	1.32 (0.34)b	0.59 (0.11)ab	0.50 (0.06)a	0.64 (0.04)ab	1.00 (0.34)ab
Cl (mg kg ⁻¹)	30.41 (14.58)a	5.08 (4.75)a	4.01 (1.32)a	0.80 (0.19)a	1.78 (0.58)a	2.14 (0.76)a	29.70 (9.99)a
P (P-Bray1) (mg kg ⁻¹)	21.39 (3.66)a	15.15 (4.34)a	152.25 (9.58)b	67.16 (19.68)a	69.08 (24.24)a	140.13 (12.97)b	44.85 (5.24)a
Organic carbon (%)	31.35 (1.11)b	11.56 (0.02)a	6.18 (1.04)a	4.65 (0.09)a	8.82 (0.03)a	10.93 (0.06)a	46.88 (1.03)c
pH	7.52 (0.07)a	7.49 (0.34)a	7.21 (0.32)a	7.19 (0.18)a	6.89 (0.31)a	6.96 (0.38)a	7.52 (0.40)a
EC (mS m ⁻¹)	53.00 (25.52)a	56.00 (27.16)a	43.67 (3.89)a	42.33 (4.14)a	48.97 (20.07)a	56.67 (27.99)a	74.67 (44.72)a
CEC (cmol(+) kg ⁻¹)	4.91 (0.22)a	14.17 (2.05)a	9.77 (1.18)bc	5.45 (0.57)a	4.81 (0.07)a	7.07 (1.93)a	17.25 (0.87)c
Base saturation (%)	99.92 (6.68)a	99.01 (5.18)a	99.48 (5.78)a	99.88 (18.10)a	91.90 (8.05)a	94.82 (22.37)a	99.68 (15.76)a
Sand (%)	67.20 (0.56)a	47.67 (4.78)a	80.27 (0.61)a	84.47 (0.76)a	81.27 (5.00)a	82.33 (4.72)a	54.13 (3.97)a
Silt (%)	13.4 (1.00)a	24.43 (3.27)a	9.93 (0.29)a	6.67 (1.77)a	7.73 (4.56)a	7.17 (3.09)a	26.57 (2.61)a
Clay (%)	19.40 (0.88)b	27.90 (2.92)c	9.83 (0.29)a	8.87 (2.49)a	11.03 (1.50)a	10.53 (1.59)a	19.30 (1.42)b
Ground Cover (%)	19.11 (3.08)ab	26.33 (2.95)abc	37.56 (3.83)bc	14.44 (6.71)a	23.00 (9.25)abc	76.00 (3.37)d	15.22 (6.30)a
Crown Cover (%)	59.00 (1.67)ab	60.67 (2.59)a	59.44 (0.98)a	40.11 (3.41)b	59.89 (6.11)a	61.56 (11.95)a	47.44 (2.24)ab

Standard error values are indicated in brackets. Data in rows with the same letters indicate no significant difference, while those with different letters indicate significant difference at $p < 0.05$ (Tukey's HSD).

EC: Electrical conductivity; CEC: Cation exchange capacity.

3.2. Enzymatic Activities

Enzyme assays were selected to represent microbial activity and a range of processes involved in decomposition and nutrient cycling (Hinojosa *et al.*, 2004). A correlation matrix (not shown) indicated significant correlations ($p < 0.05$) of dehydrogenase with NO_3 ($r = 0.79$) and microbial biomass ($r = 0.79$); β -glucosidase with organic C ($r = 0.84$) and microbial biomass ($r = 0.78$); and alkaline phosphatase with NO_3 ($r = -0.84$) and organic C ($r = -0.89$) in 2002. In 2004, dehydrogenase correlated with NO_3 ($r = 0.92$) and ground cover ($r = 0.78$); and acid phosphatase with NO_3 ($r = -0.80$). In 2005, dehydrogenase correlated with P ($r = 0.93$); β -glucosidase with P ($r = 0.93$) and ground cover ($r = 0.83$); alkaline phosphatase with NH_4 ($r = 0.86$); acid phosphatase with P ($r = 0.83$) and ground cover ($r = 0.78$); and urease with NH_4 ($r = 0.84$).

The correlations between enzymatic activities and vegetation cover is to be expected since an increase in plant biomass production is likely to stimulate soil microbial activity through the increased availability of carbon and energy, thereby resulting in stimulation of C and N cycling enzymes (Tate, 2002). In spite of the significant correlations of dehydrogenase and β -glucosidase with microbial biomass often indicated in soil studies (Aon and Colaneri, 2001; Taylor *et al.*, 2002; Knight and Dick, 2004), correlations of these enzymes with viable microbial biomass were only observed in 2002. This could be related to changes in microbial community composition since microbial activities are not constant for a given amount of biomass (Waldrop *et al.*, 2000).

B-glucosidase activity can indicate changes in soil management within 1-3 years and is a consistent indicator of soil quality (Bandick and Dick, 1999; Ndiaye *et al.*, 2000). Dehydrogenase, acid phosphatase, and alkaline phosphatase are directly involved in the transformation of soil organic matter (Sicardi *et al.*, 2004). The average enzymatic activities for dehydrogenase, β -glucosidase, and acid and alkaline phosphatases were higher in 2004 and 2005 than in 2002 for all sites, irrespective of rehabilitation age (Figure 3.2). Previous studies have indicated a relationship between decreased vegetation cover and decreased microbial activity (Aon *et al.*, 2001; Garcia *et al.*, 2002). The results obtained during this investigation, however, are more consistent with observations by Masto *et al.* (2006), which indicated increases in dehydrogenase and phosphatase activities with optimum and balanced application of fertilisers.

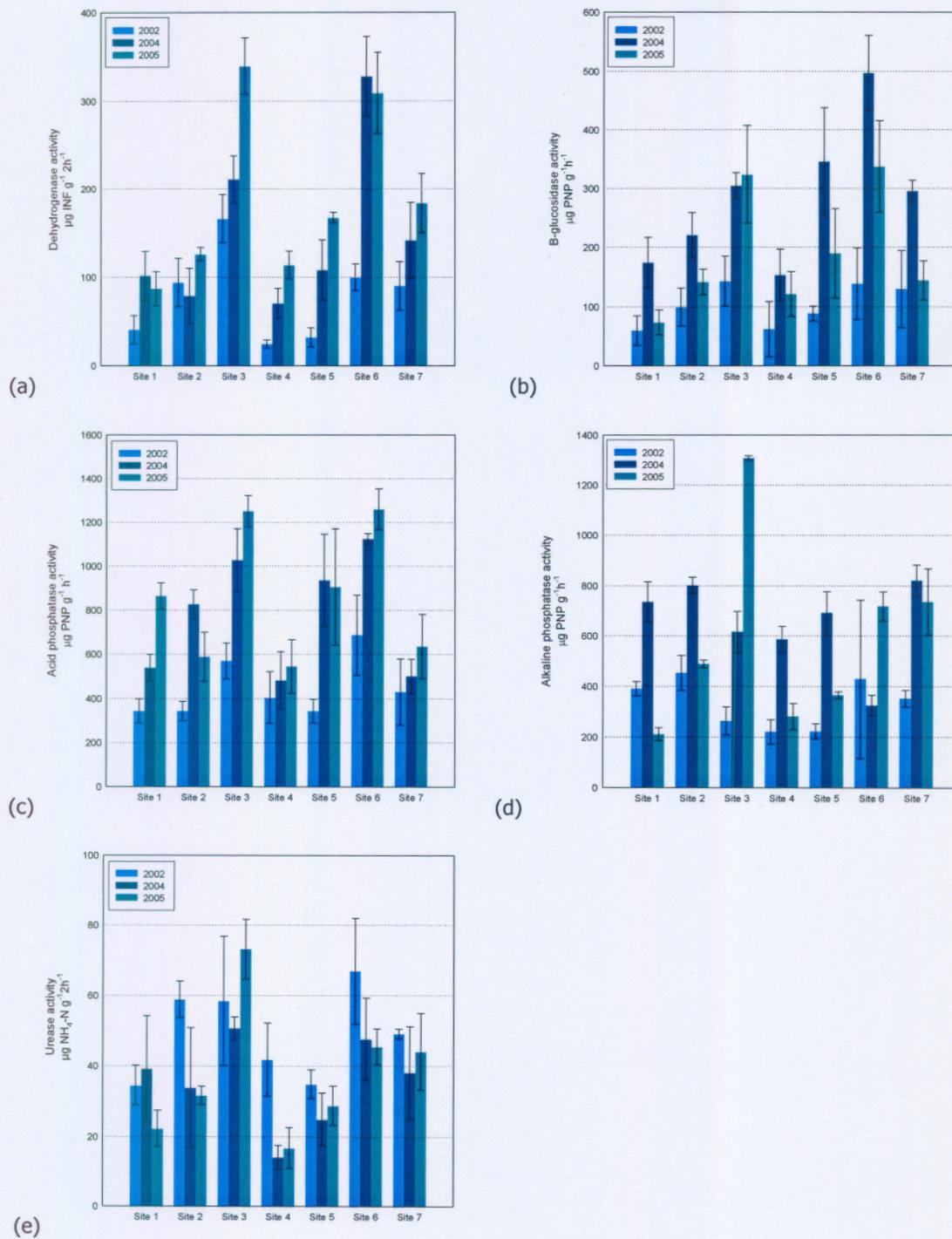


Figure 3.2. Enzymatic activities of topsoil covers from the coal discard sites (Mine A) for 2002, 2004, and 2005: (a) dehydrogenase¹, (b) β -glucosidase, (c) acid phosphatase, (d) alkaline phosphatase, and (e) urease.

¹ It was found that a second order polynomial curve ($y = ax^2 + bx + c$) was a better fit for the calibration standards for this enzyme than the straight line fit used before. Therefore, dehydrogenase activities from 2002 were re-analysed and values in this graph differs from those in Claassens *et al.*, 2006. Trends in the data remained the same.

Alkaline phosphatase activity was lower than acid phosphatase activity for some sites, irrespective of rehabilitation age (Figures 3.2 (c) and (d)). While alkaline phosphatase activity in soils is derived solely from microorganisms, acid phosphatase can also be produced by plant roots (Krämer and Green, 2000; Criquet *et al.*, 2004) and therefore would be more abundant in sites where vegetation is present. According to Lim *et al.* (1996), alkaline phosphatases may be produced by microorganisms as a stress response to unfavourable environmental conditions (Criquet *et al.*, 2004). However, the phospholipid fatty acid stress ratios determined during this investigation (discussed in Chapter 4) did not correspond to higher alkaline phosphatase activities. Inhibition of phosphatase activity has been shown in soil with low cation exchange capacity (CEC) and organic matter content (Hinojosa *et al.*, 2004). Results for Site 4 were consistent with this observation by exhibiting the lowest acid and alkaline phosphatase activities (Figures 3.2(c) and (d)) while also having lower CEC and organic C than the other sites (Table 3.1).

Urease activity has been shown to correlate with microbial biomass and is therefore considered to be of microbial origin (Klose and Tabatabai, 2000), indicating changes in soil quality associated with the N cycle (Gil-Sotres *et al.*, 2005). There was a general decrease in urease activity from 2002 to 2005 in all sites, with the exception of Site 3, which showed an increase in 2005. Although a correlation was found between urease activity and $\text{NH}_4\text{-N}$ ($p < 0.05$, $r = 0.84$) for the 2005 data, the trend in urease activity could not be explained by the chemical data for the respective sites and may be related to changes in microbial community composition.

4. CONCLUSIONS

This investigation showed no relationship between microbial activity and rehabilitation ages of the coal discard sites. Rather, management practices (including thickness of soil cover, fertiliser application, and method of defoliation of sites) caused similar changes in microbial community function in all sites, irrespective of the period of rehabilitation under which a specific site has been. This observation stresses the importance of finding suitable assessment criteria to assist land managers in decision-making and implementation of corrective management inputs.

The characterisation of microbial community function by assays of enzymatic activity could be used as part of a soil monitoring programme to determine the rehabilitation status of coal discard sites. Enzyme assays are relatively simple and

inexpensive and in the context of rehabilitation programmes managed by mining companies, these assays could be a useful tool to assist in the assessment of rehabilitation progress on rehabilitated sites. What should be kept in mind is that the evaluation of only one or some soil properties, gives an incomplete picture of the status of the soil ecosystem. To obtain an accurate representation of soil quality, it would therefore be most sensible to use a polyphasic approach – combining physical, chemical, and biological properties to assess the soil environment. Another important consideration when faced with the question of assessing rehabilitation progress is the establishment of a range of actual values of microbial activity and abundance from case studies of rehabilitated mining sites with which to compare sites under rehabilitation. This is especially critical in the absence of suitable reference sites as is the case on most mining disturbed sites in South Africa.

REFERENCES

- Alef, K., Nannipieri, P. 1995. *Methods in applied soil microbiology and biochemistry*. Boston: Academic Press. 576p.
- Aon, M.A., Colaneri, A.C. 2001. Temporal and spatial evolution of enzymatic activities and physico-chemical properties in an agricultural soil. *Appl Soil Ecol* 18:255-270.
- Aon, M.A., Sarena, D.E., Burgos, D.E., Cortassa, S. 2001. (Micro)biological, chemical, and physical properties of soils subjected to conventional or no-till management: an assessment of their quality status. *Soil Tillage Res* 60:173-186.
- Badiane, N.N.Y., Chotte, J.L., Pate, E., Masse, D., Rouland, C. 2001. Use of soil enzyme activities to monitor soil quality in natural and improved fallows in semi-arid tropical regions. *Appl Soil Ecol* 18:229-238.
- Bandick, A.K., Dick, R.P. 1999. Field management effects on soil enzyme activities. *Soil Biol Biochem* 31:1471-1479.
- Brohon, B., Delolme, C., Goudon, R. 2001. Complementarity of bioassays and microbial activity measurements for the evaluation of hydrocarbon-contaminated soils quality. *Soil Biol Biochem* 33:883-891.
- Caldwell, B.A. 2005. Enzyme activities as a component of soil biodiversity: a review. *Pedobiologica* 49:637-644.
- Criquet, S., Ferre, E., Farnet, A.M., Le Petit, J. 2004. Annual dynamics of phosphatase activities in an evergreen oak litter: influence of biotic and abiotic factors. *Soil Biol Biochem* 36:1111 – 1118.
- Dick, R.P. 1997. Soil enzyme activities as integrative indicators. In: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R., editors. *Biological indicators of soil health*. New York: CAB International. pp 121-156.
- Dick, R.P., Breakwell, D.P., Turco, R.F. 1996. Soil enzyme activities and biodiversity measurements as integrative microbiological indicators. In: Doran, J.W., Jones, A.J., editors. *Methods for assessing soil quality*, SSSA Special Publication 49, Madison: SSSA. pp 247-271.
- García, C., Hernandez, T., Roldán, A., Martín, A. 2002. Effect of plant cover decline on chemical and microbiological parameters under Mediterranean climate. *Soil Biol Biochem* 34:635-642.
- Gil-Sotres, F., Trasar-Cepeda, C., Leirós, M.C., Seoane, S. 2005. Different approaches to evaluating soil quality using biochemical properties. *Soil Biol Biochem* 37:877-887.
- Harris, J.A. 2003. Measurements of the soil microbial community for estimating the success of restoration. *Eur J Soil Sci* 54:801-808.
- Hinojosa, M.B., Carreira, J.A., García-Ruiz, R., Dick, R.P. 2004. Soil moisture pre-treatment effects on enzyme activities as indicators of heavy metal-contaminated and reclaimed soils. *Soil Biol Biochem* 36:1559-1568.
- Klose, S., Tabatabai, M.A. 2000. Urease activity of microbial biomass in soils as affected by cropping systems. *Biol Fertil Soils* 31:191-199.
- Knight, T.R., Dick, R.P. 2004. Differentiating microbial and stabilized β -glucosidase activity relative to soil quality. *Soil Biol Biochem* 36:2089-2096.
- Krämer, S., Green, D.M. 2000. Acid and alkaline phosphatase dynamics and their relationship to soil microclimate in a semiarid woodland. *Soil Biol Biochem* 32:179-188.
- Lim, C.H., Ozkanca, R., Flint, K.P. 1996. The effects of osmotic stress on survival and alkaline phosphatase activity of *Aeromonas hydrophila*. *FEMS Microbiol Lett* 137:19-24.
- Masto, R.E., Chhonkar, P.K., Singh, D., Patra, A.K. 2006. Changes in soil biological and biochemical characteristics in a long-term field trial on a sub-tropical inceptisol. *Soil Biol Biochem* 38:1577-1582.
- Milton, S.J. 2001. Rethinking ecological rehabilitation in arid and winter rainfall regions of southern Africa. *S Afr J Sci* 97:1-2.
- Mummey, D.L., Stahl, P.D., Buyer, J.S. 2002. Microbial biomarkers as an indicator of ecosystem recovery following surface mine reclamation. *Appl Soil Ecol* 21:251-259.

- Ndiaye, E.L., Sandero, J.M., McGrath, D., Dick, R.P. 2000. Integrative biological indicators for detecting changes in soil quality. *Am J Altern Agric* 15:26 – 36.
- Pascual, J.A., Garcia, C., Hernandez, T., Moreno, J.L., Ros, M. 2000. Soil microbial activity as a biomarker of degradation and remediation processes. *Soil Biol Biochem* 32:1877-1883.
- Schlöter, M., Dilly, O., Munch, J.C. 2003. Indicators for evaluating soil quality. *Agric Ecosyst Environ* 98:255-262.
- Sicardi, M., García-Préchac, F., Frioni, L. 2004. Soil microbial indicators sensitive to land use conversion from pastures to commercial *Eucalyptus grandis* (Hill ex Maiden) plantations in Uruguay. *Appl Soil Ecol* 27:125-133.
- Tate, R.L. (III). 2002. Microbiology and enzymology of carbon and nitrogen cycling. In: Burns, R.G., Dick, R.P., editors. *Enzymes in the environment - activity, ecology, and applications*. New York: Marcel Dekker Inc. p 227-248.
- Tate, R.L. (III), Rogers, B.F. 2002. Soil ecosystem properties, microbial diversity, and ecosystem assessments. In: Violante, A., Huang, P.M., Bollag, J.-M., Gianfreda, L., editors. *Developments in soil science*, volume 28B, New York: Elsevier. pp 79-93.
- Taylor, J.P., Wilson, B., Mills, M.S., Burns, R.G. 2002. Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. *Soil Biol Biochem* 34:387-401.
- Ter Braak, C.F., Šmilauer, P. 1998. *Canoco reference manual for user's guide to Canoco community ordination*, ver. 4. New York: Microcomputer Power.
- Van Rensburg, L., Maboeta, M.S., Morgenthal, T.L. 2004. Rehabilitation of co-disposed diamond tailings: growth medium rectification procedures and indigenous grass establishment. *Water Air Soil Pollut* 154:101-113.
- Waldrop, M.P., Balser, T.C., Firestone, M.K. 2000. Linking microbial community composition to function in a tropical soil. *Soil Biol Biochem* 32:1837-1846.

CHAPTER 4

SOIL MICROBIAL COMMUNITY STRUCTURE IN A POST-MINING CHRONOSEQUENCE

"The most important characteristic of an organism is that capacity for self renewal known as health. There are two organisms whose processes of self renewal have been subjected to human interference and control. One of these is man himself. The other is land."

– Aldo Leopold (1949)

Part of this material has been published in *Water, Air, and Soil Pollution* 174:355-366 (2006) by the authors S. Claassens, P.J. Jansen van Rensburg, & L. van Rensburg.

Part of this material has been submitted for publication to *Water, Air, and Soil Pollution*.

1. INTRODUCTION

A comprehensive assessment of soil microbial community characteristics is one way in which to address the incomplete picture of soil status that traditional methods have provided (Harris, 2003). Until recently, the analysis of soil microbial communities relied extensively on cultivation-dependent techniques using a variety of enriched culture media and direct viable counts (Kirk *et al.*, 2004). Although these techniques are rapid and inexpensive, they are insensitive and do not give an accurate representation of *in situ* microbial community dynamics (Hill *et al.*, 2000).

Phospholipid fatty acid (PLFA) analysis allows phenotypic fingerprinting of soil microbial communities and is a reliable tool for distinguishing ecosystem types and for assessing management effects on community structure (Tate and Rogers, 2002; Leckie, 2005). Although PLFA profiles cannot identify microbial species within a community (Ibekwe *et al.*, 2002), it provides for a quantitative, cultivation-independent manner to describe the microbial community in a particular environment at a given time. In addition, this technique offers the opportunity to gain insight into the metabolic state of the microbial community. This is accomplished by studying ratios of certain fatty acids that are formed as a response to environmental stress, thereby generating characteristic PLFA stress signatures.

During this investigation, the temporal changes in microbial community structure in a post-mining chronosequence of coal discard sites (managed by Mining Company A) of varying rehabilitation ages (1 to 11 years) was studied by means of PLFA analysis. The objective was to determine whether temporal changes in microbial community structure were related to rehabilitation age and whether PLFA analysis of community structure could indicate progress of rehabilitation.

2. MATERIALS AND METHODS

Refer to Chapter 2 for a comprehensive site description and discussion of all materials and methods used in this investigation.

2.1. Site Description and Sampling

The study was conducted on the same seven revegetated coal discard sites discussed in Chapter 3 (managed by Mining Company A). All areas were under rehabilitation and at the

start of the investigation (2002), discard sites sampled at Mine A varied in rehabilitation age from 1 to 8 years. The same sites were resampled in 2004 and 2005 to obtain a chronosequence of rehabilitation ages ranging from 1 to 11 years (chronosequence A) (site identities and rehabilitation ages are presented in Table 2.1, Chapter 2). For lipid analyses, subsamples (± 200 g) of each sample were sealed in plastic bags, frozen on site using dry ice and transported on dry ice to the laboratory, where it was stored at -80°C until lyophilisation.

2.2. Estimation of Vegetation Cover

The ground and crown vegetation cover of all the sites were estimated in three 1 m^2 quadrates randomly placed over a 50 m transect. The ground cover included all living and non-living organic material on the ground surface per area and the crown cover was regarded as the canopy cover spread of all grass species over a fixed area. Both values were expressed as a percentage per m^2 surface area (Van Rensburg *et al.*, 2004).

2.3. Physical and Chemical Soil Analysis

The extraction and analysis procedures for the physical and chemical characterisation of soil samples were conducted by an independent laboratory according to standard procedures (see Chapter 2).

2.4. Lipid Extraction, Fractionation, and Analysis

Total lipids were extracted from 5 g lyophilised soil according to a modified Bligh and Dyer procedure (Peacock *et al.*, 2001). Silicic acid column chromatography (Guckert *et al.*, 1985) was used to fractionate the total lipid extract into neutral lipids, glycolipids, and polar lipids. The polar lipid fraction was transesterified to fatty acid methyl esters (FAMES) by mild alkaline methanolysis (Guckert *et al.*, 1985). The FAMES were analysed by capillary gas chromatography with flame ionisation detection on an Agilent 6890 series II chromatograph fitted with a 60 m SPB-1 column (0.250 mm I.D., 0.250 μm film thickness). Definitive identification of peaks was undertaken using gas chromatography-mass spectrometry of selected samples using an Agilent 6890 series II chromatograph interfaced with an Agilent 5973 mass selective detector. Methyl nonadecanate (C19:0) was used as the internal standard and the PLFAs were expressed as equivalent peak

responses to the internal standard. Microbial biomass was estimated as the total extractable PLFAs (McKinley *et al.*, 2005) and microbial community composition was analysed on relative concentrations (mole percentages) of individual fatty acids. Standard fatty acid nomenclature was used (Guckert *et al.*, 1985; Allison *et al.*, 2005).

2.5. Statistical Analysis

Statistical analyses were performed and graphs generated using Statistica 7.1 (Statsoft Inc., Tulsa, Oklahoma, USA), SigmaPlot 10.0 (Systat Software Inc., San Jose, California, USA), and Canoco for Windows 4.5 (Biometris – Plant Research International, Wageningen, The Netherlands; Ter Braak and Šmilauer, 1998).

Data of physical and chemical characteristics, as well as enzymatic activities of samples were subjected to analysis of variance (ANOVA) followed by Tukey's test for mean separation where significant differences ($p = 0.05$) were indicated. A principal components analysis (PCA) (Canoco for Windows 4.5) was performed on the physical and chemical characteristics to determine whether there was differentiation between sites based on these variables.

3. RESULTS AND DISCUSSION

Throughout this discussion, coal discard sites are referred to in terms of their rehabilitation ages, with the site name and sampling year indicated in brackets (e.g. Site 1_2002). Refer to Table 2.1 (Chapter 2) for names and rehabilitation ages of all coal discard sites.

3.1. Physical and Chemical Soil Properties

See “Physical and Chemical Soil Properties”, Chapter 3 for the discussion of results obtained from physical and chemical soil analysis of samples from the seven coal discard sites that this investigation pertains to.

3.2. Phospholipid Fatty Acid Analysis

The PLFA ratios and mole percentage (mol%) fractions of the major PLFA groups are summarised in Table 4.1 indicating the variation in the lipid contributing communities between coal discard sites of different rehabilitation ages. Differential changes in the mol% values of the individual ester-linked PLFAs indicate that the relative abundance of some

functional groups within the microbial community had increased while others had declined. The concentration of total PLFA provides a quantitative measure of the soil viable (active) microbial biomass (Balkwill *et al.*, 1998) and PLFA profile analysis indicated changes in microbial community structure over time as well as differences between sites. Although there were significant differences ($p < 0.05$) between sites of different rehabilitation ages (Table 4.1), there were no trends in PLFA data related to the rehabilitation ages of the sites. A correlation matrix (not shown) indicated significant correlations ($p < 0.05$) of microbial biomass with NH_4 ($r = 0.83$) and P ($r = 0.90$) in 2002. In 2004, microbial biomass correlated with P ($r = 0.79$) and organic C ($r = 0.90$) and in 2005 mid-chain branched saturated fatty acids correlated with NH_4 ($r = -0.75$).

A general increase in normal saturated fatty acids has been shown to correlate with decreased diversity (Peacock, 2005) and such a general increase was observed in all the sites after 2002 (Table 4.1). This increase was consistent with the change in management practices from defoliation by means of cutting and baling to grazing of the sites. Monounsaturated fatty acids are indicative of Gram-negative bacteria and terminally branched saturated fatty acids of Gram-positive bacteria (McKinley *et al.*, 2005). From 2002 to 2005, there was a decrease in Gram-negative bacteria and an increase in Gram-positive bacteria in sites of all rehabilitation ages (Table 4.1). The proportion of the total community comprised of Gram-positive bacteria (Gram (+) / Total PLFA) also increased (Table 4.1, Figure 4.1). Increased proportions of Actinomycete biomarkers (mid-chain branched saturated fatty acids) were also observed in Sites 1, 4, 5, and 6. A study by Peacock *et al.* (2002), found that microbial biomass and biomarkers for microeukaryotes (polyunsaturated fatty acids) and Gram-negative bacteria decreased with increasing disturbance and soil compaction along an anthropogenic disturbance gradient. At the same time, they found an increase in the proportions of Gram-positive and Actinomycete biomarkers. Gram-positive bacteria grow slowly and are able to tolerate stress (Waldrop *et al.*, 2000), while Gram-negative bacteria are fast-growing, utilising many carbon sources and being able to adapt quickly to a variety of environments (Ponder and Tadros, 2002). While the Gram-positives are considered K-strategists, the Gram-negatives are classified as r-strategists – copiotrophic microorganisms that experience rapid reduction when resources become scarce (Sarathchandra *et al.*, 2001). This shift in the microbial communities may therefore be an indication of a reduced availability of nutrients as a consequence of the reduced application of fertilisers and manure over the study period.

Table 4.1. Phospholipid fatty acid (PLFA) composition and ratios of soil cover layers obtained from the coal discard sites managed by **Mining Company A**.

	Site_1	Site_2	Site_3	Site_4	Site_5	Site_6	Site_7
2002							
Rehabilitation age (years)	1	3	4	4	4	4	8
Viable microbial biomass (pmol g ⁻¹ dry weight)	7108.01 (1692.12)a	10315.14 (4745.73)a	29850.56 (9110.88)ab	12043.97 (4417.18)ab	15368.61 (3357.34)ab	17719.66 (2875.71)ab	16277.30 (5704.01)ab
PLFA Group (mol%)							
Normal saturated	20.46 (0.28)a	22.35 (1.35)ab	23.44 (0.63)bc	23.69 (0.57)bc	26.01 (1.08)de	25.13 (0.63)cd	27.85 (0.77)e
Mid-chain branched saturated	2.98 (0.29)bc	3.08 (0.27)c	2.11 (0.09)e	2.59 (0.06)abc	2.61 (0.20)abc	2.46 (0.90)abc	2.32 (0.16)ab
Terminally branched saturated	18.13 (0.64)a	24.09 (0.77)bc	22.84 (0.25)b	25.25 (0.72)bc	25.47 (1.68)bc	26.98 (1.07)c	22.57 (13.28)b
Monounsaturated	39.32 (0.17)bc	35.12 (0.40)a	41.82 (1.11)c	35.78 (0.82)ab	34.09 (0.45)a	35.95 (1.24)ab	33.51 (2.09)a
Polyunsaturated	17.05 (0.40)d	12.05 (0.42)c	6.89 (0.63)a	9.57 (0.65)bc	8.47 (0.83)ab	6.01 (0.85)a	10.40 (0.77)bc
Ratios							
Fungal / Bacterial ¹	0.32 (0.06)d	0.21 (0.10)bcd	0.12 (0.02)abc	0.16 (0.04)abc	0.13 (0.07)abc	0.09 (0.02)ab	0.24 (0.06)cd
Saturated / Unsaturated ²	1.00 (0.07)a	1.33 (0.13)ab	1.09 (0.04)a	1.29 (0.01)ab	1.45 (0.01)abc	1.41 (0.04)abc	1.45 (0.27)abc
Gram (+) / Total PLFA ³	0.15 (0.06)a	0.20 (0.04)ab	0.19 (0.01)c	0.20 (0.05)ab	0.22 (0.06)abc	0.23 (0.03)bc	0.18 (0.02)ab
Iso / Anteiso ⁴	0.87 (0.04)a	1.09 (0.17)abc	0.91 (0.05)ab	1.06 (0.07)abc	1.22 (0.08)c	1.21 (0.01)bc	0.97 (0.04)abc
16:1 ω 7 / 16:1 ω 7c	0.00 (0.00)a	0.00 (0.00)a	0.03 (0.02)a	0.00 (0.00)a	0.02 (0.01)a	0.01 (0.02)a	0.00 (0.00)a
18:1 ω 7 / 18:1 ω 7c	0.00 (0.00)a	0.01 (0.02)a	0.05 (0.02)a	0.00 (0.00)a	0.04 (0.03)a	0.06 (0.01)a	0.03 (0.04)a
2004							
Rehabilitation age (years)	3	5	6	6	6	6	10
Viable microbial biomass (pmol g ⁻¹ dry weight)	680.04 (178.40)a	1656.22 (600.64)ab	5216.10 (1275.29)bc	3645.77 (933.95)abc	3084.34 (596.52)abc	6362.20 (881.63)c	4178.03 (515.78)abc
PLFA Group (mol%)							
Normal saturated	49.22 (3.26)b	35.40 (8.26)ab	32.04 (0.51)a	30.16 (1.67)a	31.37 (1.10)a	30.27 (0.99)a	33.43 (2.67)a
Mid-chain branched saturated	3.00 (0.69)a	4.13 (1.42)a	2.79 (0.11)a	3.11 (0.30)a	3.86 (0.45)a	3.70 (0.35)a	2.40 (0.36)a
Terminally branched saturated	26.19 (1.96)a	26.70 (4.35)a	27.63 (1.67)a	29.54 (2.48)a	27.75 (3.77)a	29.63 (2.03)a	28.10 (2.00)a
Monounsaturated	18.27 (2.32)b	33.00 (9.03)a	30.96 (1.41)a	30.06 (4.30)a	31.02 (4.18)a	29.93 (2.43)a	30.29 (4.27)a
Polyunsaturated	13.32 (2.87)b	6.51 (0.57)a	5.85 (0.62)a	6.57 (0.78)a	5.58 (0.81)a	5.93 (0.37)a	5.26 (0.33)a
Ratios							
Fungal / Bacterial ¹	0.08 (0.02)a	0.08 (0.01)a	0.11 (0.03)a	0.12 (0.02)a	0.09 (0.03)a	0.11 (0.01)a	0.09 (0.01)a
Saturated / Unsaturated ²	4.82 (1.41)b	3.64 (1.29)ab	2.02 (0.16)a	2.16 (0.40)a	2.12 (0.47)a	2.16 (0.28)a	2.20 (0.52)a
Gram (+) / Total PLFA ³	0.17 (0.02)a	0.17 (0.01)a	0.21 (0.04)a	0.22 (0.09)a	0.19 (0.02)a	0.22 (0.04)a	0.20 (0.01)a
Iso / Anteiso ⁴	1.70 (0.13)ab	1.54 (0.11)a	1.59 (0.17)a	2.11 (0.13)b	1.63 (0.18)ab	1.41 (0.20)a	1.57 (0.21)a
16:1 ω 7 / 16:1 ω 7c	0.00 (0.00)a	0.04 (0.01)a	0.04 (0.00)a	0.07 (0.01)a	0.07 (0.02)a	0.09 (0.01)a	0.08 (0.03)a
18:1 ω 7 / 18:1 ω 7c	2.42 (0.99)a	1.08 (0.04)a	0.35 (0.12)a	0.74 (0.03)a	0.80 (0.02)a	0.06 (0.01)a	1.00 (0.18)a

	Site_1	Site_2	Site_3	Site_4	Site_5	Site_6	Site_7
2005							
Rehabilitation age (years)	4	6	7	7	7	7	11
Viable microbial biomass (pmol g ⁻¹ dry weight)	7463.66 (1947.99)a	3681.70 (1258.93)a	10705.62 (2624.10)a	6346.01 (857.26)a	14815.28 (2000.09)a	12996.82 (1813.81)a	8412.09 (1969.81)a
PLFA Group (mol%)							
Normal saturated	29.53 (1.78)ab	28.43 (0.75)ab	31.24 (0.51)ab	30.53 (1.29)ab	26.81 (0.54)a	29.64 (1.05)ab	33.08 (2.62)b
Mid-chain branched saturated	3.03 (0.27)a	3.08 (0.28)a	2.72 (0.11)a	3.76 (0.45)ab	4.96 (0.46)b	3.62 (0.34)ab	2.38 (0.40)a
Terminally branched saturated	28.93 (2.57)a	28.96 (5.02)a	26.94 (1.68)a	27.04 (3.80)a	30.26 (2.99)a	29.03 (2.06)a	27.80 (1.97)a
Monounsaturated	31.53 (4.56)a	31.77 (5.14)a	32.70 (1.48)a	32.83 (4.59)a	31.80 (2.24)a	31.38 (2.55)a	30.01 (4.30)a
Polyunsaturated	6.43 (0.80)a	5.26 (0.85)a	5.69 (0.59)a	5.42 (0.77)a	5.63 (1.06)a	5.80 (0.35)a	5.20 (0.38)a
Ratios							
Fungal / Bacterial ¹	0.09 (0.01)a	0.07 (0.02)a	0.10 (0.02)a	0.11 (0.04)a	0.09 (0.02)a	0.11 (0.03)a	0.09 (0.04)a
Saturated / Unsaturated ²	2.02 (0.38)a	1.87 (0.40)a	1.87 (0.14)a	1.95 (0.77)a	1.97 (0.98)a	2.01 (0.35)a	2.12 (0.48)a
Gram (+) / Total PLFA ³	1.53 (0.35)a	1.67 (0.58)ab	1.59 (0.13)ab	1.16 (0.40)b	1.88 (0.87)ab	1.41 (0.11)ab	1.57 (0.23)ab
Iso /Anteiso ⁴	0.19 (0.06)a	0.21 (0.04)a	0.20 (0.03)a	0.20 (0.01)a	0.23 (0.02)a	0.21 (0.04)a	0.19 (0.07)a
16:1 ω 7t / 16:1 ω 7c	0.05 (0.01)a	0.13 (0.09)a	0.03 (0.00)a	0.06 (0.02)a	0.07 (0.01)a	0.07 (0.00)a	0.06 (0.02)a
18:1 ω 7t / 18:1 ω 7c	0.69 (0.03)a	0.72 (0.15)a	0.33 (0.20)a	0.74 (0.07)a	0.62 (0.23)a	0.50 (0.01)a	0.73 (0.22)a

Standard error values are indicated in brackets. Data in rows with the same letters indicate no significant difference, while those with different letters indicate significant difference at $p < 0.05$ (Tukey's HSD).

¹ Fungi: 18:2 ω 6 (Federle, 1986; Frostegård and Bååth, 1996), Bacteria: sum of i15:0, a15:0, 15:0, i16:0, 16:1 ω 9, 16:1 ω 7t, i17:0, a17:0, 17:0, cy17:0, 18:1 ω 7, and cy19:0 (Frostegård and Bååth, 1996).

² Normal saturated to monounsaturated PLFA (McKinley *et al.*, 2005).

³ Gram-positive PLFA markers (i10me16:0, i15:0, a15:0, i16:0, and 17:0) to total PLFA (McKinley *et al.*, 2005).

⁴ Iso to anteiso branched PLFA (i15:0 + i17:0/a15:0 + a17:0) (McKinley *et al.*, 2005).

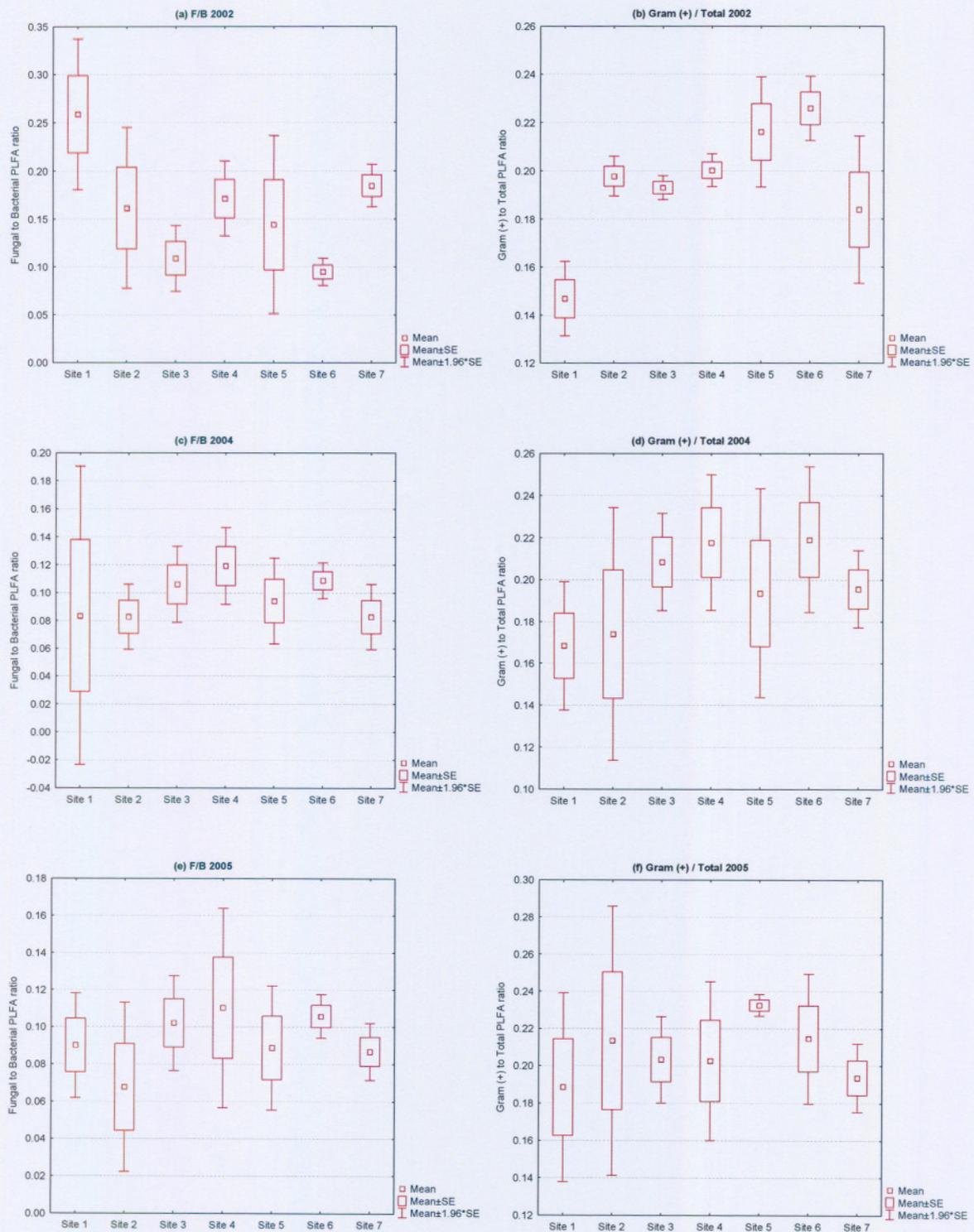


Figure 4.1. Ratios of fungal to bacterial PLFA (Frostegård and Bååth, 1996) in the coal discard sites (Mine A) for 2002 (a), 2004 (c), and 2005 (e), and Gram-positive PLFA markers to total PLFA for 2002 (b), 2004 (d), and 2005 (f). Gram-positive bacteria were i10me16:0, i15:0, a15:0, i16:0, and 17:0 (McKinley *et al.*, 2005).

A decrease in polyunsaturated fatty acids (indicative of fungi) was observed in all sites from 2002 to 2005 (Table 4.1). The ratio of fungal to bacterial (F:B) abundance was calculated using PLFA 18:2 ω 6 for fungi (Federle, 1986; Frostegård and Bååth, 1996) and PLFAs i15:0, a15:0, 15:0, i16:0, 16:1 ω 9, 16:1 ω 7t, i17:0, a17:0, 17:0, cy17:0, 18:1 ω 7, and cy19:0 for bacteria (Frostegård and Bååth, 1996). Fungi are essential to ecosystem functioning and soil quality and the relative abundance of fungi has been shown to increase with reduced disturbance and mineral fertiliser inputs (Bardgett and McAlister, 1999; Stahl *et al.*, 1999; Zeller *et al.*, 2001). Furthermore, the absolute fungal biomass is more sensitive than the total soil microbial biomass to detect the effect of management abandonment in grassland soils (Zeller *et al.*, 2001). This is of great value when comparing the long-term effects of management or environmental changes on otherwise similar sites (Bailey *et al.*, 2002). An increase in the amount of F:B PLFA was reported with increasing soil pH by Bååth and Anderson (2003). The results from the current investigation did not correspond with this observation and instead showed a decrease in F:B ratios from 2002 to 2005 for all sites (Table 4.1) despite an increase in pH (Table 3.1, Chapter 3). As for Gram-negative bacteria, the decrease of fungal biomass in this study may be related to the reduction of inputs of fertilisers and manure. However, it is suggested that the earlier high abundances of fungi and bacteria may have been due to the initial high application rates of fertilisers and organic material (manure) – stimulating vegetation growth and microbial community responses.

Assuming that the lipid composition of the microorganisms reflects a phenotypic response of the organisms to their environment, the physiological status of the microbial community can be assessed from ratios of specific biomarkers (Ponder and Tadros, 2002). Increased ratios of iso to anteiso, saturated to unsaturated, and *trans* to *cis*-monoenoic fatty acids have been associated with nutritional stress, disturbance, or changes in community composition (Kieft *et al.*, 1994; Peacock *et al.*, 2001; Fierer *et al.*, 2003; McKinley *et al.*, 2005). *Trans/cis* ratios greater than 0.1 are indicative of unfavourable growth conditions for microbial communities (Guckert *et al.*, 1986). There was an increase in saturated to unsaturated PLFAs and in *trans/cis* ratios from 2002 to 2004 with a slight decrease from 2004 to 2005 (Table 4.1). The *trans/cis* ratios were greater than 0.1 in 2004 and 2005, indicating stress in microbial communities. However, there was a considerable decrease in the ratios of iso to anteiso PLFA (indicative of nutritional stress). This shows that unfavourable growth conditions for microorganisms are caused by a stressor other than nutritional limitation and could be related to the influence of grazing of the sites by

livestock. Grazing has been shown to impact microbial community structure, although observations about the increases and/or decreases of certain microbial groups are not conclusive (Clegg, 2006).

4. CONCLUSIONS

In the absence of reference soils, as is often the case when studying mining disturbed environments in South Africa, it is important that revegetated sites be monitored over the long-term to determine trends in these ecosystems. When assessing the progress of rehabilitation on mining disturbed soils, microbial community function and structure can reveal effects of management that may not be apparent through other methods of evaluation. The coal discard sites in this investigation varied in rehabilitation ages from 1 to 11 years among sites and over the study period (2002 to 2005). As in the case of the functional data (Chapter 3), no trends consistent with rehabilitation age could be observed in the structural data, with similar changes in microbial community structure occurring in all of the sites. This absence of trends related to rehabilitation age demonstrates the considerable impact of management practices on these ecosystems. The high cost of rehabilitation of coal discard sites requires effective management practices that reach the goal of a stable ecosystem in a shorter period. In turn, such practices can only be effective if a reliable assessment of prevailing conditions can be made. Additional studies are required to establish methods for using microbial community properties in assessments of rehabilitated mining areas. Such investigations would have to determine the relationship between microbial community dynamics and specific management practices such as the thickness of the applied soil cover layer; the manner in which the soil is ameliorated; the grass seed mixture used; and the intervals at which fertilisers are applied. Future investigations will also have to account for more environmental factors that could influence microbial community function and structure and should seek to include other types of post-mining sites, such as areas disturbed by gold or platinum mining.

REFERENCES

- Allison, V.J., Miller, R.M., Jastrow, J.D., Matamala, R., Zak, D.R. 2005. Changes in soil microbial community structure in a tallgrass prairie chronosequence. *Soil Sci Soc Am J* 69:1412-1421.
- Bååth, E., Anderson, T.-H. 2003. Comparison of soil fungal/bacterial ratios in a pH gradient using physiological and PLFA-based techniques. *Soil Biol Biochem* 35:955-965.
- Bailey, V.L., Smith, J.L., Bolton Jr. H. 2002. Fungal-to-bacterial ratios in soil investigated for enhanced C sequestration. *Soil Biol Biochem* 34:997-1007.
- Balkwill, D.L., Leachs, F.R., Wilson, J.T., McNabb, J.F., White, D.C. 1998. Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate and direct counts in sub-surface aquifer sediments. *Microb Ecol* 16:73-84.
- Bardgett, R.D., McAlister, E. 1999. The measurement of soil fungal:bacterial biomass ratios as an indicator of ecosystem self-regulation in temperate meadow grasslands. *Biol Fertil Soils* 29:282-290.
- Clegg, C.D. 2006. Impact of cattle grazing and inorganic fertiliser additions to managed grasslands on the microbial community composition of soils. *Appl Soil Ecol* 31:73-82.
- Federle, T.W. 1986. Microbial distribution in soil – new techniques. In: Megusar, F., Gantar, M., editors. *Perspectives in microbial ecology*. Ljubljana: Slovene Society of Microbiology. pp 493-498.
- Fierer, N., Schimel, J.P., Holden, P.A. 2003. Variations in microbial community composition through two soil depth profiles. *Soil Biol Biochem* 35:167-176.
- Frostegård, A., Bååth, E. 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol Fertil Soils* 22:59-65.
- Guckert, J.B., Antworth, C.P., Nichols, P.D., White, D.C. 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. *FEMS Microbiol Ecol* 31:147-158.
- Guckert, J.B., Hood, M.A., White, D.C. 1986. Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increases in the *trans/cis* ratio and proportions of cyclopropyl fatty acids. *Appl Environ Microbiol* 52:794-801.
- Harris, J.A. 2003. Measurements of the soil microbial community for estimating the success of restoration. *Eur J Soil Sci* 54:801-808.
- Hill, G.T., Mitkowski, N.A., Aldrich-Wolfe, L., Emele, L.R., Jurkonie, D.D., Ficke, A., Maldonado-Ramirez, S., Lynch, S.T., Nelson, E.B. 2000. Methods for assessing the composition and diversity of soil microbial communities. *Appl Soil Ecol* 15:25-36.
- Ibekwe, A.M., Kennedy, A.C., Frohne, P.S., Papiernik, S.K., Yang, C.H., Crowley, D.E. 2002. Microbial diversity along a transect of agronomic zones. *FEMS Microbiol Ecol* 39:183-191.
- Kieft, T.L., Ringelberg, D.B., White, D.C. 1994. Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in a porous medium. *Appl Environ Microbiol* 60:3292-3299.
- Kirk, J.L., Beaudette, L.A., Hart, M., Moutoglis, P., Klironomos, J.N., Lee, H., Trevors, J.T. 2004. Methods of studying soil microbial diversity. *J Microbiol Methods* 58:169-188.
- Leckie, S.E. 2005. Methods of microbial community profiling and their application to forest soils. *For Ecol Manage* 220:88-106.
- McKinley, V.L., Peacock, A.D., White, D.C. 2005. Microbial community PLFA and PHB responses to ecosystem restoration in tallgrass prairie soils. *Soil Biol Biochem* 37:1946-1958.
- Peacock, A.D. 2005. Center for Biomarker Analysis, University of Tennessee, Knoxville. Personal communication.
- Peacock, A.D., Macnaughton, S.J., Cantu, J.M., Dale, V.H., White, D.C. 2002. Soil microbial biomass and community composition along an anthropogenic disturbance gradient within a long-leaf pine habitat. *Ecol Indic* 12:1-9.

- Peacock, A.D., Mullen, M.D., Ringelberg, D.B., Tyler, D.D., Hedrick, D.B., Gale, P.M., White, D.C. 2001. Soil microbial community responses to dairy manure or ammonium nitrate applications. *Soil Biol Biochem* 33:1011-1019.
- Ponder, Jr. F., Tadros, M. 2002. Phospholipid fatty acids in forest soil four years after organic matter removal and soil compaction. *Appl Soil Ecol* 19:173-182.
- Sarathchandra, S.U., Ghani, A., Yeates, G.W., Burch, G., Cox, N.R. 2001. Effect of nitrogen and phosphate fertilisers on microbial and nematode diversity in pasture soils. *Soil Biol Biochem* 33:953-964.
- Stahl, P.D., Parkin, T.B., Christensen, M. 1999. Fungal presence in paired cultivated and uncultivated soils in central Iowa, USA. *Biol Fertil Soils* 29:92-97.
- Tate, R.L. (III), Rogers, B.F. 2002. Soil ecosystem properties, microbial diversity, and ecosystem assessments. In: Violante, A., Huang, P.M., Bollag, J.-M., Gianfreda, L., editors. *Developments in Soil Science, Volume 28B*, New York: Elsevier. pp 79-93.
- Ter Braak, C.F., Šmilauer, P. 1998. *Canoco reference manual for user's guide to Canoco community ordination*, ver. 4. New York: Microcomputer Power.
- Van Rensburg, L., Maboeta, M.S., Morgenthal, T.L. 2004. Rehabilitation of co-disposed diamond tailings: growth medium rectification procedures and indigenous grass establishment. *Water Air Soil Pollut* 154:101-113.
- Waldrop, M.P., Balsler, T.C., Firestone, M.K. 2000. Linking microbial community composition to function in a tropical soil. *Soil Biol Biochem* 32:1837-1846.
- Zeller, V., Bardgett, R.D., Tappeiner, U. 2001. Site and management effects on soil microbial properties of subalpine meadows: A study of land abandonment along a north-south gradient in the European Alps. *Soil Biol Biochem* 33:639-649.

CHAPTER 5

MICROBIAL MEASURES OF REHABILITATION SUCCESS IN TWO POST- MINING CHRONOSEQUENCES

"To continue any longer as blind consumers of life, without learning to be visionary restorers of life, will likely insure an end to both opportunities – sooner than most of us would like to look at. Yet to fully look, in search of what is true, must surely be the first step."

— Donald A. Weaver

1. INTRODUCTION

Restoration projects in the South African mining environment usually consider vegetative stabilisation as a means to achieve rehabilitation of mine discard sites. However, revegetation of mining disturbed areas is often deterred by adverse climatic conditions characteristic to the arid and semi-arid areas of southern Africa (Milton, 2001). Assessing the progress of rehabilitation (success of management practices) based merely on aboveground indicators has therefore been problematic. For rehabilitation attempts to be successful it is necessary to realise the dynamic nature of soil as a resource and to consider the interaction between aboveground and belowground communities (Harris and Birch, 1989; Bartelt-Ryser *et al.*, 2005).

The central question facing the land manager attempting to rehabilitate land disturbed by mining activities is how to measure the success or failure of management practices applied to a particular site (Harris, 2003). It is widely accepted that the most reliable manner to measure change in an ecosystem and to gain an understanding of the basic structure and function of that ecosystem, is by long-term monitoring employing appropriate spatial and temporal scales. With reference to obtaining a realistic ecological assessment of a rehabilitation project, this implies monitoring the same site through time (Michener, 1997; Sparling *et al.*, 2003). The inherent difficulties associated with the monitoring of rehabilitation processes combined with the fact that these investigations often occur over the short-term and cannot be replicated or studied using traditional experimental approaches and statistical analyses (Michener, 1997), have necessitated the use of alternative investigative approaches to monitoring through time in order to quantitatively assess the success of rehabilitation.

One such approach is 'space-for-time' (SFT) substitution (Michener, 1997; Sparling *et al.*, 2003) – a technique used to infer a temporal trend from a study of different aged sites (Pickett, 1989). The SFT approach assumes that sites in different locations were initially similar, and that simultaneous sampling of different sites of increasing age is equivalent to resampling the same site through time. Therefore, when applying this approach, sites of different ages and stages of development at separate locations ('space') are identified to obtain a chronosequence of ages ('time') (Sparling *et al.*, 2003). Applications of SFT substitution include studies on ecosystem dynamics (Purtauf *et al.*, 2004; Dauber and Wolters, 2005; Martínez-Ruiz and Fernández-Santos, 2005) and investigations of recovery of ecosystems from various forms of disturbance (Pickett, 1989;

Sparling *et al.*, 2003).

The objective of this investigation was to apply the ‘space-for-time’ hypothesis to two chronosequences of coal discard sites under different management regimes. This included sites aged 1 to 17 years constituting two chronosequences of rehabilitation ages from 1 to 11 years and 6 to 17 years, respectively. The long-term effect of the different management regimes on the soil microbial communities was investigated in terms of microbial community function (enzymatic activities) and structure (signature lipid biomarker analysis).

2. MATERIALS AND METHODS

Refer to Chapter 2 for a comprehensive site description and discussion of all materials and methods used in this investigation.

2.1. Site Description and Sampling

The two coal mines chosen for this study offered an opportunity to apply SFT substitution and in this way to compare the management regimes of the coal discard sites. The differences in management regimes applied by the two mining companies included the application of soil cover layers of different depths. For rehabilitation of coal discard dumps, both mines applied a soil cover layer to discard material before revegetating the sites with a grass seed mixture. However, the soil cover applied at Mine B was deeper (30-60 cm) than that applied at Mine A (10-15 cm). Management practices applied after the initial revegetation of the discard sites also differed. Mining Company A applied management in the form of cutting and baling, which was replaced by grazing (in 2003) as well as annual amelioration of soil cover layers. In contrast, Mining Company B followed a less intensive management regime and only applied grazing to all sites with no application of fertilisers or organic material after initial revegetation.

At Mine A, seven discard dumps that varied between 1 and 8 years old in 2002, were sampled in 2002, 2004 and 2005 to obtain a chronosequence of ages ranging from 1 to 11 years (chronosequence A). At Mine B, seven discard dumps between the ages of 6 and 17 years old, were sampled in 2006 (chronosequence B) (Table 2.1, Chapter 2). For those ages with data from several sites (replicates), mean values and standard errors were calculated (Martínez-Ruiz and Fernández-Santos, 2005).

A random sampling design was used to obtain three composite samples per site (five cores per composite sample) of the soil cover layers from all coal discard sites. Although sites from Mine B had deeper soil cover layers (30-60 cm) than sites from Mine A (10-15 cm), the top 0-10 cm of the soil cover layer was sampled to facilitate comparison between the two chronosequences.

2.2. Estimation of Vegetation Cover

The ground and crown vegetation cover of all the sites were estimated in three 1 m² quadrates randomly placed over a 50 m transect. The ground cover included all living and non-living organic material on the ground surface per area and the crown cover was regarded as the canopy cover spread of all grass species over a fixed area. Both values were expressed as a percentage per m² surface area (Van Rensburg *et al.*, 2004).

2.3. Physical and Chemical Soil Analysis

The extraction and analysis procedures for the physical and chemical characterisation of soil samples were conducted by an independent laboratory according to standard procedures (see Chapter 2).

2.4. Assays of Enzymatic Activities

Before analyses, consolidated soil samples were passed through a 2 mm sieve. For the determination of dehydrogenase activity, soil was kept at field water content, while air-dried samples were used for determination of β -glucosidase, urease, and acid and alkaline phosphatase activities. Soil water content was determined gravimetrically after drying soil samples at 105°C overnight (Alef and Nannipieri, 1995).

Dehydrogenase and urease activities were assayed according to the procedures described in Alef and Nannipieri (1995). β -glucosidase, acid phosphatase, and alkaline phosphatase activities were based on *p*-nitrophenol release after cleavage of a synthetic substrate (*p*-nitrophenyl glucoside and *p*-nitrophenyl phosphate, respectively) (Alef and Nannipieri, 1995; Dick *et al.*, 1996). Modified universal buffers, pH 6.5 and pH 11.0, were used for acid and alkaline phosphomonoesterase, respectively.

2.5. Lipid Extraction, Fractionation, and Analysis

Total lipids were extracted from 5 g lyophilised soil according to a modified Bligh and Dyer procedure (Peacock *et al.*, 2001). Silicic acid column chromatography (Guckert *et al.*, 1985) was used to fractionate the total lipid extract into neutral lipids, glycolipids, and polar lipids. The polar lipid fraction was transesterified to fatty acid methyl esters (FAMES) by mild alkaline methanolysis (Guckert *et al.*, 1985). The FAMES were analysed by capillary gas chromatography with flame ionisation detection on an Agilent 6890 series II chromatograph fitted with a 60 m SPB-1 column (0.250 mm I.D., 0.250 µm film thickness). Definitive identification of peaks was undertaken using gas chromatography-mass spectrometry of selected samples using an Agilent 6890 series II chromatograph interfaced with an Agilent 5973 mass selective detector. Methyl nonadecanoate (C19:0) was used as the internal standard and the phospholipid fatty acids (PLFAs) were expressed as equivalent peak responses to the internal standard. Microbial biomass was estimated as the total extractable PLFAs (McKinley *et al.*, 2005) and microbial community composition was analysed on relative concentrations (mole percentages) of individual fatty acids. Standard fatty acid nomenclature was used (Guckert *et al.*, 1985; Allison *et al.*, 2005).

2.6. Statistical Analysis

Statistical analyses were performed and graphs generated using Statistica 7.1 (Statsoft Inc., Tulsa, Oklahoma, USA), SigmaPlot 10.0 (Systat Software Inc., San Jose, California, USA), GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, California, USA), and Canoco for Windows 4.5 (Biometris – Plant Research International, Wageningen, The Netherlands; Ter Braak and Šmilauer, 1998).

Data of physical and chemical characteristics, enzymatic activities, and PLFA composition of samples were subjected to analysis of variance (ANOVA) followed by Tukey's test for mean separation where significant differences ($p = 0.05$) were indicated.

Chronosequence data for Mine A and B were analysed using the *Time Series Analysis* module in Statistica 7.1 (Statsoft Inc., Tulsa, Oklahoma, USA). Missing data (missing ages) embedded in either time series was replaced by using the *predicted values from linear trend regression* option in Statistica. With this option, Statistica fits a least-squares regression line to the time series. The missing data is then replaced by the values predicted by this regression line. This method implies that the strongest feature of the

series is its linear trend across time and was chosen as the most reliable option to predict missing values in the time series even though the data was fitted with nonlinear regression.

Curve fitting was performed in GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, California, USA) by using nonlinear regression. Due to the method chosen for predicting missing data points in the chronosequences, curve fits were also performed with linear regression. The p values obtained from both linear and nonlinear regressions after applying the F-test were then compared to determine whether there was consistency in the conclusions regarding the significant differences between the curve fits. Similarity or dissimilarity of fitted curves based on the F-test is expressed in terms of an F-ratio and corresponding p value. If $p < 0.05$, there is a statistically significant difference between the curves (Motulsky, 2007).

A canonical correspondence analysis (CCA) (Canoco for Windows 4.5, Biometris – Plant Research International, Wageningen, The Netherlands) was performed to investigate the relationship between the sites from both chronosequences, enzymatic activities (microbial community function), and PLFA groups (microbial community structure).

3. RESULTS AND DISCUSSION

Results from the physical and chemical characterisation of the soil cover layers, as well as percentage vegetation cover for all sites from Mine B, are summarised in Table 5.1 (refer to Table 3.1, Chapter 3 for the physico-chemical properties and vegetation cover for sites from Mine A). As in the case of Mine A, few of these properties showed significant differences between sites ($p < 0.05$, Tukey's test) and no trends consistent with the rehabilitation ages of the respective sites were apparent from this investigation. The similarity in physical and chemical properties between sites was important in the application of SFT substitution to obtain chronosequences of rehabilitation ages. The 17-year old site from Mine B showed the highest percentage organic C of all sites sampled at this mine. It was also the oldest site sampled at either of the two mines during the investigation. However, the highest percentage organic C for all sites sampled from both mines was observed at the 11-year old site from Mine A. Although sites from both mines were grazed, sites from Mine A generally had higher organic C contents than sites from Mine B and this could be attributed to the addition of manure to sites managed by Mining Company A. Another observation that could be attributed to the different management regimes was that concentrations of P and $\text{NO}_3\text{-N}$ varied less between sites from Mine B

(Table 5.1) compared to the large variations between sites from Mine A (see Table 3.1, Chapter 3).

Overall, sites managed by Mining Company B with a less intensive management regime (only grazing applied), had higher percentages of vegetation cover (Table 5.1) than sites managed by Mining Company A, which applied annual amelioration and grazing (Table 3.1, Chapter 3). This was particularly obvious with regard to ground cover.

Enzymatic assays for dehydrogenase, β -glucosidase, acid and alkaline phosphatase, and urease activities were performed for all sites from Mine B as for sites from Mine A (see Chapters 1 and 3 for discussions on the underlying principles and associated indications of individual enzymatic activities).

Characterisation of microbial communities for sites from Mine B was carried out in the same manner as for sites from Mine A by means of PLFA analysis. The PLFA ratios and mole percentage (mol%) fractions of the major PLFA groups are summarised in Table 5.2. The sum of extracted PLFAs in each sample provides a quantitative measure of the viable microbial biomass. Variation in microbial community structure between sites is determined by PLFA profile analysis. Refer to chapters 1 and 4 for discussions on PLFA profiling.

A correlation matrix (not shown) indicated significant correlations ($p < 0.05$) of β -glucosidase with NO_3 ($r = 0.97$); alkaline phosphatase with pH ($r = 0.76$); polyunsaturated fatty acids with organic C ($r = -0.79$); and microbial biomass with NH_4 ($r = 0.78$) and organic C ($r = 0.95$).

Table 5.1. Physico-chemical properties and vegetation cover of soil cover layers obtained from the coal discard sites managed by **Mining Company B**.

	Site_1	Site_2	Site_3	Site_4	Site_5	Site_6	Site_7
Rehabilitation age (years)	6	7	10	11	15	16	17
Ca (mg kg ⁻¹)	9.15 (5.00)b	3.43 (1.61)a	2.02 (1.40)a	10.58 (4.17)ab	2.21 (0.12)a	2.67 (0.71)a	2.22 (0.75)a
Mg (mg kg ⁻¹)	7.64 (2.36)b	3.18 (1.91)ab	4.65 (2.38)ab	5.14 (1.74)ab	2.38 (0.57)ab	2.01 (0.12)a	2.28 (0.22)a
K (mg kg ⁻¹)	4.03 (1.94)a	3.64 (1.14)a	6.39 (4.52)ab	17.11 (8.08)a	3.44 (0.53)b	4.62 (0.13)ab	3.04 (0.73)a
Na (mg kg ⁻¹)	4.63 (2.06)b	1.09 (0.18)a	1.39 (0.98)ab	0.29 (0.07)a	0.46 (0.14)a	1.74 (0.56)ab	2.08 (0.24)b
SO ₄ (mg kg ⁻¹)	0.72 (0.01)a	2.42 (0.01)a	0.72 (0.25)a	0.73 (0.01)a	4.34 (0.49)ab	3.04 (0.66)c	10.63 (1.07)bc
NO ₃ -N (mg kg ⁻¹)	0.46 (0.01)a	0.47 (0.02)a	1.72 (0.53)a	1.71 (0.51)b	0.38 (0.11)a	1.55 (0.19)a	1.25 (0.18)a
NH ₄ -N (mg kg ⁻¹)	0.32 (0.05)a	0.33 (0.06)a	0.32 (0.22)a	0.31 (0.05)a	0.27 (0.00)a	0.32 (0.06)a	0.41 (0.02)a
Cl (mg kg ⁻¹)	5.71 (1.53)a	1.87 (0.33)a	3.03 (1.14)a	2.49 (0.47)a	1.15 (0.28)a	2.31 (0.39)a	3.39 (0.22)a
P (P-Bray1) (mg kg ⁻¹)	7.32 (0.34)a	7.30 (0.44)b	7.06 (2.99)a	5.95 (0.57)a	7.44 (0.54)a	10.83 (3.22)a	9.89 (0.49)a
Organic carbon (%)	0.38 (0.14)a	0.33 (0.03)a	0.71 (0.24)a	0.93 (0.15)ab	0.35 (0.06)a	0.81 (0.27)a	1.58 (0.11)b
pH	6.17 (0.93)a	5.28 (0.53)a	5.94 (0.49)a	5.63 (0.12)a	5.20 (0.57)a	5.28 (0.13)a	5.38 (0.03)a
EC (mS m ⁻¹)	0.26 (0.09)b	0.08 (0.02)ab	0.10 (0.04)ab	0.19 (0.07)ab	0.06 (0.01)a	0.07 (0.00)a	0.06 (0.02)a
Sand (%)	80.10 (3.11)ab	81.13 (2.04)a	82.87 (8.59)a	71.30 (1.46)bc	76.26 (2.73)ab	85.23 (1.14)a	66.63 (3.54)c
Silt (%)	6.87 (1.20)a	3.97 (1.02)a	6.20 (3.48)a	13.94 (1.98)b	9.83 (2.02)ab	6.23 (1.06)a	8.90 (1.11)ab
Clay (%)	13.03 (2.05)a	14.90 (1.81)a	10.97 (2.75)a	14.77 (1.23)a	13.9 (1.27)a	8.53 (0.94)a	24.50 (3.80)b
Ground Cover (%)	45.00 (4.04)a	48.00 (7.60)a	66.67 (5.76)b	78.30 (2.88)b	43.30 (4.08)a	50.00 (0.00)a	43.33 (4.08)a
Crown Cover (%)	58.00 (2.80)bc	63.00 (5.70)ab	78.33 (2.87)a	97.30 (2.51)d	45.00 (8.66)c	65.00 (5.00)ab	76.67 (8.16)a

Standard error values are indicated in brackets. Data in rows with the same letters indicate no significant difference, while those with different letters indicate significant difference at $p < 0.05$ (Tukey's HSD).

EC: Electrical conductivity.

Table 5.2. Phospholipid fatty acid (PLFA) composition and ratios of soil cover layers obtained from the coal discard sites managed by **Mining Company B**.

	Site_1	Site_2	Site_3	Site_4	Site_5	Site_6	Site_7
Rehabilitation age (years)	6	7	10	11	15	16	17
Viable microbial biomass (pmol g ⁻¹ dry weight)	4534.06 (1015.35)a	6136.43 (2484.26)ab	10950.92 (2276.17)ab	12569.54 (3589.17)ab	4247.11 (608.23)a	10874.47 (4882.96)ab	16318.15 (3600.29)b
PLFA Major Group (mol%)							
Normal saturated	33.10 (18.23)a	41.83 (13.96)a	39.94 (2.96)a	41.46 (3.72)a	40.56 (1.69)a	38.93 (0.57) a	39.76 (1.85)a
Mid-chain branched saturated	2.39 (0.41)a	3.00 (0.59)a	2.79 (0.30)a	3.76 (0.34)a	2.66 (0.12)a	4.13 (1.09)a	3.86 (0.71) a
Terminally branched saturated	15.97 (3.28)a	25.94 (11.13)a	30.81 (4.16)a	28.82 (5.47)a	23.16 (0.80)a	31.36 (3.08)a	29.07 (0.94)a
Monounsaturated	40.20 (10.73)b	24.80 (10.54)a	22.55 (0.70)ab	26.44 (1.17)ab	22.46 (3.59)ab	27.87 (3.62)ab	29.26 (1.55)ab
Polyunsaturated	5.21 (3.81)a	7.98 (3.64)a	2.97 (2.52)a	1.41 (0.55)a	7.84 (1.72)a	2.36 (0.98)a	0.79 (0.54)a
Ratios							
Fungal / Bacterial ¹	0.14 (0.07)ab	0.09 (0.04)ab	0.09 (0.07)ab	0.04 (0.01)a	0.23 (0.05)b	0.06 (0.02)ab	0.02 (0.01)a
Saturated / Unsaturated ²	1.22 (0.76)a	2.73 (1.04)a	3.14 (0.07)a	2.66 (0.20)a	2.84 (0.51)a	2.83 (0.70)a	2.35 (0.17)a
Gram (+) / Total PLFA ³	0.12 (0.01)a	0.16 (0.02)a	0.25 (0.09)a	0.25 (0.12)a	0.21 (0.13)a	0.30 (0.01)a	0.26 (0.01)a
Iso / Anteiso ⁴	0.00 (0.00)a	2.07 (0.01)a	3.76 (0.88)ab	3.01 (0.12)ab	2.69 (0.33)ab	7.26 (2.81)b	3.01 (0.11)ab
16:1 ω 7t / 16:1 ω 7c	0.02 (0.01)a	0.00 (0.00)a	0.03 (0.01)a	0.00 (0.00) a	0.03 (0.00)a	0.01 (0.00)a	0.00 (0.00)a
18:1 ω 7t / 18:1 ω 7c	0.04 (0.02)a	0.01 (0.00)a	0.02 (0.00)a	0.03 (0.02)a	0.05 (0.02)a	0.06 (0.01)a	0.00 (0.00)a

Standard error values are indicated in brackets. Data in rows with the same letters indicate no significant difference, while those with different letters indicate significant difference at $p < 0.05$ (Tukey's HSD).

¹ Fungi: 18:2 ω 6 (Federle, 1986; Frostegård and Bååth, 1996), Bacteria: sum of i15:0, a15:0, 15:0, i16:0, 16:1 ω 9, 16:1 ω 7t, i17:0, a17:0, 17:0, cy17:0, 18:1 ω 7, and cy19:0 (Frostegård and Bååth, 1996).

² Normal saturated to monounsaturated PLFA (McKinley *et al.*, 2005).

³ Gram-positive PLFA markers (i10me16:0, i15:0, a15:0, i16:0, and 17:0) to total PLFA (McKinley *et al.*, 2005).

⁴ Iso to anteiso branched PLFA (i15:0 + i17:0/a15:0 + a17:0) (McKinley *et al.*, 2005).

The intrinsic complexity associated with the monitoring of rehabilitated mining areas over the long-term, combined with the limitations of traditional statistical analyses calls for the application of alternative approaches that describe temporal trends in these ecosystems. An example of such an approach, SFT substitution, was applied to the different aged sites sampled in separate locations at Mine A and Mine B, respectively, to obtain two chronosequences of reclaimed post-mining sites. Considering prior mining activities and prevailing environmental conditions, the sites included in each of these chronosequences were considered to be comparable rehabilitations of different ages.

Graphical representations of selected microbial properties over time for the two chronosequences (Mine A and Mine B) are shown in Figures 5.1 to 5.7. The Y-axes were scaled according to the highest activities or values observed for individual samples during the study period. The embedded line graphs represent fitted curves (nonlinear regression only) for the relevant data indicated on the graph for each chronosequence. Curves were fitted with GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, California, USA) and a comparison of curves was performed between chronosequences to determine the best fit for each data set. In those cases where the same type of fit was indicated as the preferred model for both curves, the F-test was performed to compare the curves. Models fitted to the data by linear regression are not shown on the graphs and are only discussed in those instances where opposing conclusions pertaining to significant differences ($p = 0.05$) occurred. In the majority of the cases, however, models fitted by linear and nonlinear regression gave the same result regarding the similarity of the fitted curves. What should be kept in mind, is that conclusions drawn from curves fitted by a computer programme must be considered with respect to the relevant research (Motulsky and Christopoulos, 2005). It would be inappropriate to base conclusions on curve fits while ignoring the scientific principles and/or known features of the context in which the investigation is taking place.

According to Motulsky and Christopoulos (2005), one must turn the question about comparing curves into one about comparing models when attempting to compare curves derived from different data sets. In this context, the first model will be based on two distinct curves - separate curves are fit and the overall sum-of-squares (SS) is defined as the total SS for each separate curve. The second model uses global fitting - all the data is fit at once, finding one shared value of the measured parameter. In this way, the F-test can be applied to evaluate different curves and a comparison is possible between entire curves instead of only between single parameters. The F-test is an adaptation of ANOVA and is

based on the difference between the SS of two models (in this case, the two chronosequences). The question is whether the difference between SS values is greater than you would expect to see by chance. It also takes into account the number of data points and the number of parameters of each model. The result is expressed as the F-ratio from which a p value is calculated. The p value tests the null hypothesis that there is no difference between the two curves overall and any observed difference is due to chance. If all variability was random – in other words, if the two chronosequences really were the same and differences between them are due to chance, F would be near 1.0. In this case, the resulting p value would not be significant ($p > 0.05$) and a conclusion can be made that the curves did not differ in a statistically significant manner (Motulsky and Christopoulos, 2005; Motulsky, 2007).

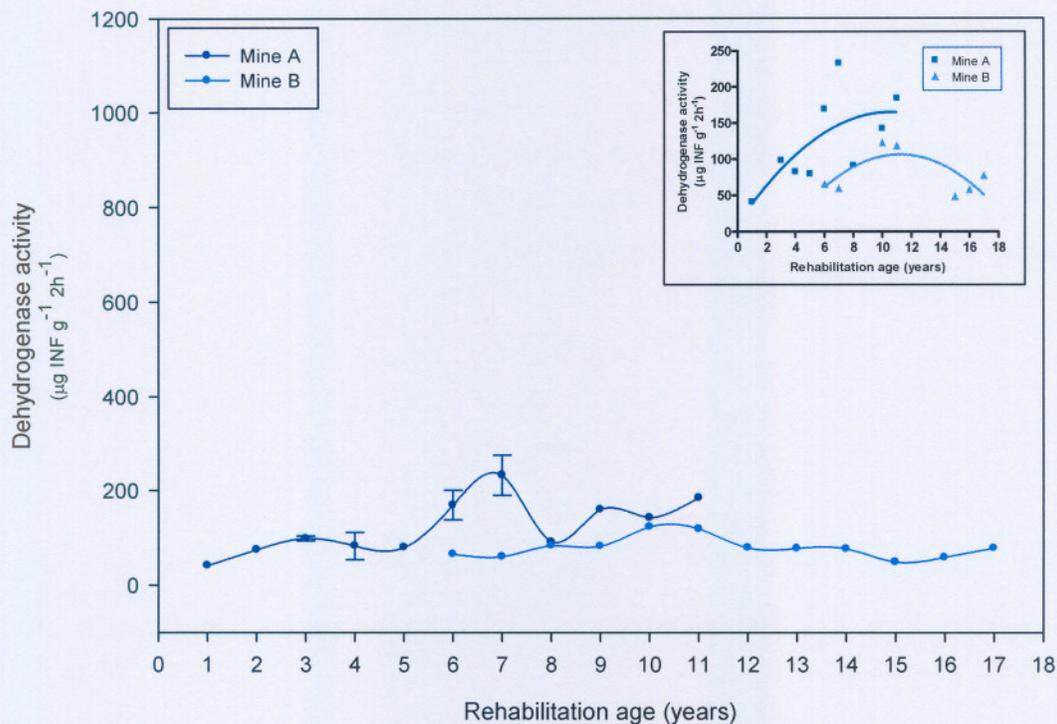


Figure 5.1. Changes in dehydrogenase activity during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence.

Dehydrogenase activity (Figure 5.1), which is an indicator of overall microbial activity (Taylor *et al.*, 2002), fluctuated in both chronosequences with higher activities observed in older sites of chronosequence A (Mine A) when compared to younger sites within the same chronosequence. Chronosequence B (Mine B) sites had higher activities during the first half of the rehabilitation period. Chronosequence A had higher dehydrogenase activities

and more pronounced fluctuations than chronosequence B, which may be related to the input of fertilisers and lime that is characteristic of the management regime applied at Mine A. A comparison of curve fits (nonlinear regression) indicated a second order polynomial ($y = a + bx + cx^2$) as the preferred model for data sets from both chronosequences. The F-test was applied to the two fitted curves and indicated no significant differences between these fits ($F = 1.98$; $p = 0.18$). It could therefore be concluded that the temporal trends in these two data sets were essentially similar.

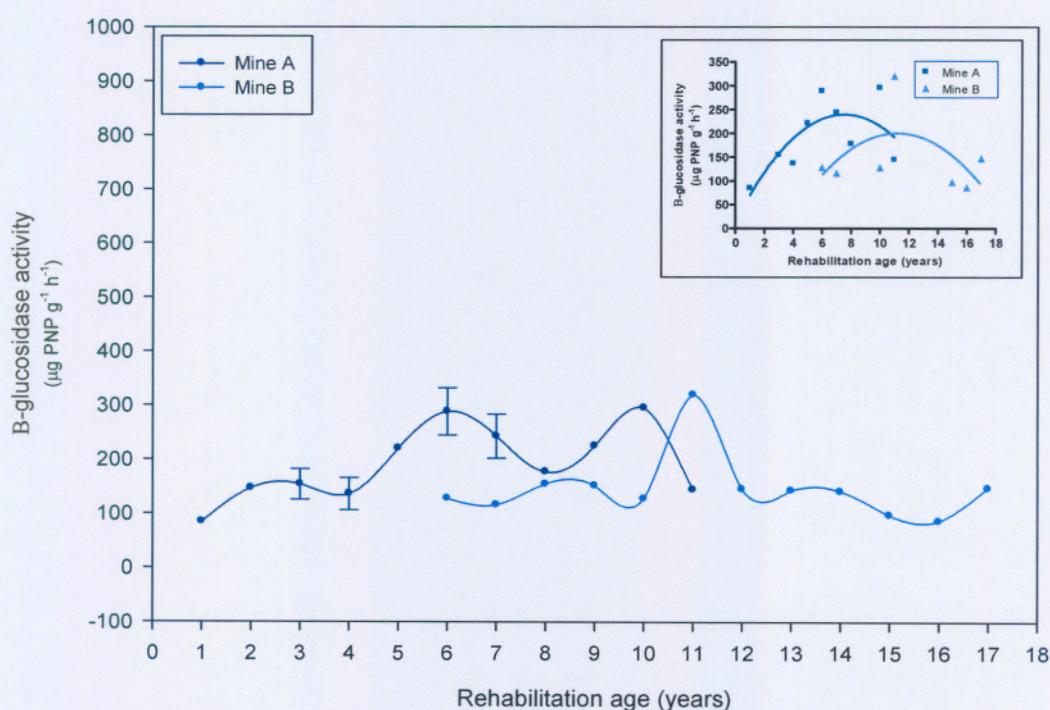


Figure 5.2. Changes in β -glucosidase activity during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence.

B-glucosidase activity (Figure 5.2) was assayed for its relevance to the C cycle (Turner *et al.*, 2002). Activities for chronosequences A and B were in the same range (84.45 – 295.33 $\mu\text{g PNP g}^{-1} \text{h}^{-1}$ and 84.53 – 319.54 $\mu\text{g PNP g}^{-1} \text{h}^{-1}$, respectively), with the exception of the 11-year old site in chronosequence B. Fluctuations in this chronosequence was less pronounced than those in chronosequence A. As in the case of dehydrogenase activity, the temporal variations in β -glucosidase activity can be ascribed to management inputs. Grazing was applied to sites from Mine B and the increased activity observed for the 11-year old site in this chronosequence may be due to higher organic C content of the sample as a result of manure at this location due to grazing by livestock. The preferred model for

both chronosequences was a second order polynomial. No significant differences existed between the two fits based on the F-test ($F = 1.20$ and $p = 0.36$). Similar temporal trends thus seem to exist in both chronosequences.

The changes in alkaline and acid phosphatase activities during rehabilitation in chronosequences A and B are shown in Figures 5.3 and 5.4, respectively. Phosphatases are involved in organic phosphorus transformations in soil (Nannipieri *et al.*, 2002) and their activities are sensitive to management practices (Aon *et al.*, 2001) and soil pH (Dick *et al.*, 2000). Alkaline phosphatases are produced by microorganisms, while acid phosphatases are mainly attributed to plant roots (Criquet *et al.*, 2004).

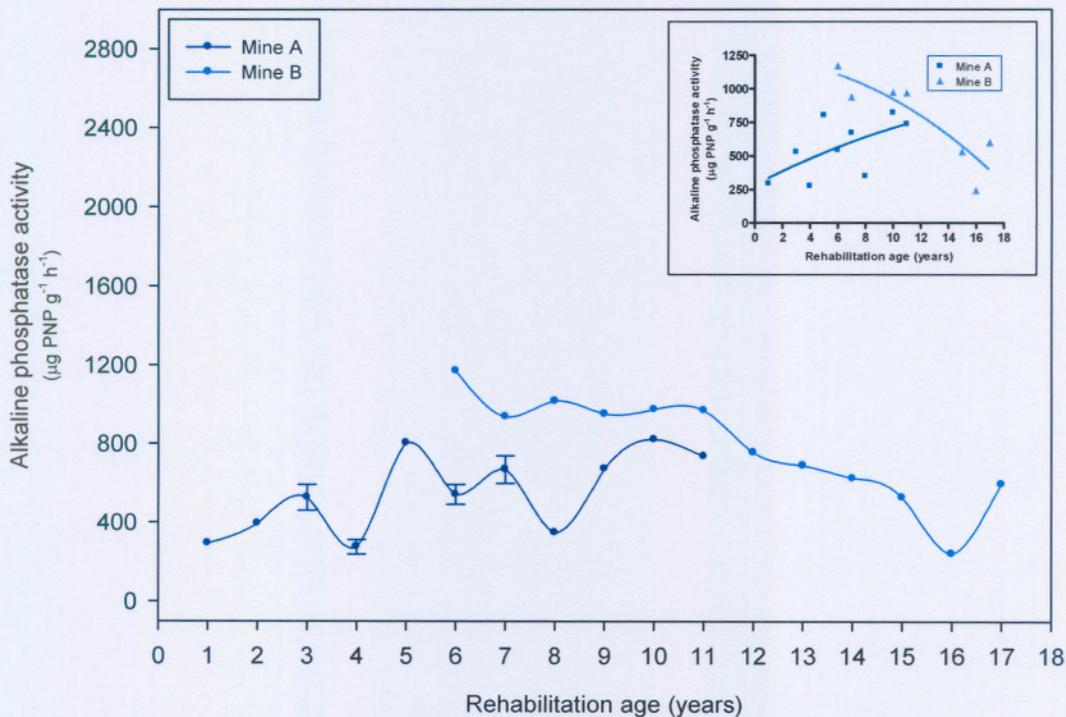


Figure 5.3. Changes in alkaline phosphatase activity during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence.

Alkaline phosphatase activity (Figure 5.3) showed fluctuations over the rehabilitation period for chronosequence A, with higher activities observed in the older sites compared to younger sites. Conversely, the activities for chronosequence B decreased with increasing age of the rehabilitation and only showed an upward tendency again in the 17-year old site. When related to the microbial origin of alkaline phosphatase, these trends may indicate the sensitivity of the microbial community to management practices. As in the case of the other enzymatic activities, a second order polynomial was indicated as the preferred curve

fit for data from both chronosequences. The curve fits obtained by nonlinear regression, visually appeared different and although the p value calculated from the F-ratio for these data was close to 0.05, strictly no significant differences existed between the two fits based on the F-test ($F = 3.49$ and $p = 0.06$). Linear regression models fitted to the two chronosequences indicated that there was a 0.14% chance of randomly choosing data points with slopes as different as these. The conclusion was that the difference between the slopes was significant ($p = 0.001$). Considering all information obtained for these data, the conclusion may be drawn that the trends that occurred in these chronosequences differed during the period of rehabilitation.

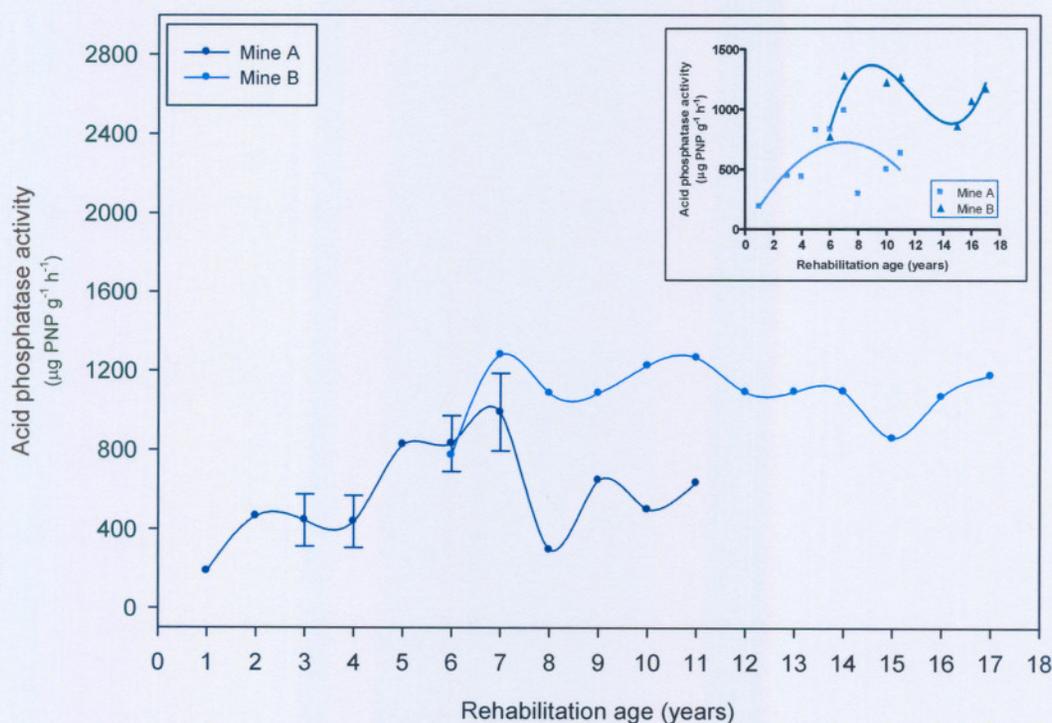


Figure 5.4. Changes in acid phosphatase activity during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence.

Chronosequence A showed clear changes in acid phosphatase activity (Figure 5.4) over the rehabilitation period, whereas activities for chronosequence B varied to a lesser extent and was higher over the rehabilitation period. Curve fit comparisons for acid phosphatase activity indicated different models as the preferred fits for each chronosequence. Chronosequence data from Mine A was fitted to a second order polynomial, while a third order polynomial ($y = a + bx + cx^2 + dx^3$) was fitted to data from Mine B. Accordingly, the temporal trends that existed in these data was different for the two chronosequences. The

higher vegetation cover observed at Mine B (compare Table 3.1, Chapter 3 and Table 5.1) and the association of acid phosphatase activity with plant roots, may be the reason for these dissimilar trends. This clearly shows the sensitivity of acid phosphatase activity to management practices. Two distinct management regimes result in two types of vegetation cover and are reflected in two sets of data with markedly different trends over the long-term.

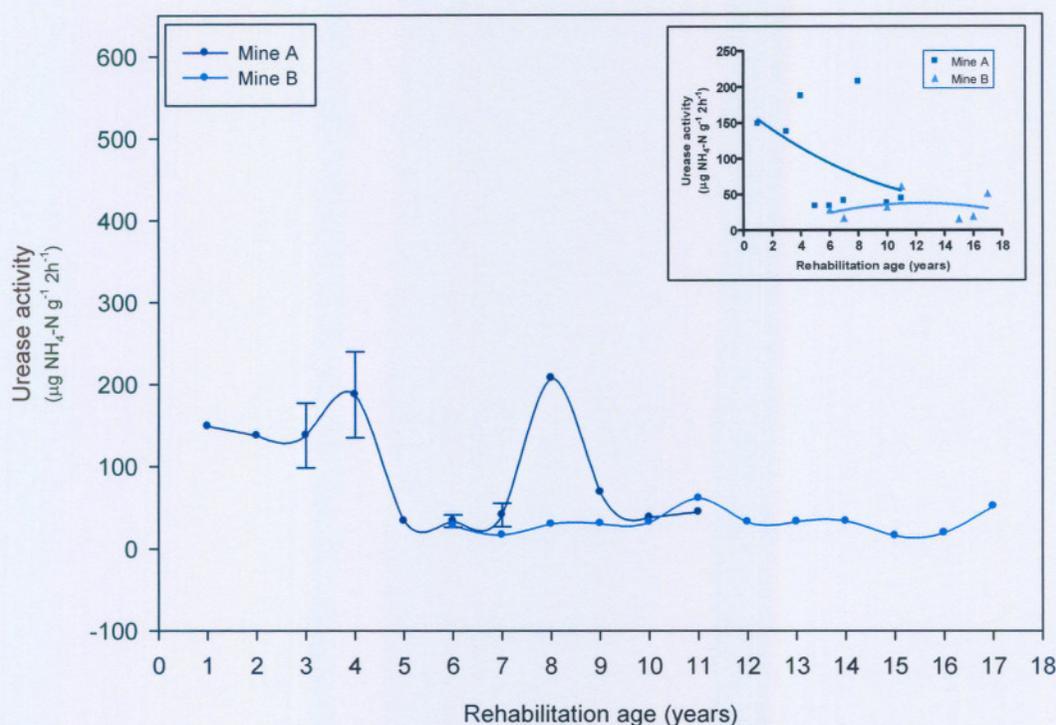


Figure 5.5. Changes in urease activity during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence.

Urease activity (Figure 5.5) in soil has been correlated with microbial biomass (Klose and Tabatabai, 2000) and is measured for its relationship to the N cycle (Gil-Sotres *et al.*, 2005). As in the case of the other enzymatic activities assayed, the changes in urease activity were greater for chronosequence A than for chronosequence B, which had relatively stable urease activities over the rehabilitation period. This could be attributed to the application of fertilisers, specifically nitrates, to sites at Mine A compared to Mine B where no fertiliser inputs occurred. Even though sites from Mine B had lower urease activities, the minimum activities for these sites were similar to the minimum activities for sites from Mine A. Despite the dissimilarity in urease activities between the two chronosequences, a comparison of curve fits indicated a second order polynomial as the

preferred model for data sets of both chronosequences. The F-test was applied to the two fitted curves and indicated no significant differences between these fits ($F = 0.61$; $p = 0.63$). This shows similar tendencies in urease activity for the different chronosequences.

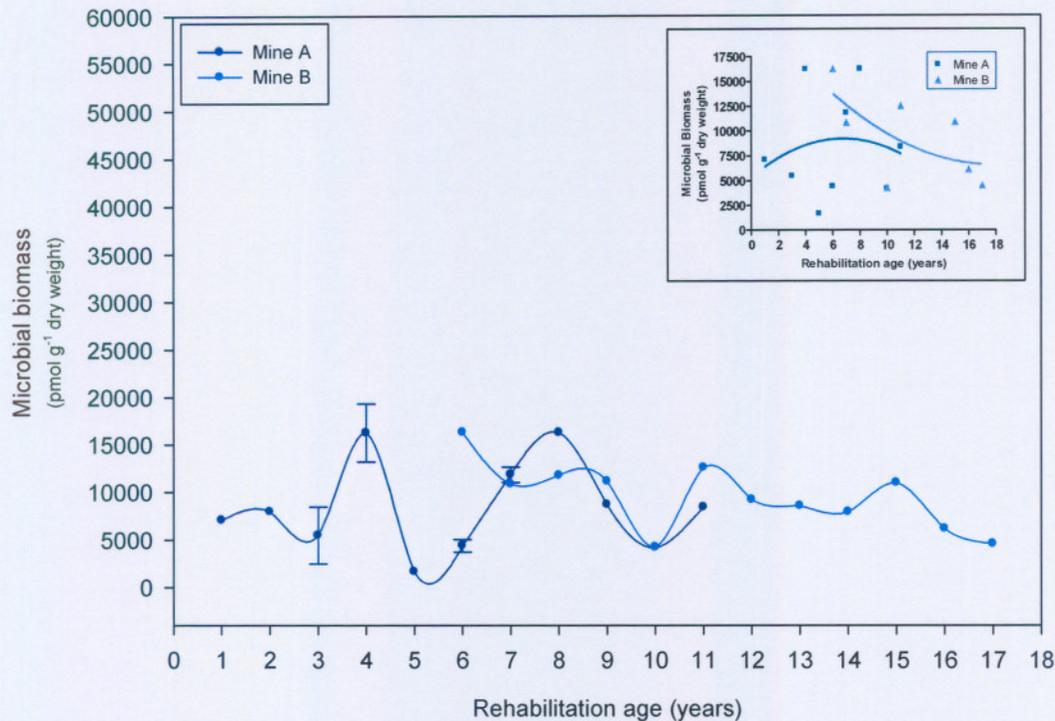


Figure 5.6. Changes in microbial biomass during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence.

The estimation of microbial biomass by means of PLFA analysis is a reliable manner in which the viable microbial community can be quantified (Calderón, *et al.*, 2000; Rütters *et al.*, 2002). In addition, PLFA profiles can signify changes in the bacterial and fungal composition of a soil (Ibekwe and Kennedy, 1998; Hill *et al.*, 2000) and the ratio of fungal to bacterial PLFA (F:B ratio) has been applied to measure soil recovery (Bardgett and McAlister, 1999).

Estimations of viable microbial biomass varied markedly between sites in individual chronosequences over the rehabilitation period – especially in chronosequence A (Figure 5.6). The biomass abundance in both chronosequences varied within a comparable range (1656.22 – 16277.30 pmol g⁻¹ dry weight for Mine A and 4247.11 – 16318.51 pmol g⁻¹ dry weight for Mine B) and curves fitted to biomass data from the respective chronosequences indicated a second order polynomial as the preferred model for both. An application of the F-test to these data indicated no statistically significant differences between the data sets

from Mine A and Mine B ($F = 0.39$; $p = 0.77$). It may therefore be concluded that the temporal trends observed for microbial biomass in the two chronosequences were also similar.

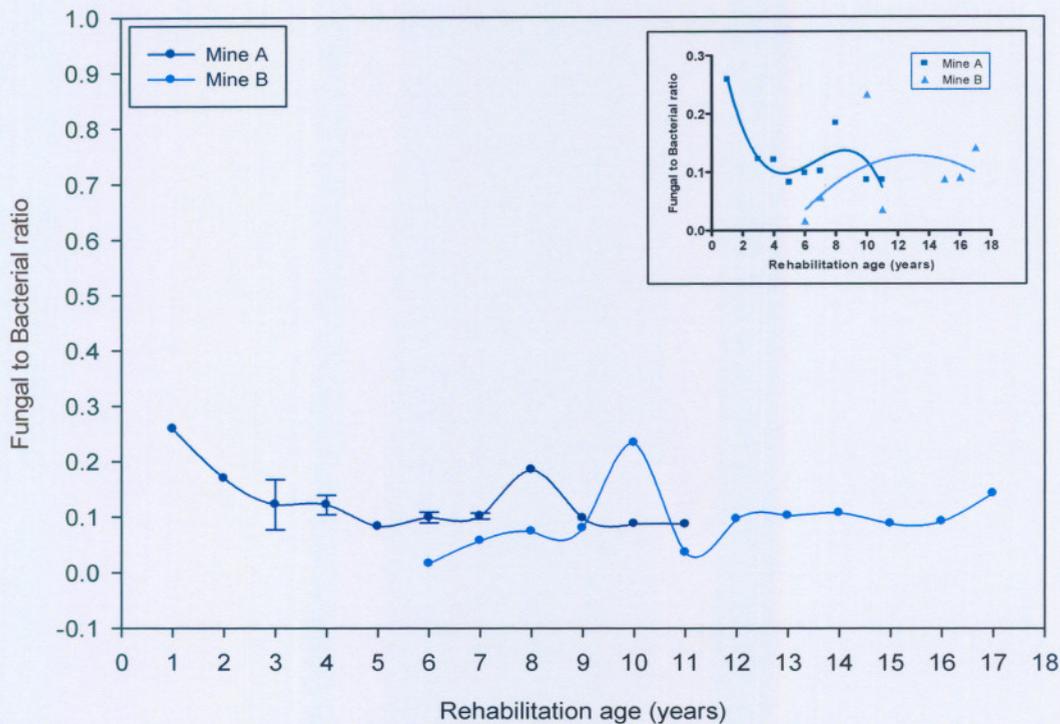


Figure 5.7. Changes in the fungal to bacterial ratio during rehabilitation in the chronosequences from Mine A and B, respectively. The embedded graph indicates the curve fits for each chronosequence.

The changes in the ratio of fungal to bacterial PLFA in chronosequence A and B over the rehabilitation period is shown in Figure 5.7. Chronosequence A showed a decreasing trend in the F:B ratio, while the same ratio was higher in the older sites than in the younger sites from chronosequence B. A comparison of curve fits indicated a second order polynomial as the preferred model for chronosequence A and a third order polynomial as the preferred model for chronosequence B. This translates to there being a significant difference between the temporal trends for the two chronosequences. On the contrary, models fitted by linear regression indicated no significant difference between the slopes ($F = 3.05$; $p = 0.11$) of the two data sets. In the context of the investigation and considering that those values in the data that cause the difference in temporal trends are real data and not predicted missing values (missing values were predicted with linear trend regression), it was decided that the curve fit comparison obtained by the nonlinear regression model was valid.

The first inclusive observation that can be made from the graphs in Figures 5.1 to 5.7 is that fluctuations in enzymatic activities and abundances of microbial communities occur in both chronosequences, despite different management regimes. It seems that the initial input of fertilisers, organic material (manure), and lime at the onset of rehabilitation brings about an increase in microbial activity and biomass. After approximately three years, activities and abundances of microorganisms decrease and without additional management inputs should reach an equilibrium level characteristic of the specific environment (in this case a mining disturbed area under rehabilitation). The prevalence of certain functional and structural characteristics of the microbial communities at the respective sites may be explained by the classification of microorganisms as r-strategists or K-strategists (Claassens *et al.*, 2005). While r-strategic microorganisms thrive in environments where nutrients are readily available, K-strategists depend on physiological adaptations to environmental resources for survival. These microorganisms are often successful in resource-limited situations (Atlas and Bartha, 1998). Therefore, decreases in enzymatic activities or biomass abundances do not necessarily signify deterioration in these ecosystems, but rather a change in the composition of the microbial communities.

The implication of the similarity in trends for the selected microbial community properties discussed here should be clarified. While similar trends exist in both chronosequences for individual microbial community measurements, the suggestion is not that the effect of the management practices on the microbial communities is exactly the same. It simply means that the common denominator in both these chronosequences is fluctuation. Similarity in temporal trends does not signify that the prevailing conditions in the two ecosystems are the same. For example, when considering microbial biomass (Figure 5.6), it means there is fluctuation in the amount of biomass over the rehabilitation period in both the chronosequences as opposed to one chronosequence having an upward (increasing) or downward (decreasing) trend in microbial biomass while the other one shows fluctuation. What does differ in these chronosequences is the amount of fluctuation – chronosequence B showed less variability in microbial community function and structure. While chronosequence B contained some older sites than chronosequence A, statistical analysis did not indicate any relationships between microbial properties and rehabilitation ages of sites. This translates into fluctuation being a reflection of management, which is why sites managed in the same way group together for the respective chronosequences when subjected to canonical correspondence analysis (CCA)

(Figure 5.8), despite the similarity in trends observed for the individual microbial properties.

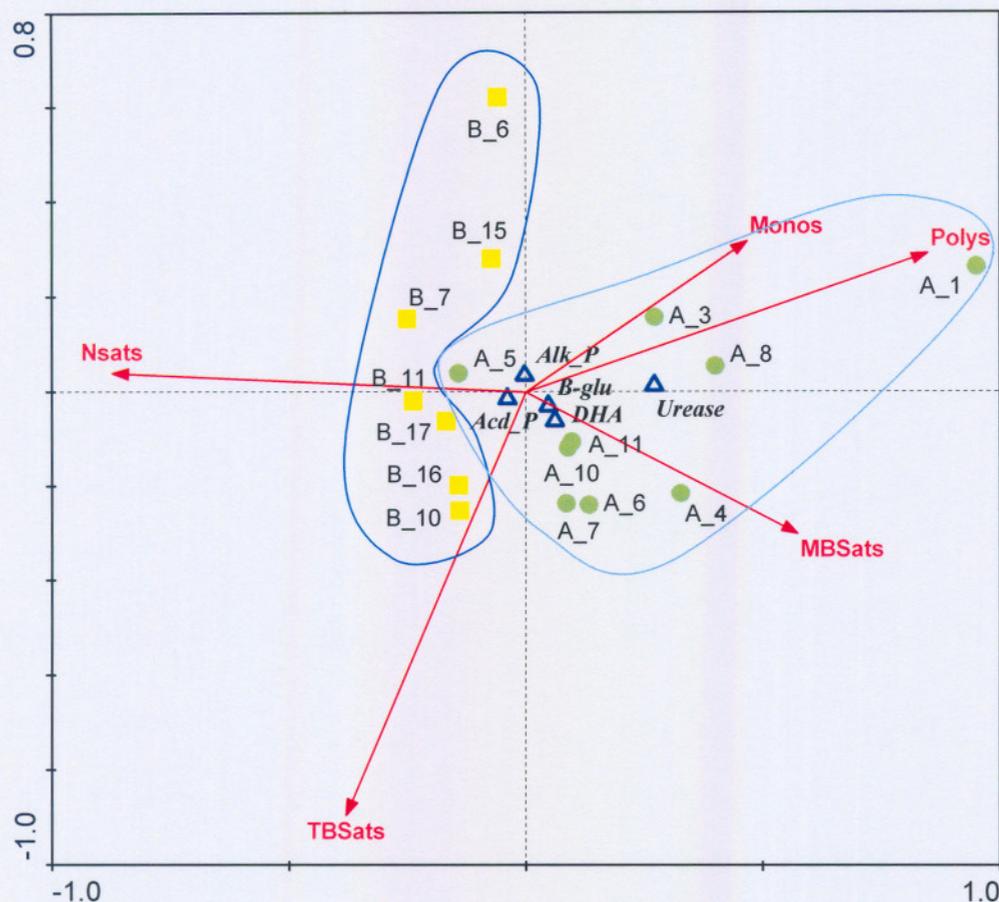


Figure 5.8. Canonical correspondence analysis (CCA) diagram illustrating the relationship between the coal discard sites based on enzymatic activities and phospholipid fatty acid (PLFA) groups. Eigenvalues for the first two axes were 0.068 and 0.014, respectively. Total observed variance of the first two axes of the CCA was 77.1%. Each site is indicated according to the chronosequence to which it belongs (A or B) followed by the rehabilitation age of the site. Key to abbreviations: DHA: dehydrogenase; B-glu: β -glucosidase; Acid-P: acid phosphatase; Alk-P: alkaline phosphatase; Nsats: normal saturated fatty acids; TBSats: terminally branched saturated fatty acids; MBSats: mid-chain branched saturated fatty acids; Polys: polyunsaturated fatty acids; Monos: monounsaturated fatty acids.

Eigenvalues for the first two axes were 0.068 and 0.014, respectively. Total observed variance of the first two axes of the CCA was 77.1%. The cumulative percentage variance of the species-environment relation was 71.6, 86.8, 99.8, and 100.0%, respectively for the four axes. The first axis was significant in explaining the variation in enzymatic activities ($p = 0.0020$), as were all four axes together ($p = 0.0020$). The first canonical axis correlated most strongly with normal saturated fatty acids ($r^2 = -0.7678$) and the second axis with

terminally branched saturated fatty acids ($r^2 = -0.6897$).

4. CONCLUSIONS

Fluctuations for chronosequence A were more pronounced than those for chronosequence B and can be related to the more intensive management regime applied at Mine A. Although the enzymatic activities and microbial abundance measurements indicated lower overall values for chronosequence B, the trends over time of rehabilitation are similar to those observed for chronosequence A. In general there was less fluctuation in chronosequence B than in chronosequence A, which is related to the regular management inputs that occurred at sites from chronosequence A. In other words, the microbial communities in the less intensively managed sites maintain their functional and structural integrity within bounds in the absence of management inputs or disturbance, without evidence of an upward or downward trend.

The fluctuations in microbial properties observed for sites from Mine A and Mine B, indicate that a final hypothetical equilibrium stage exists for each chronosequence and that functional and structural aspects of microbial populations will tend towards these equilibrium stages unless this propensity is altered by management inputs. A comparison of the two chronosequences based on minimum and maximum values and temporal trends for individual microbial community measurements indicated no bias towards management regime over the long-term. This situation might change only if management practices affected vegetation to such an extent that the feedback mechanisms that exist between aboveground and belowground communities resulted in long-term effects on microbial community function and structure.

What is important to consider in terms of rehabilitation of mining disturbed sites and the management thereof, is that the goal of rehabilitation is to obtain a stable ecosystem. Thus, while distinct management regimes may lead to similar long-term trends in rehabilitated discard sites, the question of stability remains. Since post-mining sites such as these could never reach the same status as natural soils in terms of biological communities, the goal of a stable ecosystem defined in the context of the particular environment in question is much more realistic. However, the first step in determining whether the ecosystem under management is a stable one, would be to stop interfering in that ecosystem by means of management inputs in the short term. Only in the absence of management inputs it would become obvious whether a specific ecosystem has reached a

state of equilibrium where biological parameters do not fluctuate beyond certain minimum and maximum limits. Of course, mining companies cannot simply cease all management practices at the risk of the managed ecosystems disintegrating, which is why monitoring of appropriate environmental parameters is very important in management decisions.

The possibility of achieving a stable ecosystem while lessening the amount of management inputs will translate into significant economic implications for mining companies, which is another critical aspect of rehabilitation in the South African mining context. In the end, the question that mining companies would have to answer is what the true cost of rehabilitation over the long-term is in terms of the management practices they favour.

REFERENCES

- Alef, K., Nannipieri, P. 1995. *Methods in applied soil microbiology and biochemistry*. Boston: Academic Press. 576p.
- Allison, V.J., Miller, R.M., Jastrow, J.D., Matamala, R., Zak, D.R. 2005. Changes in soil microbial community structure in a tallgrass prairie chronosequence. *Soil Sci Soc Am J* 69:1412-1421.
- Aon, M.A., Sarena, D.E., Burgos, D.E., Cortassa, S. 2001. (Micro)biological, chemical, and physical properties of soils subjected to conventional or no-till management: an assessment of their quality status. *Soil Tillage Res* 60:173-186.
- Atlas, R.M., Bartha, R. 1998. *Microbial Ecology* (4th edition). Menlo Park, California: Benjamin/Cummings Science Publishing. 694p.
- Bardgett, R.D., McAlistter, E. 1999. The measurement of soil fungal:bacterial biomass ratios as an indicator of ecosystem self-regulation in temperate meadow grasslands. *Biol Fertil Soils* 29:282-290.
- Bartelt-Ryser, J., Joshi, J., Schmid, B., Brandl, H., Balsler, T. 2005. Soil feedbacks of plant diversity on soil microbial communities and subsequent plant growth. *Perspect Plant Ecol Evol Syst* 7:27-49.
- Calderón, F.J., Jackson, L.E., Scow, K.M., Rolston, D.E. 2000. Microbial responses to simulated tillage in cultivated and uncultivated soils. *Soil Biol Biochem* 32:1547-1559.
- Claassens, S., Riedel, K.J., Van Rensburg, L., Morgenthal, T.L., Jansen van Rensburg, P.J. 2005. Soil microbial properties in coal mine tailings under rehabilitation. *Appl Ecol Environ Res* 4:75-83.
- Criquet, S., Ferre, E., Farnet, A.M., Le Petit, J. 2004. Annual dynamics of phosphatase activities in an evergreen oak litter: influence of biotic and abiotic factors. *Soil Biol Biochem* 36:1111 – 1118.
- Dauber, J., Wolters, V. 2005. Colonization of temperate grassland by ants. *Basic Appl Ecol* 6:83-91.
- Dick, R.P., Breakwell, D.P., Turco, R.F. 1996. Soil enzyme activities and biodiversity measurements as integrative microbiological indicators. In: Doran, J.W., Jones, A.J., editors. *Methods for assessing soil quality*, SSSA Special Publication 49, Madison: SSSA. pp 247-271.
- Dick, W.A., Cheng, L., Wang, P. 2000. Soil acid and alkaline phosphatase activity as pH adjustment indicators. *Soil Biol Biochem* 32:1915-1919.
- Federle, T.W. 1986. Microbial distribution in soil – new techniques. In: Megusar, F., Gantar, M., editors. *Perspectives in microbial ecology*. Ljubljana: Slovene Society of Microbiology. pp 493-498.
- Frostegård, A., Bååth, E. 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol Fertil Soils* 22:59-65.
- Gil-Sotres, F., Trasar-Cepeda, C., Leirós, M.C., Seoane, S. 2005. Different approaches to evaluating soil quality using biochemical properties. *Soil Biol Biochem* 37:877-887.
- Guckert, J.B., Antworth, C.P., Nichols, P.D., White, D.C. 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. *FEMS Microbiol Ecol* 31:147-158.
- Harris, J.A. 2003. Measurements of the soil microbial community for estimating the success of restoration. *Eur J Soil Sci* 54:801-808.
- Harris, J.A., Birch, P. 1989. Soil microbial activity in opencast coal mine restorations. *Soil Use Manage* 5:155-160.
- Hill, G.T., Mitkowski, N.A., Aldrich-Wolfe, L., Emele, L.R., Jurkonie, D.D., Ficke, A., Maldonado-Ramirez, S., Lynch, S.T., Nelson, E.B. 2000. Methods for assessing the composition and diversity of soil microbial communities. *Appl Soil Ecol* 15:25-36.
- Ibekwe, A.M., Kennedy, A.C. 1998. Phospholipid fatty acid profiles and carbon utilization patterns for analysis of microbial community structure under field and greenhouse conditions. *FEMS Microbiol Ecol* 26:151-163.
- Klose, S., Tabatabai, M.A. 2000. Urease activity of microbial biomass in soils as affected by cropping systems. *Biol Fertil Soils* 31:191-199.

- Martínez-Ruiz, C., Fernández-Santos, B. 2005. Natural revegetation on topsoiled mining-spoils according to the exposure. *Acta Oecol* 28:231-238.
- McKinley, V.L., Peacock, A.D., White, D.C. 2005. Microbial community PLFA and PHB responses to ecosystem restoration in tallgrass prairie soils. *Soil Biol Biochem* 37, 1946-1958.
- Michener, W.K. 1997. Quantitatively Evaluating Restoration Experiments: Research Design, Statistical Analysis, and Data Management Considerations. *Restor Ecol* 5:324-337.
- Milton, S.J. 2001. Rethinking ecological rehabilitation in arid and winter rainfall regions of southern Africa. *S Afr J Sci* 97:1-2.
- Motulsky, H. 2007. Curvfit.com – A complete guide to nonlinear regression. GraphPad Software Inc. [cited March 2007]; Available from http://www.graphpad.com/curvfit/1_model_2_datasets.htm
- Motulsky, H., Christopoulos, A. 2005. Fitting models to biological data using linear and non-linear regression – a practical guide to curve fitting. GraphPad Software Inc. 351p.
- Nannipieri, P., Kandeler, E., Ruggiero, P. 2002. Enzyme activities and microbiological and biochemical processes in soil. In: Burns, R.G., Dick, R.P., editors. *Enzymes in the environment - activity, ecology, and applications*, New York: Marcel Dekker Inc. pp 1-33.
- Peacock, A.D., Mullen, M.D., Ringelberg, D.B., Tyler, D.D., Hedrick, D.B., Gale, P.M., White, D.C. 2001. Soil microbial community responses to dairy manure or ammonium nitrate applications. *Soil Biol Biochem* 33:1011-1019.
- Pickett, S.T.A. 1989. Space for time substitution as an alternative to long-term studies. In: Likens, G.E., editor. *Long-term studies in ecology*. New York: Springer. pp 110-135.
- Purtauf, T., Dauber, J., Wolters, V. 2004. Carabid communities in the spatio-temporal mosaic of a rural landscape. *Landsc Urban Plan* 67:185-193.
- Rütters, H., Sass, H., Cypionka, H., Rullkötter, J. 2002. Phospholipid analysis as a tool to study complex microbial communities in marine sediments. *J Microbiol Methods* 48:149-160.
- Sparling, G., Ross, D., Trustrum, N., Arnold, G., West, A., Speir, T., Schipper, L. 2003. Recovery of topsoil characteristics after landslip erosion in dry hill country of New Zealand, and a test of the space-for-time hypothesis. *Soil Biol Biochem* 35:1575-1586.
- Taylor, J.P., Wilson, B., Mills, M.S., Burns, R.G. 2002. Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. *Soil Biol Biochem* 34:387-401.
- Ter Braak, C.F., Šmilauer, P. 1998. *Canoco reference manual for user's guide to Canoco community ordination*, ver. 4. New York: Microcomputer Power.
- Turner, B.L., Hopkins, D.W., Haygarth, P.M., Ostle, N. 2002. β -Glucosidase activity in pasture soils. *Appl Soil Ecol* 20:157-162.
- Van Rensburg, L., Maboeta, M.S., Morgenthal, T.L. 2004. Rehabilitation of co-disposed diamond tailings: growth medium rectification procedures and indigenous grass establishment. *Water Air Soil Pollut* 154:101-113.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUDING REMARKS

"What greater stupidity can be imagined than that of calling jewels, silver, and gold 'precious,' and earth and soil 'base'? People who do this ought to remember that if there were as great a scarcity of soil as of jewels or precious metals, there would not be a prince who would not spend a bushel of diamonds and rubies and a cartload of gold just to have enough earth to plant a jasmine in a little pot, or to sow an orange seed and watch it sprout, grow, and produce its handsome leaves, its fragrant flowers, and fine fruit. It is scarcity and plenty that make the vulgar take things to be precious or worthless; they call a diamond very beautiful because it is like pure water, and then would not exchange one for ten barrels of water."

— Galileo Galilei (1564 - 1642)

1. GENERAL DISCUSSION OF RESULTS

In a preliminary investigation to the current study, it was found that assays of enzymatic activities (dehydrogenase, β -glucosidase, urease, and acid and alkaline phosphatases) and analyses of phospholipid fatty acid (PLFA) profiles could discriminate between the soil covers of coal discard sites of different rehabilitation ages. During this investigation, coal discard dumps constituting two chronosequences of rehabilitation ages were investigated. These chronosequences were each managed by a different mining company and were aged 1 to 11 years (chronosequence A) and 6 to 17 years (chronosequence B), respectively. All sites constituting a chronosequence were managed identically but chronosequence A was under more intensive management than chronosequence B. The more intensive management entailed annual amelioration of sites combined with defoliation (cutting and baling which was replaced by grazing). The mining company managing sites of chronosequence B applied grazing to sites but no amelioration after the initial revegetation of the sites. To determine the extent to which different management practices favoured microbial development, the relationship between the age of the rehabilitated soil covers of the two chronosequences and their microbial community function (enzymatic activities) and structure (PLFAs) was studied.

1.1. Microbial Community Function and Structure in a Post-Mining Chronosequence

The first part of the study investigated the temporal changes in microbial community function and structure in chronosequence A (aged 1 to 11 years) obtained by sampling seven coal discard sites in 2002, 2004, and 2005. The objective was to ascertain the relationship between rehabilitation age and microbial properties and to determine whether microbial communities could be applied to indicate the progress of rehabilitation.

The most important observation made during the investigation of the microbial communities in the different aged soil covers of the rehabilitated sites, was that there was no relationship between rehabilitation age and microbial activity or abundance of certain microbial groups. What was responsible for a clear differentiation between sites was the management practices applied. The change in management (after 2002) from defoliation by cutting and baling to grazing of the sites, caused a shift in the microbial communities that was evident in the data obtained from the enzymatic assays as well as PLFA analysis.

A correlation matrix (not shown) indicated few correlations between enzymatic

activities and PLFA data. The only significant correlations ($p < 0.05$) was observed in 2002 between microbial biomass and dehydrogenase ($r = 0.79$) and microbial biomass and β -glucosidase ($r = 0.78$). The absence of a clear relationship in this study between enzymatic activities and PLFA data may be attributed to the functional redundancy of soil microorganisms (Marschner *et al.*, 2003). However, it is important to note that compositional changes in microbial communities may very well be accompanied by changes in their functional capabilities in spite of functional redundancy (Waldrop *et al.*, 2000). Therefore, functional attributes of the microbial communities in this study may be linked to community composition although a direct correlation is not evident.

A canonical correspondence analysis (CCA) (Figure 6.1) was performed to investigate the relationship between the sites, enzymatic activities (microbial community function), and PLFA groups (microbial community structure). Eigenvalues for the first two axes were 0.107 and 0.013, respectively. Total observed variance of the first two axes of the CCA was 56.2%. The cumulative percentage variance of species-environment relation was 85.0, 95.6, 99.7, and 100.0%, respectively for the four axes. The first axis was significant in explaining the variation in enzymatic activities ($p = 0.0020$), as were all four axes together ($p = 0.0020$). The first canonical axis correlated most strongly with terminally branched saturated fatty acids ($r^2 = -0.7992$) and the second axis with mid-chain branched saturated fatty acids ($r^2 = -0.4230$).

Results obtained from ordination diagrams such as this one do not indicate direct relationships or correlations between specific variables and from a traditional statistical perspective are often considered too descriptive while lacking statistical accuracy. However, results obtained from multidimensional ordination is key to facilitate the use of complex data such those obtained from measurements of microbial communities (Harris, 2003). Furthermore, these ordinations can reveal tendencies or patterns in data not shown by traditional statistical approaches. This is well illustrated by the CCA ordination diagram presented in Figure 6.1, indicating a clear separation of sites sampled in 2002 from sites sampled in 2004 and 2005 based on the combined data obtained from the characterisation of microbial community function and structure. The grouping of discard sites in this ordination indicates the influence of the change in management practices – from defoliation by means of cutting and baling to grazing, combined with the optimised application of fertilisers and organic material. Clearly, management of the sites, rather than the period of rehabilitation, had a significant impact on soil enzyme activities and the abundance of different soil microbial groups, even though there was little differentiation

between the sites based on physical and chemical soil characteristics (see Figure 3.1, Chapter 3 for the principal components analysis (PCA) ordination diagram).

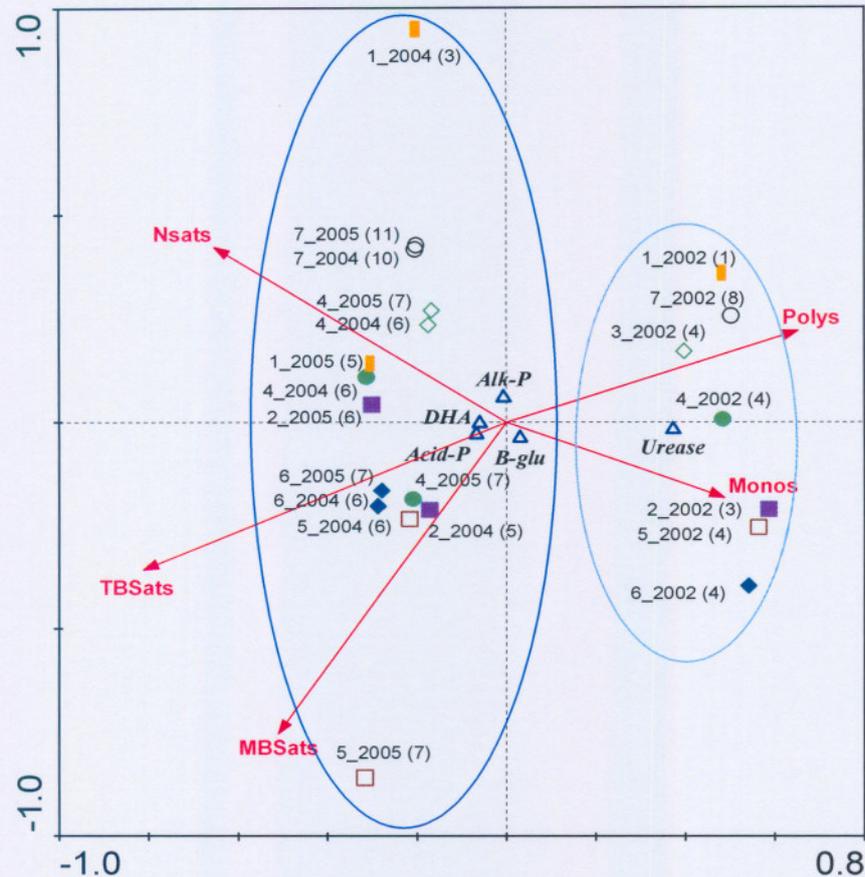


Figure 6.1. Canonical correspondence analysis (CCA) diagram illustrating the relationship between the coal discard sites based on enzymatic activities and phospholipid fatty acid (PLFA) groups. Eigenvalues for the first two axes were 0.107 and 0.013, respectively. Total observed variance of the first two axes of the CCA was 56.2%. Each site is indicated according to the name of the site, followed by the time of sampling and the rehabilitation age in brackets, e.g. Site 1 sampled in 2002 was 1 year old: 1_2002 (1). Symbols represent the same site at different sampling times (2002, 2004 and 2005). Key to abbreviations: DHA: dehydrogenase; B-glu: β -glucosidase; Acid-P: acid phosphatase; Alk-P: alkaline phosphatase; Nsats: normal saturated fatty acids; TBSats: terminally branched saturated fatty acids; MBSats: mid-chain branched saturated fatty acids; Polys: polyunsaturated fatty acids; Monos: monounsaturated fatty acids.

In 2002, sites were associated with polyunsaturated fatty acids (fungi), monounsaturated fatty acids (Gram-negative bacteria), and urease activity. A shift in the microbial community occurred after 2002, and in 2004 and 2005 sites showed a relationship to normal saturated fatty acids (a general microbial biomarker), terminally branched saturated fatty acids (Gram-positive bacteria), mid-chain branched saturated fatty acids (Actinomycetes), dehydrogenase, β -glucosidase, and acid and alkaline phosphatase

activities. Both Gram-positives and Actinomycetes are able to function in environments with limited nutrient resources (Olsson, 1999; Waldrop *et al.*, 2000) and the shift towards these microbial groups is probably related to the reduced applications of fertiliser and organic material over the rehabilitation period (refer to Chapter 2).

1.2. The Assessment of Reference Sites

For a comprehensive discussion on the sampling and availability of the reference sites referred to in this section, see Chapter 2. In brief, the nature and location of the study sites made the continuous use of reference sites during this study impractical. Comparison with rehabilitated sites was complicated by the unavailability of natural, undisturbed areas to use as reference sites. The results obtained from a once-off sampling and characterisation of the microbial community function and structure at these reference sites (Table 6.1) are briefly discussed here. However, these results were only obtained at one point in time and do not represent a chronosequence or changes over time.

Table 6.1. Enzymatic activities, phospholipid fatty acid (PLFA) composition, and PLFA ratios of soil samples obtained from reference sites in 2002.

	Reference 1	Reference 2	Reference 3
Enzymatic activities			
Dehydrogenase ($\mu\text{g INF g}^{-1} 2\text{h}^{-1}$)	482.38 (143.22)a	337.50 (60.27)a	302.62 (62.95)a
β -Glucosidase ($\mu\text{g PNP g}^{-1} \text{h}^{-1}$)	356.21 (79.41)a	433.03 (153.60)a	262.386 (84.21)a
Alkaline Phosphatase ($\mu\text{g PNP g}^{-1} \text{h}^{-1}$)	718.684 (248.11)a	675.312 (146.89)a	564.435 (323.36)a
Acid Phosphatase ($\mu\text{g PNP g}^{-1} \text{h}^{-1}$)	1294.30 (680.54)a	1239.76 (95.82)a	1358.86 (30.54)a
Urease ($\mu\text{g NH}_4\text{-N g}^{-1} 2\text{h}^{-1}$)	30.62 (5.50)a	20.36 (7.33)a	16.25 (1.09)a
PLFA Groups			
Normal saturated	31.56 (2.37)m	30.80 (5.03)m	39.38 (3.41)m
Mid-chain branched saturated	3.72 (0.29)m	2.56 (0.65)m	1.20 (0.14)m
Terminally branched saturated	33.54 (1.30)m	33.90 (1.25)m	33.17 (0.98)m
Branched monounsaturated	0.00 (0.00)m	0.00 (0.00)m	0.00 (0.00)m
Monounsaturated	26.08 (2.23)m	27.95 (3.99)m	21.64 (2.45)m
Polyunsaturated	3.98 (0.55)m	3.23 (0.56)m	3.08 (0.16)m
Viable microbial biomass (pmol g^{-1} dry weight)	49676.68 (31515.69)n	39854.20 (29537.01)mn	29503.79 (8519.65)mn
Ratios			
Fungal / Bacterial ¹	0.08 (0.04)n	0.04 (0.03)m	0.03 (0.00)m
Saturated / Unsaturated ²	2.41 (0.46)op	2.07 (0.36)nop	3.20 (0.82)p
cy17:0 / 16:1 ω 7c	0.99 (0.17)m	0.96 (0.03)m	3.03 (1.80)m
cy19:0 / 18:1 ω 7c	1.71 (1.26)m	1.20 (1.60)m	5.51 (1.60)m
16:1 ω 7t / 16:1 ω 7c	0.10 (0.02)m	0.09 (0.03)mn	1.03 (0.14)n
18:1 ω 7t / 18:1 ω 7c	0.17 (0.02)m	0.08 (0.01)m	0.29 (0.23)m

Standard error values are indicated in brackets. Data in rows with the same letters indicate no significant difference, while those with different letters indicate significant difference at $p < 0.05$ (Tukey's HSD).

¹ Fungi: 18:2 ω 6 (Federle, 1986; Frostegård and Bååth, 1996), Bacteria: sum of i15:0, a15:0, 15:0, i16:0, 16:1 ω 9, 16:1 ω 7t, i17:0, a17:0, 17:0, cy17:0, 18:1 ω 7, and cy19:0 (Frostegård and Bååth, 1996).

² Normal saturated to monounsaturated PLFA (McKinley *et al.*, 2005).

Enzymatic activities for alkaline and acid phosphatases assayed for the reference sites were in the same range as those sampled for the coal discard sites from both chronosequences (see Chapters 3 and 5). Dehydrogenase and β -glucosidase activities were slightly higher in the reference sites than in the rehabilitated sites from both chronosequences. Urease activity was higher in the rehabilitated sites from chronosequence A than in the reference sites. The urease activities obtained for the discard sites from chronosequence B, however, were similar to those of the reference sites.

Similar mole percentage (mol%) fractions of the major PLFA groups were obtained for the reference sites and discard sites from both chronosequences (see Chapters 4 and 5). Ratios of PLFA stress signatures indicated stress in microbial communities in reference and rehabilitated sites. The reference sites had higher concentrations of microbial biomass and lower fungal to bacterial (F:B) ratios than the discard sites. The lower abundance of fungi in the reference sites are in contrast with observations by other authors which has shown an increase in the relative abundance of fungi with reduced disturbance and in soils returning to a more natural state (Bardgett and McAlister, 1999; Stahl *et al.*, 1999; Zeller *et al.*, 2001; Peacock *et al.*, 2002). However, it should be kept in mind that these reference sites, although not directly impacted by mining activities were not representative of natural, undisturbed soils. It is likely that the influence of agricultural activities at these sites may have caused similar effects on microbial communities than management practices on the rehabilitated sites. It would be advisable to investigate the influence of specific management practices on microbial communities in depth and to make direct comparisons between different types of management regimes.

1.3. The Comparison of Two Chronosequences

A comparison of two chronosequences of rehabilitated coal discard sites was achieved by an application of the 'space-for-time' (SFT) hypothesis. Sites of different ages and at separate locations ('space') were identified to obtain a chronosequence of ages ('time') (Sparling *et al.*, 2003). The two chronosequences included sites aged 1 to 11 years and 6 to 17 years, respectively. Sites in the same chronosequence were managed identically, while there was a distinct difference in management regimes applied to each chronosequence. The long-term effect of the different management regimes on the soil microbial community function and structure was investigated. Minimum and maximum values of microbial activity and abundance obtained from individual sites of chronosequences A and B differed

for some properties but were within comparable ranges (Table 6.2). Fluctuations of selected microbial properties occurred in both chronosequences and similar temporal trends existed over the rehabilitation periods (Chapter 5). However, there was less fluctuation in chronosequence B over the rehabilitation period than in chronosequence A. It was therefore concluded that the microbial communities in the less managed sites maintained their functional and structural integrity within bounds in the absence of management inputs or disturbance. While there was similarity in the trends over time for individual microbial community measurements, the seemingly more stable conditions in chronosequence B is important in terms of the goal of rehabilitation.

Table 6.2. Minimum and maximum values for enzymatic activities, phospholipid fatty acid (PLFA) composition, and PLFA ratios of obtained from individual sites of chronosequences A and B over the study period (2002 – 2005).

	Chronosequence A	Chronosequence B
Enzymatic activities		
Dehydrogenase ($\mu\text{g INF g}^{-1} 2\text{h}^{-1}$)	24.34 – 339.47	47.29 – 121.85
β -Glucosidase ($\mu\text{g PNP g}^{-1} \text{h}^{-1}$)	59.72 – 496.81	84.53 – 319.54
Alkaline Phosphatase ($\mu\text{g PNP g}^{-1} \text{h}^{-1}$)	211.83 – 1308.86	241.51 – 1168.27
Acid Phosphatase ($\mu\text{g PNP g}^{-1} \text{h}^{-1}$)	343.38 – 1260.05	773.26 – 1277.70
Urease ($\mu\text{g NH}_4\text{-N g}^{-1} 2\text{h}^{-1}$)	14.12 – 73.16	15.26 – 60.96
PLFA Groups		
Normal saturated	20.46 – 49.22	33.10 – 41.83
Mid-chain branched saturated	2.11 – 4.96	2.39 – 4.13
Terminally branched saturated	18.13 – 30.26	15.97 – 31.36
Monounsaturated	18.27 – 41.82	22.46 – 40.20
Polyunsaturated	5.20 – 17.05	0.79 – 7.98
Viable microbial biomass ($\text{pmol g}^{-1} \text{dry weight}$)	680.04 – 29850.56	4247.11 – 16318.15
Ratios		
Fungal / Bacterial	0.07 – 0.32	0.02 – 0.23
Saturated / Unsaturated	1.00 – 4.82	1.22 – 3.14
Gram (+) / Total PLFA	0.15 – 1.88	0.12 – 0.30
Iso / Anteiso	0.19 – 2.11	0.00 – 7.26
16:1 ω 7t / 16:1 ω 7c	0.00 – 0.13	0.00 – 0.03
18:1 ω 7t / 18:1 ω 7c	0.00 – 2.42	0.00 – 0.06

The importance of considering the sum of microbial measurements in the context of any investigation of this nature has to be stressed. Here, multidimensional ordinations and other statistical approaches, such as neural network analysis, are of great value in facilitating the interpretation of microbial community measurements (Harris, 2003). While similar trends in individual microbial community properties may give the impression that different management practices give the same result, an ordination diagram that accounts for functional and structural measurements as well as sites from both chronosequences, indicated a distinction based on management regimes (Figure 5.8, Chapter 5). With regard

to the effect of management on microbial community function and structure and the attainment of ecosystem stability, the partiality in these two case studies was towards the less intensively managed chronosequence (B). The key here is that this chronosequence was the one to which a deeper soil cover was applied at the start of rehabilitation. It would seem that an ameliorated soil cover of sufficient depth applied at the start of rehabilitation might make the need for further amelioration unnecessary. Conversely, if the application of a soil cover is not done properly, management inputs during rehabilitation might not lead to a stable ecosystem. It is thus of the essence that mining companies will make management decisions based on more comprehensive monitoring information.

2. CONCLUDING OBSERVATIONS

“Any strategy intended to evaluate reclamation success of disturbed lands requires criteria for judging restoration progress. These criteria should reflect ecosystem viability and long-term stability.” (Mummey *et al.*, 2002a).

Even with the application of microbial community properties, which meet the criteria required for indicators of soil quality, assessing the sustainability of the soil environment has been problematic and indices in this regard is still lacking. This is especially true for post-mining landscapes and is complicated by the myriad of different and often unique environments that need to be rehabilitated and/or monitored. According to Mummey *et al.* (2002b), a complete understanding of the regulation of soil microbial community abundance and functional relationships in rehabilitated ecosystems will ultimately require analysis at a multitude of spatial and temporal scales. Even in terms of natural soils, it is difficult to answer the question of which criteria have to be met in order for soil to be considered of good quality. Some authors are of the opinion that the question cannot be answered objectively due to *“the lack of common understanding of what soil community is to be expected in ‘normal’, undisturbed soils”* (De Ruiter, 2004). In addition to the need for indicators, land managers and researchers are faced with the fact that many ecological dynamics occur over time-scales that are well beyond the duration of conventional experiments or observations. Yet, major research items are derived from the observation of fluctuations in numbers and activities in space and time (Eijsackers, 2004). Thus, in order to overcome this limitation and obtain useful information on the mechanisms behind natural dynamics in anthropogenic gradients, the limitations associated with approaches such as SFT substitution, have to be overlooked (Fukami and Wardle, 2005).

During this investigation, the application of microbial community measurements in a manner that explored temporal trends in artificial chronosequences, proved successful in revealing dominant trends as well as minimum and maximum values in rehabilitated coal discard sites of different ages. The tendencies observed in these data illustrated the prominent influence of management practices on rehabilitated sites while at the same time indicating no correlations between microbial community properties and rehabilitation age. These observations unambiguously show the potential application value of microbial communities to assess rehabilitation. Nevertheless, at this point in time such applications still depend on the use of threshold values and long-term monitoring. To moderate the requirement for long-term monitoring, more case studies are needed to compare management effects and to determine threshold values. Typically, threshold values for any indicator of rehabilitation success are determined in comparison with nearby undisturbed sites that are biologically stable. However, the pre-disturbance condition as a benchmark for evaluation of reclamation success of drastically disturbed ecosystems is questionable because many attributes of the pre-disturbance condition may be unattainable (Mummey *et al.*, 2002a). *Threshold* implies levels beyond which a system undergoes significant negative change and according to Schjonning (2005), focus should be on *thresholds* rather than on *references, baselines, or benchmarks*, often employed in the literature on soil quality indicators because thresholds have an intimate association with resilience. Thus, while it is acknowledged that some benchmark for rehabilitation is necessary, it is important that minimum and maximum values obtained from case studies of rehabilitated environments also be taken into consideration in order to determine thresholds. These thresholds will help to indicate the stability, resilience, and resistance of a system over time.

The observations made during this investigation emphasises the importance of evaluating and monitoring rehabilitated ecosystems even in the absence of reference soils or specific indices. It is of the essence that practical applications for microbial measurements be implemented in rehabilitation monitoring programmes without the primary goal being the search for an index of soil quality. These monitoring programmes should employ an integrated approach because land managers and policy makers perceive soil only at the highest level of integration – the generic responses of the soil system (Eijsackers, 2004). There is a need for a change in focus towards the effects of management. While management cannot be addressed without evaluating certain attributes of the rehabilitated soil ecosystem, there must be a shift from assessing soil quality to

managing soil quality. While many investigations have been conducted to assess soil environments, there has been little practical implementation of soil assessments to assist management decisions. A combination of the knowledge on soil functions and properties that include thresholds, with that derived from case studies on the effects of specific management practices may lead to *management thresholds*. In other words, what management inputs are required to obtain stability in a rehabilitated site. As an example, regard soil acidity. Soil pH is a soil quality indicator for which a threshold can be established, while the rate of liming (e.g. kg CaCO₃ ha⁻¹ year⁻¹) required to maintain the pH at some prescribed level represents the management threshold (Schjonning, 2005).

3. CLOSING REMARKS AND RECOMMENDATIONS

This study is a step in the right direction in terms of assessing or monitoring the progress of rehabilitation on post-mining sites. However, much research is still needed to find applicable measures for rehabilitation success. Significant contributions made by this investigation include the establishment of minimum and maximum values of microbial activity and abundance from two case studies of rehabilitated coal discard sites (Table 6.2). Contrary to expectation, the rehabilitation ages of the sites did not show any relationship with changes in microbial properties. Furthermore, the considerable impact of management practices on microbial community function and structure was illustrated. This shows that when assessing the progress of rehabilitation on mining disturbed soils, microbial community function and structure can reveal effects of management that may not be apparent through other methods of evaluation. It also shows that microorganisms in environments will distribute along gradients of relevant parameters. In other words, if the effect of management practices was the dominant factor guiding the development of the microbial community, instead of rehabilitation age, then management will cause the distribution gradient. Identifying these gradients of relevant parameters is of the utmost importance if environments under rehabilitation are to be understood and managed effectively.

Further study is required to establish assessment criteria as a practical tool that will be applicable for land managers in mining environments and that will assist in decision-making and implementation of corrective inputs. Recommendations for future investigations include the following:

- Specific management practices with recorded details of quantifiable management inputs have to be compared in terms of the effect of the different management practices on the rehabilitated ecosystems. These management practices include the depth of the applied soil cover layer; the manner in which the soil is ameliorated; the grass seed mixture used; if sites are defoliated and by what methods; and the intervals at which fertilisers are applied.
- Investigations should be conducted on comparable sites with different soil cover depths for which a replicate site is available; for example, two or three rehabilitated sites with a thick soil cover should be compared to two or three sites with a thin soil cover.
- More case studies of management regimes have to be evaluated and information should be made available to the managing companies in order for assessments to contribute knowledge to practice.
- Threshold or baseline values from case studies of rehabilitated sites have to be established and the information provided by these values have to be combined with benchmark values for natural areas in order to obtain a realistic reference for rehabilitation.
- Investigations will have to account for more environmental factors that could influence microbial communities and vegetation such as rainfall and temperature.
- Different types of post-mining sites, such as areas disturbed by gold or platinum mining, should be investigated to determine whether rehabilitation practices on these sites are comparable.

REFERENCES

- Bardgett, R.D., McAlister, E. 1999. The measurement of soil fungal:bacterial biomass ratios as an indicator of ecosystem self-regulation in temperate meadow grasslands. *Biol Fertil Soils* 29:282-290.
- De Ruiter, P.C. 2004. Balance and stability in vital soils. In: Doelman, P., Eijsackers, H., editors. *Vital soil: function, value, and properties. Developments in soil science, volume 29*. Amsterdam: Elsevier. pp 197-214.
- Eijsackers, H. 2004. Leading concepts towards vital soil. In: Doelman, P., Eijsackers, H., editors. *Vital soil: function, value, and properties. Developments in soil science, volume 29*. Amsterdam: Elsevier. pp 1-20.
- Federle, T.W. 1986. Microbial distribution in soil – new techniques. In: Megusar, F., Gantar, M., editors. *Perspectives in microbial ecology*. Ljubljana: Slovene Society of Microbiology. pp 493-498.
- Frostegård, A., Bååth, E. 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol Fertil Soils* 22:59-65.
- Fukami, T., Wardle, D.A. 2005. Long-term ecological dynamics: reciprocal insights from natural and anthropogenic gradients. *Proc R Soc B Biol Sci* 1577:2105-2115.
- Harris, J.A. 2003. Measurements of the soil microbial community for estimating the success of restoration. *Eur J Soil Sci* 54:801-808.
- Marschner, P., Kandeler, E., Marschner, B. 2003. Structure and function of the soil microbial community in a long-term fertilizer experiment. *Soil Biol Biochem* 35:453-461.
- McKinley, V.L., Peacock, A.D., White, D.C. 2005. Microbial community PLFA and PHB responses to ecosystem restoration in tallgrass prairie soils. *Soil Biol Biochem* 37:1946-1958.
- Mummey, D.L., Stahl, P.D., Buyer, J.S. 2002a. Microbial biomarkers as an indicator of ecosystem recovery following surface mine reclamation. *Appl Soil Ecol* 21:251-259.
- Mummey, D.L., Stahl, P.D., Buyer, J.S. 2002b. Soil microbiological properties 20 years after surface mine reclamation: spatial analysis of reclaimed and undisturbed sites. *Soil Biol Biochem* 34:1717-1725.
- Olsson, P.A. 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. *FEMS Microbiol Ecol* 29:303-310.
- Peacock, A.D., Macnaughton, S.J., Cantu, J.M., Dale, V.H., White, D.C. 2002. Soil microbial biomass and community composition along an anthropogenic disturbance gradient within a long-leaf pine habitat. *Ecol Indic* 12:1-9.
- Schjonning, P. 2005. The soil quality concept as a tool for exposing values in science and promoting sustainability considerations. [cited March 2007]; Available from <http://orgprints.org/00003357>.
- Sparling, G., Ross, D., Trustrum, N., Arnold, G., West, A., Speir, T., Schipper, L. 2003. Recovery of topsoil characteristics after landslip erosion in dry hill country of New Zealand, and a test of the space-for-time hypothesis. *Soil Biol Biochem* 35:1575-1586.
- Stahl, P.D., Parkin, T.B., Christensen, M. 1999. Fungal presence in paired cultivated and uncultivated soils in central Iowa, USA. *Biol Fertil Soils* 29:92-97.
- Waldrop, M.P., Balser, T.C., Firestone, M.K. 2000. Linking microbial community composition to function in a tropical soil. *Soil Biol Biochem* 32:1837-1846.
- Zeller, V., Bardgett, R.D., Tappeiner, U. 2001. Site and management effects on soil microbial properties of subalpine meadows: A study of land abandonment along a north-south gradient in the European Alps. *Soil Biol Biochem* 33:639-649.

APPENDIX A

MICROBIAL COMMUNITY FUNCTION AND STRUCTURE ON COAL MINE DISCARD UNDER REHABILITATION

Published in *South African Journal of Plant and Soil* 23(2):105-112 (2006).

Microbial community function and structure on coal mine discard under rehabilitation

S. Claassens*, K.J. Riedel, L. Van Rensburg, J.J. Bezuidenhout and P.J. Jansen van Rensburg

School of Environmental Sciences and Development, North-West University, Potchefstroom Campus, Private Bag X6001, Potchefstroom, 2520, South Africa.

Accepted 29 August 2005

The rehabilitation of coal mine discard is problematic, especially since the lack of measures to evaluate the success of rehabilitation in terms of the self-sustainability of soil ecosystems established on these sites. In this study, the potential for using microbial community function and structure as such a measure was investigated using enzymatic assays (dehydrogenase, β -glucosidase, urease and acid and alkaline phosphatase) and signature lipid biomarkers (PLFAs). Samples from seven coal discard sites in South Africa currently under rehabilitation, were investigated and the relationship between soil properties, vegetation cover and microbial properties analysed using PCA and RDA ordination techniques. Although the discard sites had different rehabilitation ages (one to eight years), no statistically significant differences existed between these sites based on physical and chemical characteristics. Differentiation was possible based on enzymatic activities and PLFA profiles. Sites with relatively higher vegetation cover and organic carbon content had a positive association with enzymatic activities and microbial biomass. Organic carbon content correlated significantly with β -glucosidase ($r = 0.80$, $P < 0.05$), urease ($r = 0.96$, $P < 0.05$), acid phosphatase ($r = 0.76$, $P < 0.05$), dehydrogenase ($r = 0.69$, $P < 0.10$) and microbial biomass ($r = 0.73$, $P < 0.10$). The characterisation of microbial community function and structure holds potential for evaluating rehabilitation progress on mine discard sites.

Keywords: Coal discard, Enzymatic activity, Microbial community, PLFA, Rehabilitation

*To whom correspondence should be addressed (E-mail: mkbsc@puk.ac.za)

Introduction

South African legislation, such as the Environment Conservation Act (73/1989) (Anonymous, 1989) and the Minerals Act (50/1991) (Anonymous, 1991), requires developers to rehabilitate damaged environments. One of the more critical aspects of the rehabilitation process is the improvement of mine discard material to sustain plant growth by the creation of a suitable growth medium. A control measure frequently applied for the containment of coal discard material is the application of a soil cover layer, which assists in the prevention of further sulphur oxidation and facilitates the subsequent revegetation of the site. In spite of the annual addition of fertilisers, establishment of self-sustaining vegetation on most mine waste sites is problematic.

The characterisation of soil quality requires the selection of properties most sensitive to management practices and environmental stress. Microbial communities are critical to soil function (Mummey, Stahl & Buyer, 2002) and are responsive to small changes due to soil ecological stress or restoration processes (Badiane *et al.*, 2002). Assessment of soil microbial properties and microbial diversity can therefore be used as sensitive indicators of both short and long-term changes (Hill *et al.*, 2000) that occur in the soil environment. Studies in agricultural environments have shown that enzymatic activities give an indication of the diversity of functions that can be assumed by the microbial community (Bandick & Dick, 1999; Aon & Colaneri, 2001; Brohon, Delolme & Gourdon, 2001). The use of a culture-independent method (Hill *et al.*, 2000), such as phospholipid fatty acid (PLFA) analysis circumvents many of the problems frequently associated with conventional culture dependent techniques and provides a more comprehensive view of the structure of complex microbial communities (Ibekwe & Kennedy, 1998; Pinkart, Deveraux & Chapman, 1998). Recent studies suggest that

PLFA profiles could be used as 'fingerprints' indicative of successful restoration of soil communities and as indicators of responses to management practices and changes in soil quality, and that changes in PLFA profiles could reflect past and present management practices (Ponder & Tadros, 2002; Steenwerth *et al.*, 2003).

This study was undertaken to determine whether microbial enumeration techniques, enzymatic assays, and PLFA analysis of microbial community structure could differentiate between coal discard sites of varying rehabilitation ages.

Material and methods

Site details

The study was conducted on seven vegetated coal discard sites under rehabilitation and managed by Ingwe Mine Closure Operations, Ingwe Mines, South Africa (site identities are presented in Table 1). The soil used as cover was excavated from adjacent borrow pits or stripped from the sites before mining. All the coal discard sites were vegetated with a grass seed mixture mostly dominated by the following commercially available grasses: annual teff [*Eragrostis tef* (Zuccagni) Trotter]; weeping lovegrass [*Eragrostis curvula* (Schrader) Nees]; rhodesgrass (*Chloris gayana* Kunth); common fingergrass (*Digitaria eriantha*); bermudagrass [*Cynodon dactylon* (L.) Pers. var. *dactylon*]; and Kikuyu grass [*Pennisetum clandestinum* Chiov]. As a management practice, all sites are defoliated at the end of each growing season. Amelioration of soil covers was conducted according to lime requirements, fertiliser recommendations, and organic carbon content, based on the results of soil physical and chemical analyses obtained by standard procedures (Van Rensburg *et al.*, 1998). Macro-elements (Ca, Mg and K) were always added as nitrates and phosphorus was supplemented using

super-phosphate. Sites were annually treated with variable amounts of lime, inorganic fertilisers, and well-cured manure so that conditions would be optimal for vegetation growth after amelioration. The average application rates per site over

a four-year period are indicated in Table 2. All areas were located within the grassland biome of South Africa and receive an average annual rainfall between 700-800 mm.

Table 1 Physical and chemical properties of the topsoil covers obtained from the seven coal discard sites.

PROPERTIES	SITES						
	Newcastle	Ermelo	Hendrina	Bethal	Witbank 1	Witbank 2	Ogies
Grid Reference	27°51'14"S 29°57'23"E	26°29'41"S 29°45'34"E	26°20'4"S 29°53'19"E	26°10'8"S 29°21'2"E	25°53'42"S 29°10'27"E	25°54'35"S 29°11'21"E	26°01'22"S 29°06'06"E
Rehabilitation age (years)	8	3	4	1	4	4	4
Chemical Properties							
Ca (mg kg ⁻¹)	49.30 ± 13.04	7.36 ± 0.27	3.73 ± 1.68	25.10 ± 9.72	2.92 ± 0.53	3.13 ± 0.27	5.65 ± 1.29
Mg (mg kg ⁻¹)	15.77 ± 3.40	2.08 ± 0.22	1.47 ± 0.64	7.89 ± 4.41	0.92 ± 0.18	0.98 ± 0.16	1.22 ± 0.49
K (mg kg ⁻¹)	6.29 ± 1.71	5.31 ± 0.78	25.08 ± 8.32	5.90 ± 1.96	4.92 ± 1.26	12.59 ± 6.51	24.59 ± 9.31
Na (mg kg ⁻¹)	2.08 ± 0.30	1.85 ± 0.12	1.21 ± 0.44	1.56 ± 0.44	0.93 ± 0.12	0.87 ± 0.27	1.21 ± 0.17
SO ₄ (mg kg ⁻¹)	239.00 ± 72.57	28.27 ± 2.17	13.77 ± 4.72	122.04 ± 71.69	25.62 ± 12.21	26.34 ± 6.06	32.62 ± 15.90
NO ₃ (mg kg ⁻¹)	2.45 ± 1.23	6.38 ± 1.30	16.19 ± 6.59	6.38 ± 5.15	1.31 ± 0.16	6.05 ± 3.50	11.45 ± 7.31
NH ₄ (mg kg ⁻¹)	0.39 ± 0.07	0.26 ± 0.07	0.73 ± 0.23	0.30 ± 0.04	0.43 ± 0.09	0.64 ± 0.07	0.47 ± 0.21
Cl (mg kg ⁻¹)	2.59 ± 0.18	12.75 ± 5.20	5.71 ± 1.96	5.35 ± 0.71	1.69 ± 0.32	4.01 ± 1.35	3.03 ± 1.03
Fe (mg kg ⁻¹)	33.44 ± 18.89	81.48 ± 16.06	5659.82 ± 2273.28	105.93 ± 89.03	5937.56 ± 2048.50	11956.04 ± 743.35	2126.28 ± 878.29
Mn (mg kg ⁻¹)	837.25 ± 480.20	16.45 ± 15.02	47.96 ± 14.59	5.25 ± 5.05	19.90 ± 5.57	75.32 ± 6.21	29.99 ± 6.24
Cu (mg kg ⁻¹)	0.96 ± 0.16	3.99 ± 1.03	31.29 ± 4.05	6.86 ± 1.94	8.14 ± 2.10	19.63 ± 8.16	8.62 ± 5.44
Zn (mg kg ⁻¹)	16.94 ± 9.69	6.74 ± 6.50	19.08 ± 4.42	5.59 ± 2.77	4.28 ± 3.79	21.88 ± 6.96	17.27 ± 0.57
B (mg kg ⁻¹)	35.36 ± 15.28	2.72 ± 0.84	0.00 ± 0.00	32.64 ± 16.32	130.54 ± 64.76	89.75 ± 63.38	127.83 ± 67.34
P (mg kg ⁻¹)	18.97 ± 7.53	5.60 ± 0.84	146.43 ± 65.07	18.23 ± 9.75	27.37 ± 7.27	38.13 ± 16.77	96.77 ± 17.27
pH (H ₂ O)	5.51 ± 0.27	6.20 ± 0.26	7.03 ± 0.03	7.41 ± 0.44	7.15 ± 0.50	6.24 ± 0.41	6.90 ± 0.37
Electrical Properties							
Conductivity (mS m ⁻¹)	117.33 ± 46.25	21.67 ± 4.10	29.00 ± 6.27	37.00 ± 4.36	26.33 ± 2.03	18.67 ± 6.23	35.00 ± 8.02
Organic Properties							
Carbon (%)	0.69 ± 0.14	0.52 ± 0.10	2.20 ± 0.74	0.31 ± 0.16	0.32 ± 0.08	0.67 ± 0.01	2.72 ± 1.36
Cation Exchange Capacity							
cmol(+).kg ⁻¹	24.34 ± 1.60	17.55 ± 1.92	9.34 ± 1.56	7.06 ± 1.77	4.19 ± 0.61	4.90 ± 1.01	9.92 ± 3.95
S-value cmol(+).kg ⁻¹	13.12 ± 0.17	12.80 ± 3.59	9.58 ± 2.81	8.46 ± 3.43	4.18 ± 1.07	3.23 ± 1.20	95.17 ± 14.20
Base Saturation (%)	54.33 ± 3.07	70.13 ± 12.70	98.94 ± 15.35	112.29 ± 17.54	96.49 ± 13.94	61.28 ± 13.34	6.90 ± 0.37
Lime requirement (t.ha ⁻¹)	3.55 ± 2.21	8.05 ± 0.19	0.00 ± 0.00	0.00 ± 0.00	0.62 ± 0.17	1.62 ± 1.04	0.67 ± 0.30
Soil Water Content (%)							
	6.61 ± 0.35	9.87 ± 2.13	6.84 ± 0.57	4.35 ± 0.27	1.68 ± 0.04	2.59 ± 0.29	3.02 ± 0.75
Physical Properties							
Sand (%)	79.22 ± 5.53	39.80 ± 2.62	68.18 ± 2.53	58.70 ± 1.00	61.23 ± 1.62	68.12 ± 2.61	67.46 ± 0.91
Silt (%)	8.46 ± 1.96	42.66 ± 1.76	23.42 ± 2.84	34.27 ± 1.54	26.27 ± 2.61	20.93 ± 2.92	25.25 ± 2.83
Clay (%)	12.31 ± 3.81	17.55 ± 0.86	8.39 ± 0.51	7.02 ± 1.63	12.51 ± 1.10	10.95 ± 1.75	7.29 ± 2.26
Biological Properties							
Ground Cover (%)	77.42 ± 17.71 ^{abc}	63.21 ± 23.72 ^a	71.25 ± 19.10 ^{cd}	41.57 ± 15.67 ^{ab}	28.92 ± 12.30 ^{ab}	86.75 ± 16.59 ^d	60.91 ± 14.97 ^{bcd}
Crown Cover (%)	56.29 ± 19.85 ^b	38.75 ± 12.36 ^b	80.13 ± 12.95 ^b	45.71 ± 15.97 ^a	42.50 ± 22.11 ^a	84.83 ± 14.27 ^b	65.91 ± 19.98 ^{ab}

^aAll values ± SEM represents the results obtained from three independent samples (n=3) at a sampling depth of 0-10 cm.

^bSites with the same combination of superscript alphabetic letters indicate no significant differences among sites.

Table 2 Average application rates (kg. ha⁻¹) of fertiliser amendments per site over a four year period.

AMENDMENT	APPLICATION RATE (kg. ha ⁻¹)
CaNO ₃	121.00 ± 17.62
MgNO ₃	55.90 ± 7.48
KNO ₃	89.10 ± 21.27
Super-phosphate	183.75 ± 29.79
Manure	6361.11 ± 2362.71
Lime	6800.00 ± 3117.69

^aAll values ± SEM.

Sampling procedure

A random sampling design was used to obtain three composite samples per site (five cores per composite sample) of the soil cover from seven coal discard sites (n = 21) (Alef & Nannipieri, 1995; Dick, Breakwell & Turco, 1996). All samples were obtained mid-summer (November) in the same quadrates used for the assessment of vegetation growth. A soil auger was used to obtain volume samples with a minimum of 1 kg of soil per sampling area. The top 0-10 cm of the soil cover layer was sampled since most of the discard sites had an effective soil cover depth of only 15 cm. Samples were obtained using aseptic techniques as described by Dick *et al.* (1996). Soil samples were placed in tightly sealed plastic bags and kept at 4°C (for enzymatic analyses) to keep them at field water content and to preserve biological properties. For lipid analyses, samples were sealed in plastic bags, frozen on site using dry ice and transported on dry ice to the laboratory, where it was stored at -86°C. Composite (consolidated) samples were mixed thoroughly to contain equal weights of individual samples (Alef & Nannipieri, 1995). Each consolidated sample was analysed for enzymatic activity within five days of sampling. Subsamples (± 200 g) of each sample were lyophilised before PLFA extraction, fractionation, and analysis.

Chemical and physical analysis

The extraction and analysis procedures for the chemical characterisation of soil samples was conducted according to standard procedures that have previously been extensively described (Van Rensburg *et al.*, 1998; Van Rensburg & Maboeta, 2004). Quantification of the particle-size distribution was conducted according to the procedures advocated by the American Society for Testing and Materials (ASTM, 1961). Soil water content was determined gravimetrically after drying soil samples at 105°C (Alef & Nannipieri, 1995). Organic carbon was determined according to the Walkley-Black procedure described by Nelson & Sommers (1982).

Estimation of vegetation cover

The ground and crown vegetation cover of all the sites were estimated in three 1 m² quadrates randomly placed over a 50 m transect. The ground cover included all living and non-living organic material on the ground surface per area and the crown cover was regarded as the canopy cover spread of all grass species over a fixed area. Both values are expressed as a

percentage per m² surface area (Van Rensburg, Maboeta & Morgenthal, 2004).

Microbial counts

Viable bacterial and fungal populations were determined by plating the dilution series onto selective media. Total heterotrophic bacteria were enumerated using cycloheximide agar, R2A, and soil extract agar (SEA). Sodium pyrophosphate (Na₂PO₄·7H₂O; pH 7.0) (0.1% m/v) solution was used as diluent for the bacteria (Frederickson & Balkwill, 1998). Soil extract agar and R2A are selective media for oligotrophic and copiotrophic heterotrophic bacteria, respectively (Taylor *et al.*, 2002). Soil extract agar was prepared specifically for each of the seven sites using soil from the relevant sites. Fungi were enumerated by plating the dilution series onto rose bengal-streptomycin (RBS) agar (Fredrickson & Balkwill, 1998). Dextrin (0.2% m/v) and dextrose (0.2% m/v) solutions were used as diluents for suspension and dilution of the soil, respectively (Alef & Nannipieri, 1995). All inoculated media were incubated at room temperature (22±3°C) for ca. 4-10 days. All microbial enumerations were carried out in duplicate. Data are reported as CFU g⁻¹ dry soil.

Soil enzymatic activities

Before analyses, consolidated soil samples were passed through a 2 mm sieve. For the determination of dehydrogenase activity, soil was kept at field water content, while air-dried samples were used for determination of β-glucosidase (EC 3.2.1.21), urease (urea amidohydrolase, EC 3.5.1.5), and acid (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2, pH 6.5) and alkaline (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, pH 11.0) phosphatase activities (Alef & Nannipieri, 1995). All analyses were carried out in triplicate.

Dehydrogenase and urease activities were assayed according to the procedure described by Alef & Nannipieri (1995). β-glucosidase as well as acid and alkaline phosphatase activities were all based on *p*-nitrophenol release after cleavage of a synthetic substrate (*p*-nitrophenyl glucoside and *p*-nitrophenyl phosphate, respectively) (Alef & Nannipieri, 1995; Dick *et al.*, 1996). Modified universal buffer pH 6.5 and pH 11.0 were used for acid and alkaline phosphomonoesterase, respectively.

Total lipid extraction, fractionation, and analysis

All glassware used for lipid analyses was washed with phosphate-free soap, rinsed five times with tap water and five times with distilled water, then air dried and heated in a muffle furnace at 450°C for a minimum of 4 hours to remove any lipid contaminants. Total lipids were extracted from 5 g lyophilised soil according to a modified Bligh and Dyer procedure (White & Ringelberg, 1998). Silicic acid column chromatography (Guckert *et al.*, 1985) was used to fractionate the total lipid extract into neutral lipids, glycolipids, and polar lipids. The polar lipid fraction was transesterified to the fatty acid methyl esters (FAMES) by mild alkaline methanolysis (Guckert *et al.*, 1985). The FAMES were analysed by capillary gas chromatography with flame ionisation detection on a Hewlett-Packard 6890 series II chromatograph fitted with a 60 m SPB-1 column (0.250 mm I.D., 0.250 m film thickness).

Definitive identification of peaks was undertaken using gas chromatography-mass spectrometry of selected samples using a Hewlett-Packard 6890 series II chromatograph interfaced with a Hewlett-Packard 5973 mass selective detector. Methyl nonadecanoate (C19:0) was used as the internal standard and the PLFAs were expressed as equivalent peak responses to the internal standard. Standard fatty acid nomenclature was used (Ibekwe & Kennedy, 1998).

Statistical analysis

All samples ($n = 21$) were analysed in triplicate. The relationship between soil physical and chemical characteristics, the microbial and the vegetation variables was investigated using Principal Components Analysis (PCA) and Redundancy Analysis (RDA) multivariate ordination techniques using Canoco (Canoco for Windows Version 4.5, GLW-CPRO ©). Principal Components Analyses were conducted on the soil physical and chemical variables, as well as on the vegetation cover and microbial properties analysed to determine how these variables were interrelated. An RDA was subsequently performed with the activities of the five enzymes assayed, the microbial counts obtained using conventional techniques, and the percentage ground and crown cover as species dependent variables and the most significant soil properties as independent environmental factors. The most significant soil physical and chemical variables were selected through the forward selection procedure provided in Canoco, thereby ensuring that only the most pertinent environmental gradients were investigated.

Results and discussion

Results from the physical and chemical characterisation of the soil samples, as well as the percentage vegetation cover are summarised in Table 1. The results obtained from the chemical analyses, showed high standard error values for most of the elements, indicating the heterogeneous nature of the topsoil layer or possible mixing of topsoil with underlying discard material. A Principal Components Analysis (PCA) (not shown) indicated that the soil used as cover varied markedly between sites based on physical and chemical characteristics, although this variation was not statistically significant. The variation observed could have a significant impact on the structural diversity of the bacteria and fungi present, as well as on the rehabilitation and establishment of a self-sustaining vegetation cover at these sites.

Grass species showed great variation in their growth form - some had a small basal (ground) cover but a large crown cover (Table 1). The dominance of tufted grasses with a higher crown cover and a lower basal cover at Bethal and Witbank 1, could be the reason for the low percentage ground vegetation cover (41.57%) observed at these sites. A degraded plant cover generally results in lower soil organic matter content. Our results correspond with observations by other authors (Aon *et al.*, 2001; Garcia *et al.*, 2002) that decreased availability of organic matter relates to lower microbial activity. Bethal was characterised by a lower percentage vegetation cover (45.71%) and related to lower enzymatic activity (Table 3) and estimated viable biomass (Table 4) compared to the other sites.

Table 3 Microbial counts and enzymatic activity of topsoil covers obtained from the seven coal discard sites.

PROPERTIES	SITES						
	Newcastle	Ermelo	Hendrina	Bethal	Witbank 1	Witbank 2	Ogies
Microbial counts (log CFU g ⁻¹ dry soil)							
Soil extract agar*	6.29 ± 6.05 ^a	5.23 ± 4.84 ^a	6.17 ± 6.08 ^{ad}	5.17 ± 4.82 ^a	6.35 ± 6.09 ^a	6.18 ± 5.89 ^a	6.86 ± 6.75 ^a
R2A*	7.03 ± 6.69 ^a	7.26 ± 7.05 ^a	7.61 ± 7.39 ^a	7.70 ± 7.34 ^a	6.67 ± 6.60 ^a	7.33 ± 7.30 ^a	6.56 ± 6.26 ^a
Cycloheximide*	6.66 ± 6.47 ^a	6.88 ± 6.45 ^a	7.70 ± 7.49 ^a	7.30 ± 7.16 ^a	8.37 ± 8.19 ^a	7.09 ± 6.72 ^a	6.63 ± 6.57 ^a
Rose Bengal-	4.49 ± 4.15 ^a	3.82 ± 3.47 ^a	4.54 ± 3.34 ^a	4.04 ± 3.27 ^a	3.13 ± 2.38 ^a	3.28 ± 2.66 ^a	3.82 ± 3.47 ^a
Streptomycin agar*							
Enzymatic activities							
Dehydrogenase*	285.92 ±	315.83 ±	578.51 ±	139.93 ±	67.53 ±	107.80 ±	316.00 ±
(g INF g ⁻¹ 2h ⁻¹)	159.45 ^a	42.63 ^{ad}	222.38 ^b	22.68 ^a	28.69 ^a	40.38 ^{ac}	237.53 ^{ad}
β-Glucosidase**	129.76 ±	99.51 ±	143.20 ±	59.72 ±	62.08 ±	88.70 ±	138.77 ±
(mg PNP g ⁻¹ h ⁻¹)	64.85 ^a	32.16 ^{ab}	41.83 ^a	25.36 ^b	47.47 ^b	12.66 ^a	63.62 ^a
Alkaline Phosphatase**	352.11 ±	453.55 ±	264.64 ±	391.20 ±	221.37 ±	223.29 ±	428.73 ±
(mg PNP g ⁻¹ h ⁻¹)	33.09 ^a	68.87 ^{ab}	55.24 ^{ac}	28.11 ^{ab}	48.07 ^{ac}	30.25 ^{ac}	313.57 ^{ab}
Acid Phosphatase**	430.06 ±	343.46 ±	569.53 ±	343.38 ±	404.60 ±	344.14 ±	687.22 ±
(mg PNP g ⁻¹ h ⁻¹)	149.32 ^a	43.71 ^a	80.40 ^{ab}	54.49 ^a	117.25 ^a	53.33 ^a	182.05 ^a
Urease**	49.20 ±	58.95 ±	58.48 ±	34.59 ±	41.90 ±	34.93 ±	66.94 ±
(g NH ₄ -N g ⁻¹ 2h ⁻¹)	1.37 ^a	25.02 ^{ab}	8.64 ^{ab}	5.03 ^{ac}	11.22 ^a	3.92 ^{ac}	15.26 ^{ab}

^aAll values ± SEM represents the results obtained from three independent samples ($n=3$) at a sampling depth of 0-10 cm.

^b*Significant at $P=0.05$ probability level

^c**Significant at $P=0.01$ probability level

^dSites with the same combination of superscript alphabetic letters indicate no significant differences among sites.

^eINF: iodinitrotetrazolium chloride-formazan; PNP: para-nitrophenol

Table 4 Percentage fractions of PLFA groups, estimated viable biomass and ratios of specific lipid biomarkers from samples taken at the different coal discard sites.

PLFA Major Group	SITES						
	Newcastle	Ermelo	Hendrina	Bethal	Witbank 1	Witbank 2	Ogies
Normal saturated	27.85 ± 0.77 ^c	22.35 ± 1.35 ^{ab}	23.44 ± 0.63 ^{bc}	20.46 ± 0.28 ^a	23.69 ± 0.57 ^{bc}	26.01 ± 1.08 ^{de}	25.13 ± 0.63 ^{cd}
Mid-chain branched saturated	2.32 ± 0.16 ^{ab}	3.08 ± 0.27 ^c	2.11 ± 0.09 ^e	2.98 ± 0.29 ^{bc}	2.59 ± 0.06 ^{abc}	2.61 ± 0.20 ^{abc}	2.46 ± 0.90 ^{abc}
Terminally branched saturated	22.57 ± 13.28 ^b	24.09 ± 0.77 ^{bc}	22.84 ± 0.25 ^b	18.13 ± 0.64 ^a	25.25 ± 0.72 ^{bc}	25.47 ± 1.68 ^{bc}	26.98 ± 1.07 ^c
Branched monoun saturated	3.24 ± 0.20 ^{bcd}	2.90 ± 0.20 ^{bc}	2.77 ± 0.08 ^b	2.06 ± 0.01 ^a	3.11 ± 0.10 ^{bcd}	3.35 ± 0.10 ^{cd}	3.47 ± 0.15 ^d
Monounsaturated	33.51 ± 2.09 ^a	35.12 ± 0.40 ^a	41.82 ± 1.11 ^c	39.32 ± 0.17 ^{bc}	35.78 ± 0.82 ^{ab}	34.09 ± 0.45 ^a	35.95 ± 1.24 ^{ab}
Polyunsaturated	10.40 ± 0.77 ^{bc}	12.05 ± 0.42 ^c	6.89 ± 0.63 ^a	17.05 ± 0.40 ^d	9.57 ± 0.65 ^{bc}	8.47 ± 0.83 ^{ab}	6.01 ± 0.85 ^a
Estimated viable biomass (pmol g ⁻¹ dry weight)	16277.30 ± 5704.01 ^{ab}	10315.14 ± 4745.73 ^a	29850.56 ± 9110.88 ^{ab}	7108.01 ± 1692.12 ^a	12043.97 ± 4417.18 ^{ab}	15368.61 ± 3357.34 ^{ab}	17719.66 ± 2875.71 ^{ab}
Ratios							
Fungal / Bacterial*	0.24 ± 0.06 ^{cd}	0.21 ± 0.10 ^{bcd}	0.12 ± 0.02 ^{abc}	0.32 ± 0.06 ^d	0.16 ± 0.04 ^{abc}	0.13 ± 0.07 ^{abc}	0.09 ± 0.02 ^{ab}
cy17:0 / 16:1ω7c	0.52 ± 0.05 ^a	0.49 ± 0.05 ^a	0.46 ± 0.04 ^a	0.50 ± 0.07 ^a	0.57 ± 0.12 ^a	0.62 ± 0.07 ^a	0.66 ± 0.24 ^a
cy19:0 / 18:1ω7c	0.44 ± 0.37 ^a	0.36 ± 0.21 ^a	0.69 ± 0.38 ^a	0.37 ± 0.13 ^a	0.72 ± 0.28 ^a	0.68 ± 0.40 ^a	0.67 ± 0.72 ^a
Saturated / Unsaturated							
16:1ω7t / 16:1ω7c	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.03 ± 0.02 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.02 ± 0.01 ^a	0.01 ± 0.02 ^a
18:1ω7t / 18:1ω7c	0.03 ± 0.04 ^a	0.01 ± 0.02 ^a	0.05 ± 0.02 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.04 ± 0.03 ^a	0.06 ± 0.01 ^a

All values ± SEM represents the results obtained from three independent samples (n=3) at a sampling depth of 0-10 cm.

^bSites with the same combination of superscript alphabetic letters indicate no significant differences among sites.

^c*Calculated according to Frostegard and Bååth (1996)

Statistical analysis of the microbial counts indicated no significant differences ($P > 0.05$) between sites based on microbial enumeration using a variety of culture media (Table 3). It is hypothesised that the abundance of microorganisms in the different sites could be classified along an r-K gradient. The r-strategists rely upon high reproductive rates for survival within a community and prevail in environments where nutrients are readily available (copiotrophic), whereas the K-strategists depend upon physiological adaptations to the environmental resources (Atlas & Bartha, 1998). When resources become scarce, r-strategists experience rapid reduction, whereas K-strategists tend to be successful in resource-limited situations (oligotrophic) (Sarathchandra *et al.*, 2001).

Enzymatic activities

The average activities of the enzymes assayed are presented in Table 3. The results indicate a lack of relationship between the type of phosphatase and soil pH. This suggests that a variation in pH (H_2O) (5.51 – 7.41) was not a critical factor governing the predominance of acid or alkaline phosphatase activity in these ecosystems. An RDA ordination diagram illustrating the association between the dominant environmental variables, microbial enzymatic activities, microbial counts, and percentage ground and crown cover is presented in Figure 1. Eigenvalues for the first two axes were 0.531 and 0.256, respectively. Total observed variance of the first two canonical axes was 78.7%. The first canonical axis correlated strongly with percentage organic carbon (%C) ($r^2 = 0.8815$)

and the second axis correlated with NH_4^+ ($r^2 = 0.7817$). According to a Monte Carlo Permutation test conducted with 499 permutations the first canonical axis was not statistically significant ($P = 0.054$). The overall effect of the chosen environmental variables on the microbial enzymatic activities, however, was statistically significant ($P = 0.0060$).

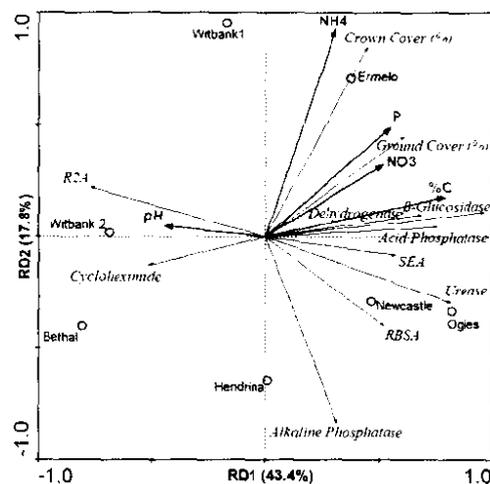


Figure 1 Redundancy Analysis (RDA) ordination diagram illustrating the relationship between the dominant environmental variables, vegetation cover, and soil microbial and enzymatic properties of the seven discard sites. The environmental physical/chemical parameters are represented by bold vectors.

Dehydrogenase activity was assayed as an estimation of overall microbial activity due to its presence in all microorganisms (Taylor *et al.*, 2002). β -glucosidase activity is related to the carbon cycle, fulfils a central role in the cycling of organic matter, is the most abundant of the three enzymes involved in cellulose degradation, and is rarely substrate limited (Turner *et al.*, 2002). Urease and phosphatase are often measured because of their importance in the nitrogen and phosphorus cycles, respectively (Aon & Colaneri, 2001). Enzymatic activities in relation to the cycling of nitrogen (ammonification, nitrification, denitrification) or phosphorus (release of inorganic phosphorus) in soil have been used to evaluate the fertility of the soil or to describe the functioning of the ecosystem (Aon & Colaneri, 2001; Brohon *et al.*, 2001).

The Hendrina, Newcastle, and Ogies sites were grouped to the right of the first ordination axis (Figure 1) and were associated with higher overall enzymatic activity and vegetation cover. This may be attributed to the higher organic carbon content (0.69%) (Table 1) present in these sites in comparison with the sites grouped to the left of the ordination diagram. The Bethal and Witbank 1 sites were characterised by lower organic carbon content (0.32%) and associated lower ground cover (41.57%). The RDA also showed these sites to have a negative/weak association with enzymatic activities. These results correspond with observations of Aon *et al.* (2001) and Garcia *et al.* (2002), which indicated a relationship between reduced aboveground plant diversity, lower soil organic matter, and decreased microbial diversity. The observation that organic carbon content had a significant correlation with β -glucosidase ($r = 0.80$, $P < 0.05$), acid phosphatase ($r = 0.96$, $P < 0.05$) and urease ($r = 0.76$, $P < 0.05$) (results not shown), emphasises the importance of organic matter as soil amendment during rehabilitation.

Phospholipid fatty acid (PLFA) analyses

The mole percentage (mol%) fractions of the major PLFA groups are summarised in Table 4. Results revealed differences in the PLFA compositions and in turn, indicated the differences in the microbial community structures present in the study sites. The estimated viable biomass ranged from 7 108.01 to 29 850.56 pmol g^{-1} dry weight (Table 4).

Frostegård & Bååth (1996) suggested the use of the sum of PLFAs considered to be predominantly of bacterial origin (i15:0, a15:0, i16:0, 16:1 ω 9, 16:1 ω 7, i17:0, a17:0, 17:0, cy 17:0, 18:1 ω 7 and cy 19:0) as an index of the bacterial biomass (BactPLFA). The quantity of 18:2 ω 6 was used as an indicator of fungal biomass (FungPLFA), since it is mainly of fungal origin (Merilä, Strömmer & Fritze, 2002). The results indicate significant differences between sites based on F:B ratios (Table 4). All the discard sites investigated showed F:B ratios of < 1.0 , with the highest ratio observed at the Bethal site, thus corresponding with the observation that this site had the highest concentration of polyunsaturated fatty acids.

Previous studies have indicated increased ratios of saturated to unsaturated fatty acids (Guckert, Hood & White, 1986), increased ratios of trans- to cis-monoenoic fatty acids (Keweloh & Heipieper, 1996; White, Stair & Ringelberg, 1996), and increased ratios of cyclopropyl fatty acids to their monoenoic precursors to be indicative of stress in natural microbial communities (Guckert *et al.*, 1986; Kieft, Ringel-

berg & White, 1994). Ratios greater than 0.1 are considered indicative of stress (White *et al.*, 1996), while non-stressed microbial communities in the exponential growth phase have ratios of less than 0.05 (Smith *et al.*, 2000). Based on these ratios, the results indicate stress in all the sites sampled.

An RDA triplot illustrating the association between the dominant environmental variables, microbial PLFA profiles, and percentage ground and crown cover is presented in Figure 2. Eigenvalues for the first two axes were 0.454 and 0.237 respectively. Total observed variance of the first two canonical axes was 69.1%. The first canonical axis correlated strongly with NH_4^+ ($r^2 = -0.6132$) and the second axis with P ($r^2 = 0.7276$). According to a Monte Carlo Permutation test conducted with 499 permutations the first canonical axis was not statistically significant ($P = 0.314$) and neither was the overall effect of the chosen environmental variables on the phospholipid fatty acid groups ($P = 0.088$).

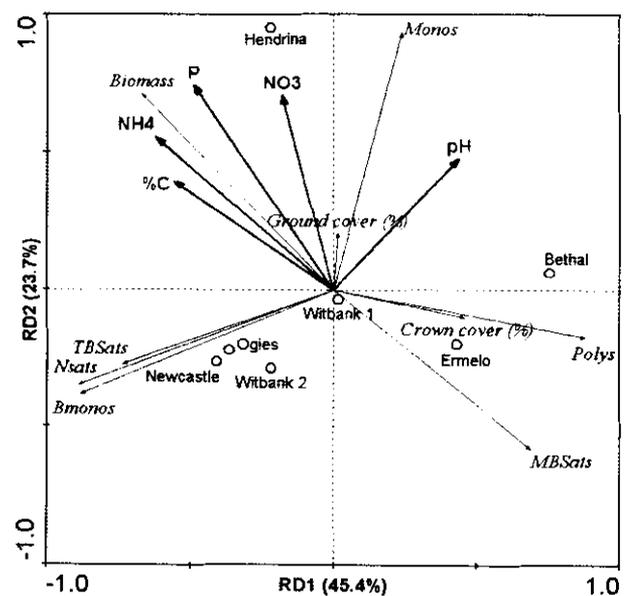


Figure 2 Redundancy Analysis (RDA) ordination diagram illustrating the relationship between the dominant environmental variables, vegetation cover, and major PLFA groups of the seven discard sites. The environmental physical/chemical parameters are represented by bold vectors. Key to major PLFA groups: Nsats (normal saturated), MBSats (mid-chain branched), TBSats (terminally branched saturated), Bmonos (branched monounsaturated), Monos (monounsaturated), Polys (polyunsaturated).

Estimated viable biomass had a significant correlation with NH_4^+ ($r = 0.83$, $P < 0.05$), P ($r = 0.90$, $P < 0.05$), NO_3 ($r = 0.74$, $P < 0.1$) and %C ($r = 0.73$, $P < 0.1$). The Hendrina site was associated with all these environmental variables and was characterised by the highest viable biomass, monounsaturated fatty acids, and high ground cover. Monounsaturated fatty acids are indicative of gram-negative bacteria (Ratledge & Wilkinson, 1988) and the RDA shows this group as the predominant contributor to the biomass abundance. The high biomass abundance in the Hendrina site could be attributed to the response of r-strategists to the presence of excessive nutrients due to the current management practices applied. It is, however, evident that this condition is not sustainable, and that the microbial community within this site would experi-

ence a significant reduction in estimated viable biomass once the resources in this site become depleted. Actinomycetes and fungi should be more able to colonise harsh environments than bacteria, due to their ability to span interstitial spaces to collect water and nutrient sources (Olsson, 1999). The Bethal and Ermelo sites are the youngest sites (one and three years, respectively) in terms rehabilitation age and the soil covers of these sites should have the least suitable growth conditions. Both these sites were associated with higher abundances of those PLFA groups indicative of fungi (polyunsaturated fatty acids) and actinomycetes (mid-chain branched saturated fatty acids) (Ponder & Tadros, 2002).

Conclusions

The relevance of the results must be understood in the context of a search for indicators of sustainable management for the successful rehabilitation and revegetation of coal discard sites. Results obtained from the multifactorial analysis of microbial properties illustrate that these properties are all interrelated and influenced by the physical and chemical characteristics of the soils studied. The lower microbial activity and biomass in the soils with degraded plant cover may result in decreased mineralisation rates and, hence, slower nutrient recycling. In the long term, these factors could result in decreased soil quality, which could adversely affect the sustainable revegetation of discard sites. It is evident that the characterisation of microbial function and structure is sufficiently sensitive to differentiate between the soil covers of various coal discard sites and could be used as assessment criteria to determine the rehabilitation status of sites associated with discard mining material. It also seems that a trend could exist between the progress of rehabilitation and r- and K- strategic microorganisms, with r-strategists favouring new sites or sites under fertiliser treatment and K-strategists favouring more stable environments. Clearly, a more detailed sampling study and in-depth analysis, including spatial and temporal monitoring, as well as appropriate molecular analysis, is needed to investigate the observations made during this study.

Acknowledgements

This research was performed with the financial support of Ingwe Mine Closure Operations and the National Research Foundation (NRF), South Africa.

References

- ALEF, K. & NANNIPIERI, P. (eds.), 1995. *Methods in Applied Soil Microbiology and Biochemistry*, Academic Press, New York.
- AMERICAN SOCIETY FOR TESTING AND MATERIALS (ASTM), 1961. Tentative method for grain-size analysis of soils. In: *The 1961 book of ASTM standards*, ASTM, Philadelphia, PA.
- ANONYMOUS., 1989. *Environment Conservation Act, No. 73, 1989*, Government Printer, Pretoria, South Africa.
- ANONYMOUS., 1991. *The Minerals Act, No. 50, 1991*, Government Printer, Pretoria, South Africa.
- AON, M.A. & COLANERI, A.C., 2001. Temporal and spatial evolution of enzymatic activities and physico-chemical properties in an agricultural soil. *Appl. Soil Ecol.* 18, 255-270.
- AON, M.A., SARENA, D.E., BURGOS, D.E. & CORTASSA, S., 2001. (Micro)biological, chemical, and physical properties of soils subjected to conventional or no-till management: an assessment of their quality status. *Soil Till. Res.* 60, 173-186.
- ATLAS, R.M. & BARTHA, R., 1998. *Microbial ecology*. 4th ed. Benjamin/Cummings Science Publishing, Menlo Park, California.
- BADIANE, N.N.Y., CHOTTE, J.L., PATE, E., MASSE, D. & ROULAND, C., 2001. Use of soil enzyme activities to monitor soil quality in natural and improved fallows in semi-arid tropical regions. *Appl. Soil Ecol.* 18, 229-238.
- BANDICK, A.K. & DICK, R.P., 1999. Field management effects on soil enzyme activities. *Soil Biol. Biochem.* 31, 1471-1479.
- BROHON, B., DELOLME, C. & GOURDON, R., 2001. Complementarity of bioassays and microbial activity measurements for the evaluation of hydrocarbon-contaminated soils quality. *Soil Biol. Biochem.* 33, 883-891.
- DICK, R.P., BREAKWELL, D.P. & TURCO, R.F., 1996. Soil enzyme activities and biodiversity measurements as integrative microbiological indicators. p 247-271. In: Doran, J.W., Jones, A.J. (eds.), *Methods for assessing soil quality*, SSSA Special Publication 49, SSSA, Madison, WI.
- FREDERICKSON, J.K. & BALKWILL, D.L., 1998. Sampling and Enumeration Techniques. p 239-252. In: Burlage, R.S., (ed.), *Techniques in microbial ecology*, Oxford University Press, Inc., New York.
- FROSTEGÅRD, A. & BÅÅTH, E., 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol. Fertil. Soils* 22, 59-65.
- GARCIA, C., HERNANDEZ, T., ROLDAN, A. & MARTIN, A., 2002. Effect of plant cover decline on chemical and microbiological parameters under Mediterranean climate. *Soil Biol. Biochem.* 34, 635-642.
- GUCKERT, J.B., ANTORTH, C.P., NICHOLS, P.D. & WHITE, D.C., 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. *FEMS Microbiol. Ecol.* 31, 147-158.
- GUCKERT, J.B., HOOD, M.A. & WHITE, D.C., 1986. Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increases in the *trans/cis* ratio and proportions of cyclopropyl fatty acids. *Appl. Environ. Microbiol.* 52, 794-801.
- HILL, G.T., MITKOWSKI, N.A., ALDRICH-WOLFE, L., EMELE, L.R., JURKONIE, D.D., FICKE, A., MALDONADO-RAMIREZ, S., LYNCH, S.T. & NELSON, E.B., 2000. Methods for assessing the composition and diversity of soil microbial communities. *Appl. Soil Ecol.* 15, 25-36.
- IBEKWE, A.M. & KENNEDY, A.C., 1998. Phospholipid fatty acid profiles and carbon utilization patterns for analysis of microbial community structure under field and greenhouse conditions. *FEMS Microbiol. Ecol.* 26, 151-163.
- IBEKWE, A.M., KENNEDY, A.C., FROHNE, P.S., PAPIERNIK, S.K., YANG, C.H. & CROWLEY, D.E., 2002. Microbial diversity along a transect of agronomic zones. *FEMS Microbiol. Ecol.* 39, 183-191.
- KEIFT, T.L., RINGELBERG, D.B. & WHITE, D.C., 1994. Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation of porous media. *Appl. Environ. Microbiol.* 60, 3292-3299.
- KEWELOH, H. & HEIPIEPER, H.J., 1996. *Trans*-unsaturated fatty acids in bacteria. *Lipids* 31, 129-135.
- MERILÄ, P., STRÖMMER, R. & FRITZE, H., 2002. Soil microbial activity and community structure along a primary succession transect on the land-uplift coast in western Finland. *Soil Biol. Biochem.* 34, 1647-1654.
- MUMMEY, D.L., STAHL, P.D. & BUYER, J.S., 2002. Soil microbiological properties 20 years after surface mine reclamation: spatial analysis of reclaimed and undisturbed sites. *Soil Biol. Biochem.* 34, 1717-1725.
- NELSON, D.W. & SOMMERS, L.E., 1982. Total carbon, organic

- carbon, and organic matter. p 539-579. In: Page, A.L., Miller, R.H., Keeney, D.R. (eds.), *Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties*, ASA-SSSA, Madison, WI.
- OLSSON, P.A., 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. *FEMS Microbiol. Ecol.* 29, 303-310.
- PINKART, H.C., DEVEREUX, R. & CHAPMAN, P.J., 1998. Rapid separation of microbial lipids using solid phase extraction columns. *J. Microbiol. Methods* 34, 9-15.
- PONDER, F. (JR.) & TADROS, M., 2002. Phospholipid fatty acids in forest soil four years after organic matter removal and soil compaction. *Appl. Soil Ecol.* 19, 173-182.
- RATLEDGE, C. & WILKINSON, S.G., 1988. An overview of microbial lipids. p 3-22. In: Ratledge, C., Wilkinson, S.G. (eds.) *Microbial Lipids, Volume 1*, Academic Press, London.
- SARATHCHANDRA, S.U., GHANI, A., YEATES, G.W., BURCH, G. & COX, N.R., 2001. Effect of nitrogen and phosphate fertilisers on microbial and nematode diversity in pasture soils. *Soil Biol. Biochem.* 33, 953-964.
- SMITH, C.A., PHIEFER, C.B., MACNAUGHTON, S.J., PEACOCK, A., BURKHALTER, R.S., KIRKEGAARD, R. & WHITE, D.C., 2000. Quantitative lipid biomarker detection of unculturable microbes and chlorine exposure in water distribution system biofilms. *Water Res.* 34, 2683-2688.
- STEENWERTH, K.L., JACKSON, L.E., CALDERÓN, F.J., STROMBERG, M.R. & SCOW, K.M., 2003. Soil microbial community composition and land use history in cultivated and grassland ecosystems of coastal California. *Soil Biol. Biochem.* 35, 489-500.
- TAYLOR, J.P., WILSON, B., MILLS, M.S. & BURNS, R.G., 2002. Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. *Soil Biol. Biochem.* 34, 387-401.
- TURNER, B.L., HOPKINS, D.W., HAYGARTH, P.M. & OSTLE, N., 2002. β -Glucosidase activity in pasture soils. *Appl. Soil Ecol.* 20, 157-162.
- VAN RENSBURG, L., DE SOUSA CORREIA, R.I., BOOYSEN, J. & GINSTER, M., 1998. Revegetation on a coal fine ash disposal site in South Africa. *J. Environ. Qual.* 27, 1479-1486.
- VAN RENSBURG, L., MABOETA, M.S. & MORGENTHAU, T.L., 2004. Rehabilitation of co-disposed diamond tailings: growth medium rectification procedures and indigenous grass establishment. *Water Air Soil Pollut.* 154, 101-113.
- WHITE, D.C. & RINGELBERG, D.B., 1998. Signature lipid biomarker analysis. p 255-272. In: Burlage, R.S., Atlas, R., Stahl, D., Geesey, G., and Sayler, G. (eds.), *Techniques in microbial ecology*, Oxford University Press, New York.
- WHITE, D.C., STAIR, J.O. & RINGELBERG, D.B., 1996. Quantitative comparisons of *in situ* microbial biodiversity by signature biomarker analysis. *J. Ind. Microbiol.* 17, 185-196.