ASSESSMENT AND MONITORING OF REHABILITATION EFFORTS BY ANALYSIS OF SOIL MICROBIAL COMMUNITIES AS BIOLOGICAL INDICATORS OF SUSTAINABILITY

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"Ek kan, ek wil en ek sal"

DECLARATION

The experimental work conducted and discussed in this dissertation was carried out in the School for Environmental Sciences and Development, Microbiology, North-West University, Potchefstroom Campus, South Africa. This study was conducted during the period 2004 to 2005 under the supervision and co-supervision of Mr. P.J. Jansen van Rensburg and Ms. S. Claassens, respectively.

The study represents original work undertaken by the author and has not been previously submitted for degree purposes to any other university. Appropriate acknowledgements in the text have been made where the use of work conducted by other researchers have been included.

Leon Liebenberg

OPSOMMING

Wegdoening van soliede afvalmateriaal gegenereer deur mynbou aktiwiteite kan ernstige omgewingsversteuring ten opsigte van grondkwaliteit. Wetgewing wêreldwyd, asook in Suid Afrika, verplig mynmaatskappye om hierdie versteurde gebiede tot volhoubare status ekologies te rehabiliteer. Rehabilitasie van hierdie afvalmateriaal by Finsch myn (De Beers) behels die gebruik van organiese materiaal (vermi kompos), chemiese behandeling en grassaadmengsels. Optimum hoeveelhede chemiese toevoegings is vasgestel deur eksperimentele proewe voor die uitleg van die huidige veld proewe by die myn. Die doel van hierdie studie was om die rehabilitasie te evalueer deur analise van grondmikrobiese gemeenskappe wat dien as biologiese indikatore van volhoubaarheid. Grondkwaliteit kan deur fisiese, chemiese en biologiese eienskappe geëvalueer word. Chemiese analise van die grond is uitgevoer deur 1:2 water ekstrak, asook ammonium asetaat ekstrak tegnieke. Ensiematiese analises (dehidrogenase, β-glukosidase, alkaliese en suurfosfatase, urease en ariel-sulfatase) asook substraat-geïnduseerde respirasie (SIR) is gebruik om die grondmikrobiesegemeenskapsfunksie te bepaal. Grondmikrobiesegemeenskapstruktuur is geëvalueer deur analise van spesifieke mikrobiese lipiedbiomerkers. Die plantegroei respons tot rehabilitasie is geëvalueer deur bepaling van plantegroei frekwensie, digtheid en biomassa opnames. Plant vitaliteit is bepaal deur analise van fotosintese wat as proses baie sensitief teenoor omgewingsversteurings is. In terme van algehele ensiematiese aktiwiteit (dehidrogenase) het die toevoeging van 90 ton/ha organiese materiaal die beste resultate gelewer. Hierteenoor het die behandeling met 60 ton/ha organiese materiaal die beste resultate gelewer in terme van plant vitaliteit en plant biomassa. Substraat geïnduseerde respirasie het nie statisties betekenisvolle verskille getoon nie. Die bepaling van die grondmikrobiese gemeenskapstruktuur het getoon dat fungi tot bakterieë verhoudings stelselmatig verhoog oor die proef tydperk alhoewel die verhoudings nog steeds minder as een is, en dat die bakteriese gemeenskape in die eksperimentele persele domineer.

SUMMARY

The disposal of solid waste material produced by mining activities can have vastly negative environmental impacts with regards to soil ecosystems. Global and South African legislation requires the mining company to rehabilitate the disturbed area in such a manner that sustainable ecosystem stability is achieved. Rehabilitation of co-disposed diamond tailings at Finsch mine (De Beers) entails the application of organic matter in the form of vermi-compost, organic and inorganic fertilizers, as well as different grass seed mixtures. Optimal amounts of chemical ameliorants were determined in a previous experimental trial prior to the current field study at the mine. The aim of this study was to evaluate the rehabilitation progress by analysis of soil microbial communities as biological indicators of sustainability. Soil quality can be determined by the analysis of physical, chemical and biological soil properties. Chemical analysis of the tailings material was done by 1:2 water extract, as well as ammonium acetate extraction techniques. Enzymatic analysis (dehydrogenase, acid and alkaline phosphatase, β-glucosidase and urease) as well as substrate induced respiration (SIR) was used to determine the soil microbial community function. Soil microbial community structure was evaluated by the analysis of specific microbial lipid biomarkers. The vegetation response towards the rehabilitation effort was evaluated by determination of subjective ratings for plant species frequency and density, and plant biomass. Plant vitality was determined by analysis of photosynthetic activity, which is known to be extremely sensitive towards environmental disturbance. In terms of overall enzymatic activity (dehydrogenase), the application of 90-ton/ha vermi-compost in the experimental plots showed the best results. No statistically significant differences in SIR results were noted in the experimental plots. However, the plots treated with 60-tons/ha vermi-compost showed the best results in terms of plant vitality and plant biomass. The determination of soil microbial community structure showed that fungal to bacterial ratios were increasing over time although actual ratio values are much less than one, and that overall bacterial population dominance is evident in the experimental plots.

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Chapter 1

Introduction

1.1. Soil ecosystems

Environmental ecosystems consist of many integrated factors which all contribute to form a balanced and functional unit (Nortcliff, 2002). Natural resource ecosystems involve a complex interaction between soil, vegetation, water and air components together with all the organisms that exist in these biomes. Soil seems to be the most important factor with regards to primary productivity whereby all plants and biogeochemical processes have their origin in soil ecosystems. Soil has a direct influence on plant productivity and soil functions include life support processes like plant anchorage and nutrient supply, water retention and conductivity, support of food webs, and environmental regulatory functions, such as nutrient cycling, source of microbial diversity and remediation of environmental perturbations (Van Bruggen and Semenov, 2000). The soil environment is a very dynamic entity and consists of physical, chemical and biological properties (Nortcliff, 2002), all of which need to be actively integrated in a correct functioning soil (Gil-Sotres *et al.*, 2004). Therefore any effort to define or assess soil quality should combine all of these aspects to form an accurate representation of the soil ecosystem. Soil quality is defined as "the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation" (Karlen *et al.*, 1997).

The biological component of soil consists mainly of soil microorganisms, which are actively involved in transformations and biogeochemical cycling of nutrients needed for uptake by plants as substrates for biomass accumulation and photosynthesis (Schloter *et al.*, 2003). Soil biological properties also react very sensitively to any environmental disturbances and can therefore be used as environmental indicators of soil quality in any land management projects or for rehabilitation purposes. Biogeochemical cycles in soil systems are mediated by microbial populations, especially bacteria and fungi, and therefore the microbial community functional and structural diversity could explain occurrences in plant growth and establishment, as well as soil chemical properties (Liu *et al.*, 2002). Functional diversity of soil microorganisms refers to the potential activity, or capability of the microbial community to adapt metabolically to the addition of substrates (Preston-Mafham *et al.*, 2002). Common indicators of microbial community function include microbial enzymatic activity and microbial respiration.

Soil microbial activity can be measured in terms of potential enzymatic activity. Enzymes catalyse all biochemical reactions in soil ecosystems and are an integral part of nutrient cycling in the soil (Bandick and Dick, 1999). They are involved in various chemical transformations and are therefore commonly used as indicators of the effects of environmental changes on microbial activity and fertility status of soils (Pati and Sahu, 2003), especially in remediation operations or particular types of land management (Schloter *et al.*, 2003). There are many methods to estimate microbial activity in soils, but of these methods, substrate induced respiration (SIR) is one of the simplest and most rapid techniques (Cheng and Coleman, 1988). The rate at which fixed C substrates are oxidised to CO_2 in a soil sample is proportional to the quantities of organisms mediating the reaction (Tate, 2000). Substrate induced respiration reflects the size of the active microbial biomass (Bailey *et al.*, 2002) since it evaluates the maximum potential activity occurring for the residue at the time of sampling (Schomberg and Steiner, 1997).

Microbial community structure can accurately be analysed by molecular techniques such as phospholipid fatty acid (PLFA) analyses. Analyses of PLFAs provide a quantitative measure of the microbial biomass that contains intact cellular membranes and is thus representative of the active microbial population because PLFAs are rapidly turned over (Hill et al., 2000; White *et al.*, 1996). Phospholipid fatty acid profiling is used to describe microbial communities and uses the cell membrane lipids within microorganisms as biomarkers for specific groups of organisms, thereby creating a profile or fingerprint of the microbial community as well as differentiating among environmental samples (Carpenter-Boggs *et al.*, 1998; Steenwerth *et al.*, 2003). These PLFA profiles are also good indicators of environmental changes or disturbances because such disturbances cause rapid changes in soil microbial community structure (Calderon *et al.*, 2000).

Subjective vegetation ratings of crown and basal cover, species frequency and plant biomass as well as qualitative analyses of photosynthetic activity in plants are used to reflect any stresses causes by environmental disturbances. During their development, plants are subjected to various environmental stresses such as drought, salinity or temperature extremes (Parvanova *et al.*, 2004). Plants by their very nature, being embedded in the soil, are unable to escape exposure to these environmental extremes and therefore must respond to survive (Taylor *et al.*, 2003). Therefore physiological and morphological changes are apparent in plants after such environmental changes occur. One of the most important environmental factors that inhibit photosynthesis is water stress, and many studies have shown how water stress resulted in damage to the oxygen-evolving complex of photosystem II (PS II) (Lu and Zhang, 1998). These vegetation responses are an indication of soil status since all primary productivity stems from the soil ecosystem.

1.2. Problem statement

South Africa reached a record level of diamond production output in 2003, which was mainly sourced from kimberlite mines producing 90% of the country's diamonds. South Africa is the world's fourth largest producer of diamonds in terms of value and supplies nine percent of global production (Chamber of Mines, 2003). Finsch diamond mine, located 160km North West of Kimberley, is one of De Beers' seven South African operations. Discovered in 1961 during exploration for asbestos, the deposit was first developed as an open pit. Since 1991, production has come from the underground mine beneath the old pit. Production in 2002 totalled 2.4Mct from 5.1Mt of kimberlite ore, giving a recovered grade of 46.6ct per 100t of ore (Mining-technologies.com, 2004). The millions of tones of waste rock material produced annually is stockpiled or pumped into slime dams (Van Rensburg and Maboeta, 2002).

Current legislation in South Africa (South African Environmental Conservation Act 73:1989; National Environmental Management Act (NEMA) 107:1998; South African Minerals Act 50:1991) requires the responsible party to restore and rehabilitate the disturbed environment in such a manner that it is ecologically sustainable and similar to the original surrounding environment. Traditional rehabilitation methods of the past have relied too heavily on vegetation analysis, and healthy vegetation was taken to be a sign of successful rehabilitation. This is however not a reliable method because soil microbial communities are not considered, and if they are not flourishing then plant life will also at a later stage deteriorate. Vegetation is dependent on biogeochemical cycling of nutrients in the soil by microbial communities. Thus vegetation alone is not a reliable indicator of the success of rehabilitation (Ludwig *et al.*, 2003), and the main focus must be more on sustainability (Mummey *et al.*, 2002). The question must be asked: "even if plant life is flourishing, will it be sustained in the future, and are all the components of the ecosystem in balance?" Rehabilitation will only be sustainable if soil quality is at the desired level, or if the resource quality is maintained or improved (Garcia and Hernandez 1997). Rehabilitation must be done in such a manner that it leads to a self-sustaining ecosystem similar to the surrounding landscapes. This is achieved by implementation of physical structures and processes such as reshaping of waste rock piles and contours, which support the developing ecosystem until biological systems are in place (Ludwig *et al.*, 2003).

The main problem faced during the rehabilitation process was the high pH of the medium and the high levels of Na. The concentration of Na in the soil medium was four times that which it should be for normal plant growth to occur (8.22mmolL^{-1} instead of <2mmolL^{-1})(Van Rensburg and Maboeta, 2002). This means that there is lower water potential outside the plants in the growing medium and plants will have to increase their solute concentrations inside the plants as an adaptation for water to be taken up (Schachtman and Liu, 1999). This process however requires more energy and could lead to nutrient deficiencies in the plant. K uptake is vital for

plant growth but in saline soils Na competes with K for uptake across the plasma membrane of plant cells. This can result in high Na:K ratios that reduce plant growth by creating an energy deficit (Van Rensburg and Maboeta, 2002). The element K is involved in synthesis of proteins, carbohydrates and chlorophyll and is also involved in anionic uptake at the roots. However, plants cannot distinguish between the Na and the K ion because they have the same charge and the same particle size. Thus a high level of Na competitively inhibits the uptake of K by the plants even if there are sufficient levels of K in the surrounding soil environment. One of the purposes of fertiliser application in the field trials was to rectify the pH imbalance. Super-phosphate was added because it releases the acidic SO_4^{2-} ion when dissolved, and this in turn lowers the pH. The application of super-phosphate was at a much higher quantities than usual, to release enough sulphate ions so that the pH would be sufficiently lowered.

More K was released into the soil by addition of KNO_3 to rectify the Na:K ratio so that K would no longer be competitively inhibited by Na. Even if K ions were present in the soil in sufficient quantities, larger concentrations of Na would cause the plants to experience physiological drought stress. Nitrogen was added in the form of NH_4^+ because this also assists in the lowering of the soil pH. For every NH_4 molecule taken up by the plant, one H^+ ion is released into the surrounding environment, thus lowering the pH.

Most solid waste material, especially kimberlite or diamond tailings contain very low levels of organic matter and are of no nutritional benefit to any forms of vegetation. Kimberlite is very sandy in nature and is very difficult to rehabilitate due to the properties of sand. Because of low organic content, these tailings also have a low cation exchange capacity. The problem faced in most cases of rehabilitation of solid waste material is the sustainability thereof. In order to ensure sustainability, all aspects of the soil environment in which plants grow need to be in equilibrium, especially the ratios of elemental ions in the soil. Thus correct chemical amelioration is necessary in the soil before soil microbial communities and in turn the vegetation will thrive. Rehabilitation outcomes cannot be termed self-sustainable unless the progress of the rehabilitation is monitored over a certain length of time and the results noted that the soil quality is actually improving (Garcia and Hernandez, 1997).

An ecologically-based approach to assessing soil quality can include investigations of individual populations of soil organisms if they have been recognised as an important part of the sustainable biodiversity in a soil ecosystem or an important agent in soil ecological processes, e.g. in nitrogen fixation or carbon mineralisation (Filip, 2002). One of the methods used in analysis of the sustainability of rehabilitation is improvement of the soil quality, which as previously mentioned, can be defined as the capacity of a soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality and promote plant and animal health (Doran and Parkin, 1994). Soil structure can be divided into three

classes' namely biological, chemical and physical characteristics (Nortcliff, 2002). Biological assessment techniques are used in some cases to monitor soil quality because of the sensitive response of microbial communities to any environmental change or disturbance (Pascual *et al.*, 2000). Microbial communities in the soil environment are involved in most of the biogeochemical processes like the cycling of carbon, nitrogen and phosphorus, the decomposition and transfer of organic matter as well as the soil structure and composition (van Bruggen and Semenov, 2000). Soil quality is thus an important tool in the assessment of rehabilitation efforts and the sustainability thereof.

1.3. Research objectives

The aim of this study was to compare the data from different soil rectification procedures based on a field trial, which was done at Finsch mine in the Northern Cape, South Africa. This was done by characterisation of the tailings material of the sites in terms of chemical, physical and biological properties of the soil. Biological soil properties were represented by the structural and functional diversity of microbial communities in the soil. The different field trials were assessed in terms of soil quality to see if the soil was aggrading or degrading or if it is just in a stable state (Karlen *et al.*, 2003). Biological parameters such as SIR with selective inhibition and enzyme activity were used to analyse microbial community function, and quantification of signature lipid biomarkers such as PLFAs were used as indicators of microbial community structure. Independent companies undertook physical and chemical characterisation of the soil samples (Eco-Rehab, Potchefstroom).

Specific objectives include:

- Comparison of different rehabilitation field trials by evaluation of the topsoil quality in terms of physical, chemical and biological properties, as well as subjective vegetation analyses such crown cover, species frequency, density and basal cover measurements.
- Characterisation of the physical and chemical properties of the soil by 1:2 water extractions.
- Characterisation of microbial activity by measurement and analysis of the end products of potential enzymatic activity and substrate-induced respiration with selective inhibition.
- Characterisation of structural diversity of the microbial communities by analysis and quantification of PLFAs.
- Multivariate statistical analysis of the interaction between soil physical and chemical parameters and biological parameters such as functional and structural diversity of the microbial community.
- Correlation of microbial function and structure to physical and chemical characteristics of the soils in the different experimental sites.

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Chapter 2

LITERATURE REVIEW

2.1. MINING AND SOLID WASTE MATERIAL

2.1.1. Environmental impacts of mining

Solid waste material, in terms of chemical and physical characteristics, can have vast negative effects on the environment. If there are large chemical imbalances in the elemental ratios in the waste material, which is almost always the case, the material could become toxic and highly unfavourable to the vegetation in the area (Adriano *et al.*, 1980). Mining is one of the main contributing factors to the global economy and is also responsible for most solid waste material. South Africa is a major global producer of minerals, which has been the cornerstone of the South African economy (Chamber of Mines SA, 2003). Finsch mine in the Northern Cape alone produces more than five million tonnes of kimberlite ore per annum, which is processed and discarded as waste rock material (Mining-technology.com, 2004). The biologically inactive solid waste is piled and stored, and new biogeochemical interactions will start to develop as shown in the scheme of Harris *et al.* (1989), presented in Figure 1.

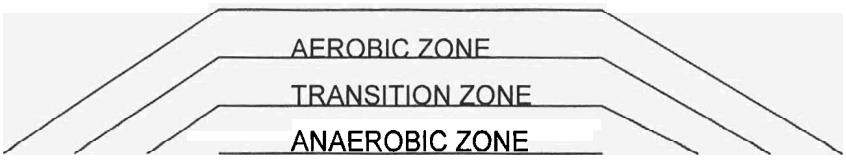

Figure 1. Schematic diagram showing a cross section through a soil store, indicating three zones (Harris *et al*, 1989).

Stockpiling of the solid waste material can lead to negative environmental impacts on underlying soil and ground water such as soil compaction, reduced water-holding capacity, and decreases in aggregate formation due to anaerobic conditions deep inside these stockpiles (Harris *et al.*, 1989). These adverse conditions often limit spontaneous restoration processes at mine reclamation sites. Restoring nitrogen (N) cycling is one of the major goals of ecosystem recovery in these disturbed environments, and adding organic materials to soil promotes nutrient cycling and accelerates soil recovery (Coyne *et al.*, 1997). The viability of the seed bank in these piles rapidly diminishes after prolonged dormancy (approximately 10 years) and continual compaction (Harris *et al.*, 1989). During their development plants growing on solid waste material are subjected to various environmental stresses such as drought, salinity or temperature extremes (Parvanova *et al.*, 2004).

Criteria for judging reclamation success of these disturbed soil systems largely encompasses only visually distinguishable aboveground indicators such as vegetation coverage, and fail to account for the health and composition of the soil microbial populations, which are the basis for all terrestrial ecosystems (Mummey *et al.*, 2002). Edgerton *et al.*, (1995), found a clear indication of a parallel increase in microbial biomass and aggrading soil quality in subsoil material restored after opencast mining.

2.1.2. Finsch mine in the Northern Cape, South Africa

The African continent is the largest producer of diamonds and has contributed to more than 75% of the world's total value in diamonds. Until the year 2001, South Africa produced 10.6 million carats, which comes primarily from De Beers operated mines (Chamber of Mines, 2003). One of these mines is Finsch diamond mine in the Northern Cape. The extraction of the diamonds includes the grinding of the rock and ores, as well as the physical and chemical extraction of the diamonds. At Finsch mine, the grounded ore material is first passed through a high rate thickener to remove all the excess water, which is then recycled and used again. The remaining material is pumped to a screen with an aperture of 1.6mm. The overflow (>1.6mm) is dumped onto a coarse heap and used for the re-extraction of diamonds. The underflow (<1.6mm) is pumped out onto a tailings dam and this process produces waste material that needs to be rehabilitated. The process is also known as the co-disposal of diamond tailings (Mining-technology.com, 2004; Van Rensburg *et al.*, 2002).

2.1.2.1. Chemical characteristics of co-disposed tailings

Kimberlite diamond tailings usually contain high levels of sodium (Na) due to the nature of the material (Van Rensburg *et al.*, 2002). The concentration of Na in the experimental plots at Finsch mine was four times higher than normal concentrations to favour plant growth (8.22mmol.L^{-1} instead of <2mmol.L^{-1},which is the benchmark value) (Van Rensburg and Maboeta, 2002). The exchangeable sodium percentage (ESP) was found to be 25%, rendering the environment sodic since any value above 15% shows Na dominance. The co-disposed material was considered saline because of the higher than normal levels of electrical conductivity (EC) (Van Rensburg and Maboeta, 2002). Sodic soils are either saline or non-saline depending on the salt concentration in soil solution, and high sodicity creates a nutritional imbalance, which affects plant physiological processes as well as soil drainage (Khan and Abdullah, 2003). Sodic soils tend to be impermeable to water thereby posing the question of whether leaching is a possible option for lowering high salt concentrations in rehabilitation projects (Shannon, 1998). The major adverse effect of soil salinity is the reduction in availability of soil water to plants. This is due to the presence of salt in water and increases the necessary energy that the plant must generate to extract water from the soil solution (Schachtman and Liu, 1999). Plants under salinity stress reduce their solute potential by accumulating organic and inorganic solutes to maintain continuous water absorption at low soil water potential (Morant-Monceau, 2004). Many plants develop mechanisms either to exclude salt

from their cells or to tolerate its presence within the cells (Parida and Das, 2005). These osmotic adjustments are often made in plants to tolerate drought or salinity stress (Souza, 2004), but occur at the expense of plant growth and biomass production (Lee *et al.*, 2004). Passive nutrient uptake is also related to water uptake and therefore any decrease in water availability would immediately be reflected in the condition of the plant (Jurinak *et al*, 1987).

Potassium uptake is vital for plant growth but in saline soils Na competes with potassium (K) for uptake across the plasma membrane of plant cells, which result in high Na:K ratios that reduce plant growth and eventually become toxic to the plant if such a high ratio is maintained (Schachtman and Liu, 1999). Past experiments have shown that K uptake declines in the presence of excess Na (Morant-Monceau, 2004), and a lack of adequate calcium (Ca) can enhance substitution of Na for K in plants. It has also been suggested that the ability of a cell to retain K in the presence of high Na concentrations could be an important regulating mechanism in salt tolerant species (Jurinak *et al.*, 1987). The K ionic concentrations are higher than normal in the tailings material and this is advantageous as it improves the ratio of K to Na. Even though the concentrations of all the above mentioned elements may be high, it is more important for them to be in the correct proportion to each other, because more important than the absolute concentration of an element is the balance in ratio among all the elements present in the soil (Van Rensburg and Maboeta, 2002).

2.1.2.2. Physical characteristics of co-disposed tailings

Considering the texture, co-disposed diamond tailings were found to consist of about 75% sand, 10% loam and 15% clay. Due to the high sand content in the tailings material, low water retention was evident. This is particularly disadvantageous in the arid climatic region of the Northern Cape where Finsch mine is situated. The rate at which water infiltrates into soil bears importantly on the amount of erosion and sedimentation encountered during soil rehabilitation, as well as the water that moves into the root zone. Due to the smectite nature, there is 2:1 layering, meaning that two tetrahedral sheets fold around an octahedral sheet causing the material to be loosely bound at molecular levels. This type of clay is also prone to isomorphic substitution, which results in water uptake between the layers and causes swelling. Thus, shrinking and swelling occurs when there is change in moisture levels and this renders the material very unstable, especially on sloping surfaces (Van Rensburg and Maboeta, 2002).

2.2. SOILS AND ECOSYSTEM STABILITY

2.2.1. Soil quality

Soil quality has been defined in terms of sustaining plant and animal productivity, maintaining or enhancing water and air quality, and supporting human health (Karlen *et al.*, 1997). Soil quality can also be described as an integral value of compositional structures and natural functions of soil in relation to soil use and environmental conditions on site (Filip, 2002). Soil quality involves many aspects and has many characteristics, which can be divided into three categories namely physical, chemical and biological characteristics (Nortcliff 2002), all of which need to be actively integrated in a correct functioning soil (Gil-Sotres *et al.*, 2004).

Chemical and physical soil parameters such as organic matter and nutrient status have been used to measure soil quality, but these parameters change very slowly over time and therefore it may take several years to measure any significant environmental changes in a specific area. Soil biological and biochemical properties however, are responsive to small changes that occur in soil, thereby providing immediate and accurate information on changes in soil quality (Pascual *et al.*, 2000; Gil-Sotres *et al.*, 2004). Because soil microorganisms are so sensitive to environmental change, measures of microbial activity in soil could serve as indicators of rehabilitation progress throughout the duration of rehabilitation projects (Mummey *et al.*, 2002; de Mora *et al.*, 2005). Many biological indicators of soil quality are measures of the processes of nutrient mineralisation (Knoepp *et al.*, 2000) and the use of microorganisms as bio-indicators of environmental impact is already well established (Pankhurst *et al.*, 1995). The quality of soil is defined by the natural composition of the soil as well as many other critical factors in the surrounding environment (Masciandaro and Ceccanti, 1999).

Soil is a critically important component of the earth's biosphere, functioning not only in the production of food and fiber (Karlen *et al.*, 2003), but also in the maintenance of local, regional, and global environmental quality. It is also the basis of agricultural and of natural plant communities (Doran and Zeiss, 2000). Soil has a direct influence on plant productivity, and soil functions include life support processes like plant anchorage and nutrient supply, water retention and conductivity, support of food webs and environmental regulatory functions, such as nutrient cycling, source of microbial diversity and remediation of environmental perturbations (Van Bruggen and Semenov, 2000).

The ideal soil microbiological and biochemical indicator used to determine soil quality should be simple to measure, work equally well in all environments, and reliably reveal which problems existed where. It is however highly unlikely that a sole indicator can be defined with a single measure (Schloter *et al.*, 2003). Indicators should also be relevant to the type of disturbance and accurately reflect the significance thereof (Doran and Zeiss, 2000). The selection of indicators that enable the quantification of soil quality is also important. According to Karlen *et al.* (1997), the evaluation of soil quality is based on four critical soil functions: (1) allowing water entry; (2) retaining and supplying water to plants; (3) resisting degradation; and (4) supporting plant growth.

2.2.2. Organic matter in tailings material

Organic matter in any soil environment is a key attribute of soil quality, as it is a nutrient sink, enhances soil structural conditions, promotes biological activity, and makes the soil resilient to environmental disturbances (Doran and Parkin, 1994). Organic matter is also important for air and water infiltration, water retention, erosion and the transport or immobilisation of pollutants (Knoepp *et al.*, 2000; Bulluck *et al.*, 2002). Organic matter is one of the most critical components of the soil-plant ecosystem and depletion of organic matter causes a loss in water holding capacity; poor aggregation; acceleration in soil erosion; poor retention of applied nutrients; and reduced soil biological and enzymatic activities (Ghani *et al.*, 2003). The natural remediation processes that occur in soil, for example sorption, precipitation, and complexation reactions, can be accelerated by the addition of organic amendments (Bolan and Duraisamy, 2003). Therefore, the applications of amendments, as well as the development of plant cover, play an important role in the restoration of the physical, chemical and biological properties of disturbed soils (De Mora *et al.*, 2005). The total organic matter content of soil changes slowly and is difficult to measure. However, the soil microbial biomass responds much more rapidly than organic matter as a whole to changes in management and therefore changes in biomass measured over relatively short periods can indicate trends in total organic matter content long before these changes can be detected by chemical analysis (Powlson *et al.*, 1987). Although low soil organic matter (SOM) content is generally less important to plant reestablishment from a soil chemical point of view, than N availability, SOM is very important to sustained nutrient-cycling and establishment and maintenance of soil physical characteristics (Mummey *et al.*, 2002).

2.2.3. Sustainability of soil quality

Sustainability of soil quality refers to the longevity or the health of a land use system, and the ability of this system to maintain a productive capability (Hannam, 2001). In terms of the environment, sustainability signifies maintaining the productivity and potential of an ecosystem used by humans with time. Human intervention in the landscape almost always has a strong impact on resources, which become depleted or degraded (Paoletti, 1999). The increasing human population is, however, a growing threat to global sustainability, and to sustain agriculture and the world for future

generations, it is necessary to act now to develop production systems which rely less on non-renewable energy sources but more on renewable resources like sun energy (Doran, 2002). Soil degradation is one of the main antagonistic forces inhibiting sustainability and several factors such as water erosion, wind erosion, waterlogging, and excess salts can contribute to soil degradation (Hannam, 2001). The capacity of a soil to continue to support similar potential range of uses in future that it supports today depends on both its resistance to degradation and on its resilience following degradation (Herrick, 2000). Not only is soil linked to plant productivity, but also to water and air quality (Doran, 2002). Soil also serves as one of the most important biogeochemical regulators of the flow of substances into, through, and out of the ecosystem. The disturbance of soils can lead to critical changes in the biosphere, which could be negatively impact many forms of life (Snakin *et al.*, 1996). Karlen *et al.* (2003) links agricultural sustainability to soil quality as presented in Figure 2:

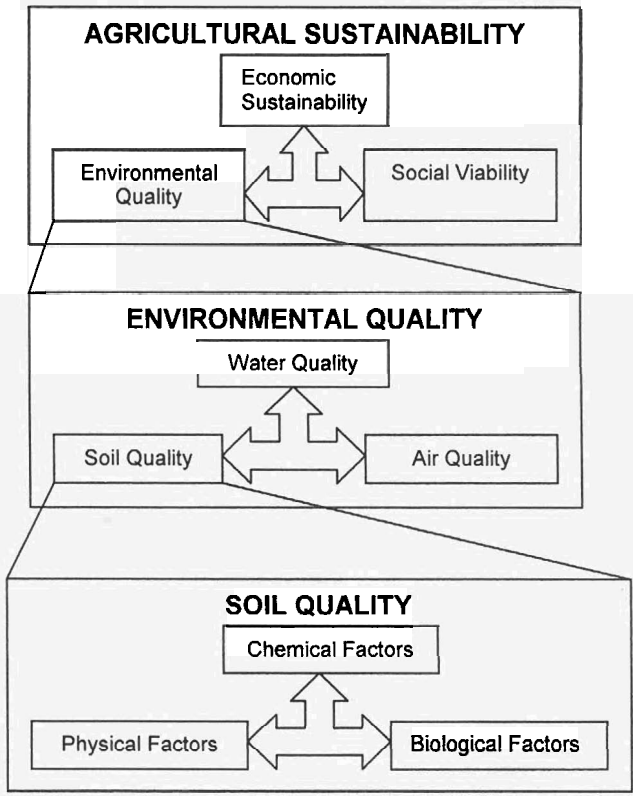

Figure 2. Hierarchical relationship of soil quality to agricultural sustainability (Karlen et al. 2003)

2.2.4. Soil compaction

Soil compaction has a major negative effect on soil structure, resulting in deterioration of the plant root environment and reduced decomposition rate of organic matter (Breland and Hansen, 1995). Compaction tends to reduce the total soil pore volume and alters the pore size distribution towards a higher percentage of small pores, thus isolating organic materials from microbial decomposition (Breland and Hansen, 1995), and reducing absorbance of rainwater (Whalley *et al.*, 1995). The reduction of the total soil pore volume after compaction increases the probability of anaerobic conditions. Furthermore, biogeochemical processes that occur in these spaces, such as N mineralisation (Jordan *et al.*, 2003) are negatively impacted upon by compaction (Jensen *et al.*, 1996). Compaction lowers the water holding capacity and causes aggregation deep in stockpiles due to the anaerobic conditions (Harris *et al.*, 1989). Compaction also reduces seedling establishment and growth because N is needed for effective plant growth and is often the most limiting factor in such anaerobic environments (Jordan *et al.*, 2003).

2.3. ROLE OF MICROORGANISMS IN SOIL ENVIRONMENTS

2.3.1. Microbial activity and populations

The soil biological component consists mainly of the soil microbial biomass. Bacteria and fungi are the most important with regards to energy flow and nutrient transfer in ecosystems, and dominate within the microbial biomass (Schloter *et al.,* 2003). Microbial activity is a general term and includes all metabolic reactions conducted in the soil. The importance of microbial activity in cycling organic matter and regulating active nutrient pools in soils suggests that the effect of disturbance on soil microorganisms is fundamentally related to ecosystem productivity (Brohon *et al.*, 2001). The soil microbial community is one of the most important (functional) components of the soil biota, influencing productivity and nutrient cycling in soil (Villar *et al.*, 2004) and could also be responsible for the mineralisation and mobilisation of pollutants in soil (Schloter *et al.*, 2003). High quantity, activity and diversity of the soil microorganisms are therefore the prerequisite of good soil quality, soil fertility, and tolerance of the soil to stress factors. Changes in the soil microbial parameters can serve as an early warning for decreasing soil quality (Hofman *et al.*, 2002). Soil microbial populations are very dynamic and react sensitively to disturbances and therefore they can be of great advantage in monitoring environmental changes (Schomberg and Steiner, 1997).

2.3.2. Microbial communities represented by microbial biomass (carbon)

Determination of organic carbon allows for the quantities of microbial biomass in the vegetative microflora of soils to be estimated (Anderson and Domsch, 1980). The quantities of C in the microbial biomass of soils and the relative contribution of bacteria and fungi to the biomass can be determined using respiratory and selective inhibition methods as described by Anderson and Domsch (1973; 1975). Microbial biomass is an important indicator of soil fertility and its

measurement is often essential for developing management strategies to improve soil fertility (Wang *et al.*, 2003). Any insight into the immobilisation and mobilisation of bioelements in soil requires knowledge of the amounts stored in the microbial biomass (Beck *et al.*, 1997). Soil microbial biomass is the living component of SOM and performs at least two critical functions for plant production in the ecosystem. It is a labile source and sink of C, N, P, and S, and an agent of nutrient transformation and pesticide degradation in soil ecosystems (Dalal, 1998; Bolan *et al.*, 2003). In addition, microorganisms form symbiotic associations with roots; act as biological agents against plant pathogens; and participate in soil formation (Dalal, 1998).

Although soil microbial biomass C is a sensitive indicator of change, and despite it's proven usefulness as an indicator in soil management, biomass measurements must not be used as a sole indicator of environmental change (Hargreaves *et al.*, 2003). It is more advantageous to use a suite of variables that characterise C availability, such as carbon dioxide (CO_2) efflux, microbial biomass C (C_{mic}), and respiratory quotient (qCO_2) to evaluate soil quality (Knoepp *et al.*, 2000) rather than a single environmental parameter. There are many methods to estimate microbial biomass in soils, but of these methods, substrate-induced respiration (SIR) is one of the simplest techniques with the most rapid outcome (Cheng and Coleman, 1988). The rate at which fixed C substrates are oxidised to CO_2 in a soil sample is proportional to the quantities of organisms mediating the reaction (Tate, 2000), therefore, SIR, according to Lin and Brookes (1999), seems to be a reliable method to represent the living component of the microbial biomass. There is a direct correlation between the amount of nutrients held in the microbial biomass and the amount of mineralisable nutrients in the soil, emphasising that the microbial biomass in soil plays a key role in maintaining soil fertility (Tarafdar *et al.*, 2001).

2.3.3. Biogeochemical cycles

Soil microbial biomass is responsible for degradation and decomposition of organic matter and is therefore sensitively influenced by changes in the quantity and quality of SOM (Tarafdar *et al.*, 2001). Although microbial biomass usually makes up a small percentage of the soil organic matter, it carries out many critical functions in the soil ecosystem, among which the following could be pointed out: it is both a source and sink for nutrients, it participates in the C, N, P and S transformations, and plays an active role in the degradation and immobilisation of soil contaminants (Gil-Sotres *et al.*, 2004). The diversity of microorganisms in soil is critical for the maintenance of good soil health as such a wide range of microbes conduct these important soil functions (Ibekwe *et al.*, 2002). The important role that soil microorganisms play in the nutrient and energy-flow relationships (Waldrop *et al.*, 2000) of natural and man-made environments have created the need for biological parameters such as biogeochemical cycles in soil ecosystems to be quantified (Yan *et al.*, 2003). Increasing concern over problems of environmental pollution and disturbance has stimulated research to evaluate the possible impact and change that these stresses have on

ecosystems. The importance of microbial activity in cycling organic matter and regulating active nutrient pools in soil suggests that the effects of pollution on soil microorganisms are related to the effects on natural vegetation and ecosystem productivity (Brohon *et al.*, 2001).

2.4. ASSESSMENT OF REHABILITATION

2.4.1. Methods of assessment

2.4.1.1. Soil microbial functional diversity

Functional diversity of soil microorganisms refers to the potential activity, or capability of the microbial community to adapt metabolically to the addition of substrates (Preston-Mafham *et al.*, 2002). Soil microbial activity can be measured in terms of potential enzymatic activity. Enzymes catalyse all biological reactions and are an integral part of nutrient cycling in the soil (Bandick and Dick, 1999). They are involved in various chemical transformations and are therefore commonly used as indicators of the effects of environmental changes on microbial activity and fertility status of soils (Pati and Sahu, 2003), especially in remediation operations or particular types of land management (Schloter *et al.*, 2003). Microbial enzymatic activities in tailings material are closely correlated with soil chemical and biological parameters (Liu *et al.*, 2002), and data collected from measurements of potential tailings enzymatic activity can be used in modeling biogeochemical cycles (Li and Sarah, 2003). Enzyme assays are a measure of microbial activity and they indicate microbial response to environmental change. Enzymes catalyse reactions involved in the biogeochemical transformations of C, P, N and S, and are likely to be an essential component of any assessment of soil microbial activity and substrate mineralisation (Pascual *et al.*, 2002; Aon *et al.*, 2001).

β-glucosidase (EC 3.2.1.21); urease (EC 3.5.1.5); phosphomonoesterases, namely acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1); and dehydrogenase are enzymes that carry out specific hydrolyses and are commonly assayed because it catalyses biogeochemical transformations and are likely to be an essential component of any assessment of soil microbial activity and substrate mineralisation (Aon *et al.*, 2001; Pascual *et al.*, 2002). These enzymes in the soil are believed to be primarily of microbial origin but also stem from plants and animals (Bandick and Dick, 1999). Enzymes present in the soil profile are known to show intracellular and extracellular existence, whereby a fraction of the soil enzyme activity is associated with living organisms, and the rest with abiotic components, which are then classified as extracellular (Li and Sarah, 2003).

One of the most difficult problems in research on soil enzymes in soil has been to distinguish between extracellular activities and activities associated with living organisms (Klose and Tabatabai, 1999). Potential enzymatic activity in soil can be associated with active cells such as dehydrogenase (Dick, 1994), or dead cells, or can exist in complexes (Burns, 1982; Rao *et al.*, 2000).

With regard to enzymes involved in the C cycle, β-glucosidase has been commonly used in the evaluation of soil environments (Turner *et al.*, 2002) because it is negatively impacted by land management practices and is sensitive to any environmental changes in the soil (Gil-Sotres *et al.*, 2004). β-glucosidase regulates the supply of an important energy source for microorganisms and breaks down cellulase chains into smaller glucose molecules which can then be utilised by microbes (Tabatabai, 1994; Turner *et al.*, 2002).

Urease is involved in the N cycle (Bandick and Dick, 1999) and is considered an important agent for N mineralisation in terrestrial and aquatic systems (Sinsabaugh *et al.*, 2000). It catalyses the hydrolysis of urea to CO_2 and ammonium (NH_4) (Tabatabai, 1994) and its activity is generally assayed by measurement of the rate of NH_4 produced. The cycling of N is carried out in part by ammonia-oxidising bacteria. This group of bacteria is responsible for the conversion of ammonia (NH_3) to nitrite (NO_2), which is the form of N readily available to plants (Ibekwe *et al.*, 2002). Urease is of great importance from an agricultural point of view because of the common use of urea fertilisers and its role in the decomposition of these substances (Tabatabai 1994; Sinsabaugh *et al.*, 2000).

Dehydrogenase activity can be considered a direct measure of microbial activity (Trasar-Cepeda *et al.*, 2000; Li and Sarah, 2003) as it is involved in biological oxidation of organic compounds (Taylor *et al.*, 2002), which forms the basis of all dehydrogenation processes. This enzyme is present in all microorganisms and the assays are considered to be an accurate measure of the microbial oxidative activity of the soil and should have a direct relationship to total viable microorganisms (Tabatabai, 1994; Taylor *et al.*, 2002).

In soil, the phosphomonoesterases are extracellularly secreted by plants and microorganisms, and play a key role in the P cycle. These enzymes are usually not free in solution but associated with soil constituents (Rao *et al.*, 2000). Acid and alkaline phosphatases hydrolyse a variety of phosphomonoesters, changing organic P into inorganic P compounds (Li and Sarah, 2003). Among the hydrolases, acid phosphomonoesterase activity has been the most frequently used for estimating changes in soil quality. It is also a good index of the quality and quantity of organic

matter in soil (Gil-Sotres *et al.*, 2004). The enzymes are classified as acid and alkaline because they show optimum activities in acid and alkaline conditions, respectively (Tabatabai, 1994).

Arylsulfatase is also important in nutrient cycling because it releases plant available sulphates (SO_4) (Bandick and Dick, 1994), by the hydrolysis of organic S compounds to inorganic S compounds (Li and Sarah, 2003).

It is, however, unlikely that the activity of a single enzyme could holistically represent the more complex functions such as total microbial activity or soil quality, which depend on many reactions and properties (Gil-Sotres *et al.*, 2004). As with all enzyme assays, the incubation conditions determine the rate of substrate catalysis, and the use of buffers represents a potential maximal activity for the enzyme, which differs from activity of enzymes in the natural environment (Taylor *et al.*, 2002). Another approach is to rely on the inherent buffering capacity of soil itself using purified water, and to measure enzyme activity at the natural pH of the soil. This would produce a lower rate of substrate catalysis and is more likely to accurately reflect activity found in the natural environment (Taylor *et al.*, 2002).

2.4.1.2. Substrate induced respiration

It is known that microbes in the soil are actively involved in respiration, whether it be aerobic or anaerobic. Soil respiration is the process whereby CO_2 emanates from the soil surface generated from the metabolic activity of soil microbes (Frank *et al.*, 2005). Substrate induced respiration may be used to measure the maximal respiratory levels of the active microorganisms in soil. A substrate that is readily used by most microorganisms and which stimulates microbial growth and enzymatic activity, such as glucose (Dilly, 2003), is added to soil, and after incubation, CO_2 levels are measured to determine the maximal respiratory response of the microbial populations to the substrate addition (Lin and Brookes, 1999). Substrate induced respiration is also strongly influenced by soil water content because all biochemical reactions occur in solution (Lin and Brookes, 1999; Cheng and Coleman, 1989). For efficient distribution of the glucose in the soil samples, addition in solution produces better results, and enzymatic activity also increases with enough moisture present. This also prevents moisture from being a limiting factor to soil respiration (West and Sparling, 1986). Glucose metabolism consists of primary and secondary oxidation. The initial slow mineralisation rate of glucose is considered to be due to the enzymes originally present in the soil microbial biomass, and possibly extra-cellular enzymes. The fact that respiration then increases exponentially could be due to the synthesis of glucose-induced enzymes in secondary oxidation (Lin and Brookes, 1999). The magnitude of the SIR response of microorganisms over 0-6hrs is characteristic of the initial microbial community in soil before growth of organisms occurs on the added substrates (Degens and Harris, 1997).

Of all the methods used to estimate microbial biomass in soils, SIR is one of the most effective, non-complex and rapid techniques (Cheng and Coleman, 1988). The rate at which fixed C substrates are oxidised to CO_2 in a soil sample is proportional to the quantities of organisms mediating the reaction (Tate, 2000). Substrate induced respiration reflects the size of the active microbial biomass (Bailey *et al.*, 2002) since it evaluates the maximum potential activity, not the actual activity, occurring for the residue at the time of sampling (Schomberg and Steiner, 1997). One of the advantages of SIR is the ability to separately measure the contributions of bacterial and fungal biomass to SIR by making use of selective inhibitors (antibiotics) (Lin and Brookes, 1999). The glucose and inhibitor concentrations selected for the soils, need to be determined in a stepwise sequence of experiments as described by Anderson and Domsch (1973).

The soil pH can also influence the equilibration of gaseous and liquid phase CO_2. With a soil solution pH $\leq$ 6.0 the theoretical distribution of CO_2 between these phases favours the gaseous phase by a ratio $\geq$ 10:1 at equilibrium. It is therefore suggested that the modified SIR method should be limited to use on acidic soils with a pH $\leq$ 6.5 (West and Sparling, 1986). However, in the case of soil with a pH > 6.5, calibration can be performed to account for the CO_2 dissolved in the soil solution, against other CO_2 measurements of relatively acidic soil (Lin and Brookes, 1996).

Another disadvantage of headspace sampling is that CO_2 accumulates during the course of incubation, and CO_2 is retained at high pH in the soil solution, which results in underestimation of biomass C. Only systems with continuous aeration of the headspace with ambient air do not have these limitations (Beck *et al.*, 1997).

The original SIR method has several technical problems. Firstly, the method is restricted to soil samples with a narrow moisture range, and secondly, the CO_2 flushing is non-continuous and subject to experimental error, especially with a pH > 6.0. When the soil water content is higher than the optimal, the lower oxygen supply may inhibit aerobic activities of microbes, decreasing the SIR (Cheng and Coleman, 1989). According to Schomberg and Steiner (1997), SIR should reflect the effect of temperature, water availability and resource quality on microbial communities at a given time because optimum conditions result in growth, whereas periods of stress result in reduced growth and death. The SIR method being relatively simple and rapid, identifies the metabolically active component of the microbial community. Substrate induced respiration used in combination with selective inhibitors allows for separation of prokaryotic and eukaryotic contributions to the total respiratory response. Comparisons with direct biomass procedures allow determination of a glucose inducible or potentially active microbial biomass (Beare *et al.*, 1990).

2.4.1.2.1. Selective inhibition

To measure the metabolic contributions of bacteria and fungi respectively, the overall metabolism of the active microbial population is stimulated and maximised by the addition of a readily usable substrate, as described for SIR. During SIR, the soil samples are simultaneously mixed with inhibitors, which are highly specific to either bacteria or fungi (Anderson and Domcsh, 1975). Applying selective inhibitors to the SIR method can distinguish between prokaryotic and eukaryotic respiration. This method, substrate-induced respiration inhibition (SIRIN), has been used to assess bacterial and fungal contributions to glucose mineralisation in soil respiration (Johnson *et al.*, 1996). Selective inhibition of bacteria and fungi is carried out during the metabolism of the C source to determine the ratio of bacteria to fungi according to the different respiratory results. Bacterial respiration is inhibited by addition of the antibiotic streptomycin. Fungal respiration is inhibited by addition of the antibiotic cycloheximide, which is specific to the respiration of fungi (Ingham and Coleman, 1984).

Cycloheximide is a glutarimide antibiotic that inhibits protein biosynthesis in 80s ribosomes (Raubach *et al.*, 2005). It is used in a wide range of eukaryotic organisms for protein synthesis inhibition, and not only for fungi (Lin and Brookes, 1999). Streptomycin blocks synthesis of protein on the 70s ribosomes and is a basic antibiotic which can be strongly bound and inactivated by soil (Atlas, 1997). Both of these antibiotics are involved in the blocking of protein synthesis by attaching to the 30s subunit of the ribosomes involved in protein synthesis. Because streptomycin and cycloheximide function by inhibiting protein synthesis, microorganisms must be actively growing in order for these antibiotics to be effective (Johnson *et al.*, 1996).

Selective inhibitory techniques used together with SIR have the advantages of identifying an active microbial mineralisation potential and differentiating fungal and bacterial components (Beare *et al*, 1990). There are however, certain limitations to the use of selective inhibition. The antibiotics are often not specific enough, they are often inactivated by soil or degraded by microorganisms (Lin and Brookes, 1999), and even if the antibiotics do suppress the target communities, additional energy becomes available to the non-suppressed organisms for metabolism (Anderson and Domsch, 1975).

2.4.1.2.2. Fungal/bacterial ratios

An important indicator of reestablishment of soil microbial community stability and, hence, ecosystem self-regulation, is the relative proportions of bacterial and fungal biomass (Bardgett and McAlister, 1999). In most soil ecosystems fungi are more prevalent than bacteria and their respiratory contribution is mostly much higher than that of bacteria in the same environment (Anderson and Domsch, 1975; 1980). High fertility and nutrient availability favours the bacterial community, whereas low soil fertility favours fungal dominance. Thus, variations in the soil microbial

communities can be attributed to quantitative and qualitative differences in substrate supply (Grayston *et al.*, 2001). Fungal-to-bacterial ratios increase with decreasing soil pH, therefore respiratory activity of fungi increases under acidic conditions while that of bacteria decreases. Fungi can tolerate low pH better than bacteria, use available CO_2 substrates more efficiently, and play a dominant role in the early decomposition process (Evgenia *et al.*, 1998).

2.4.1.3. Soil microbial community structure

All intact cells contain polar lipids, and polar lipids in microbes are primarily phospholipids. Modern molecular techniques such as the measurement of phospholipids fatty acids (PLFAs) in soil are applied to environmental samples to produce detailed descriptions of the microbial community structure (Peacock *et al.*, 2001). Analyses of PLFAs provide a quantitative measure of the microbial biomass that contains intact cellular membranes and is thus representative of the active microbial population because PLFAs are rapidly turned over (Hill *et al.*, 2000; White *et al.*, 1996). Phospholipid fatty acid profiling is used to describe microbial communities and uses the cell membrane lipids within microorganisms as biomarkers for specific groups of organisms, thereby creating a profile or fingerprint of the microbial community as well as differentiating among environmental samples (Carpenter-Boggs *et al.*, 1998; Steenwerth *et al.*, 2003). These PLFA profiles are also good indicators of environmental changes or disturbances as such disturbances cause rapid changes in soil microbial community structure (Calderon *et al.*, 2000). Phospholipid fatty acid analysis includes the whole microbial community, regardless of activity providing a spectrum of structural diversity (Waldrop *et al.*, 2000). Biodiversity is also an important soil property necessary for recovery from perturbations (Griffiths *et al.*, 2001).

Changes that occur after soil disturbances are usually quite complex in these microbial community structures. Bacteria are known to alter their membrane fatty acid components in response to environmental stresses, thereby generating characteristic PLFA stress signatures such as an increase in the ratio of saturated to unsaturated fatty acids, an increase in the ratio of trans- to cis-monoenoic fatty acids, and an increase in the ratio of cyclopropyl fatty acids to their monoenoic precursors (Kieft *et al.*, 1994). In particular, the proportion of cyclopropyl and trans-fatty acids can increase with changes in environmental or growth conditions, such as starvation, low oxygen tension, or increased temperature (Findlay and Dobbs, 1993). Phospholipid fatty acid analysis can be used to estimate the relative size of fungal, actinomycete, anaerobe, Gram-positive and Gram-negative communities (Waldrop *et al.*, 2000) and does not rely upon the cultivation of microorganisms (Findlay and Dobbs, 1993). Lipids are extracted directly from an entire sample, thus rendering the analysis free from any bias such as that of culturing techniques (Vestal and White, 1989). As such, lipid analysis offers insight into microbial ecology and provides useful information about community structure (Pinkart *et al.*, 1998). According to Findlay and Dobbs (1993) the advantages of lipid analyses are:

- Viable microbial biomass can be assessed from the same sample.
- Results integrate across the entire microbial community.
- There are none of the difficulties inherent in enumerative studies.
- These techniques can be used in sediments not amendable to analysis by microscopy (e.g. coarse sands).
- Relative to visual and molecular techniques, these biochemical methods are time and cost competitive and allow for experimental designs involving scores of analyses.
- The techniques have high precision and as few as three to five replicate samples per treatment may be sufficient.
- If desired, further biochemical characterisation may be made using the samples extracted in these protocols.

Despite the usefulness of the method, appropriate signature PLFAs are not known for all organisms in a soil sample, and since the method relies heavily on signature fatty acids to determine gross community structure, any variation in these signatures could give rise to false community estimates created by artifacts in the method (Haack *et al.*, 1994). Soil is a complex and heterogenous ecosystem and its characterisation poses a significant challenge. Environmental conditions and perturbations are likely to affect population structures and their functions in soils, which may in turn result in a change in overall soil properties (Ibekwe and Kennedy, 1998; Widmer *et al.*, 2001).

2.4.1.4. Vegetation response to environmental perturbations

Any environmental factor that limits productivity or destroys biomass such as salinity, water availability and temperature, are referred to as a stress or disturbance (Grime, 1979). During their development, plants are subjected to various environmental stresses such as drought, salinity or temperature extremes (Parvanova *et al.*, 2004). Plants by their very nature, being embedded in the soil, are unable to escape exposure to these environmental extremes and therefore must respond to survive (Taylor *et al.*, 2003). Soil salinity is one of the major stresses for vegetation, especially in arid and semi-arid regions, and can severely limit plant productivity (Shanon, 1998). The negative effects of salinity on plant growth are associated with low osmotic potential of soil solution (water stress), nutritional imbalance, ion concentration imbalances, or a combination of these factors (Ashraff, 1994; Marschner, 1995). Despite a great deal of research into tolerance of plants to high salinity conditions, the adaptive mechanisms utilised by plants to survive saline stress are still not well understood. Osmotic adjustment in plants subjected to salt stress can occur by the accumulation of high concentrations of either inorganic ions or low molecular weight organic solutes (Ashraff and Harris, 2004), which increase the ability of the plant to retain water but not disturb normal cellular functions (Yancey *et al.*, 1982).

Certain environmental influences can affect photosynthetic activity in plants. One of the most important environmental factors that inhibit photosynthesis is water stress, and many studies have shown how water stress resulted in damage to the oxygen-evolving complex of photosystem II (PS II) (Lu and Zhang, 1998). After drought stress, the photosynthetic rate of the plant is never fully recovered, even after rewatering (Miyashita *et al.*, 2005). The inhibition of photosynthesis by excess light exposure is also a common occurrence in many plants and is caused by visible light at wavelength 400-700nm, which is the same light that drives photosynthesis. This is also referred to as photoinhibition. This phenomenon (photoinhibition) is always characterised by a reduction in efficiency of PS II, and often a significant decrease in rates of photosynthesis in leaves and chloroplasts are observed (Critchley and Russell, 1994).

Plants are also very often sensitive to low temperatures, which slow down the plant metabolism and modify membrane lipids as well as other key molecules (Foyer *et al.*, 2002). For plants that retain and maintain leaves during the winter months, exposure to low temperatures in combination with excessive light can impose a considerable level of stress on the photosynthetic apparatus, thereby lowering the overall rate at which photosynthesis can proceed and the excess energy that cannot be dissipated results in photoinhibition (Adams *et al.*, 1994). In many areas where there are prolonged periods of low temperature as well as short-term chilling, the crucial stages of germination and early vegetative growth are impacted negatively. The ultimate consequence of such unfavourable temperature conditions will be reduced productivity (Haldimann, 1998).

Every environmental change forces the photosynthetic system to adapt by changing its physiological state. This is also reflected in the shape of the fast polyphasic fluorescence transient, which has been shown to change under altered environmental conditions, such as light intensity, temperature, drought, or chemical influences (Strasser *et al.*, 2000). *Chlorophyll-a* fluorescence has often been used as a screening tool for stress tolerance and provides valuable information about the response of the photochemical reaction to stress (Van Heerden *et al.*, 2004). The ability of the chlorophyll molecule not only to absorb light and to carry out the primary photochemical reaction, but also to dissipate part of this energy as fluorescence, makes the fluorescence a very convenient probe for the state of photosynthetic apparatus (Goltsev *et al.*, 2003). All photosynthetic material such as chlorophyll a, exhibit a polyphasic rise of chlorophyll fluorescence transient during the first exposure to illumination. These phases are labeled as O, J, I, P (Strasser *et al.*, 1995). We can therefore evaluate the modifications in PS II photochemistry in water-stressed plants through the JIP test by measuring the polyphasic rise of fluorescence transients (Lu and Zhang, 1998).

Use of chlorophyll fluorescence from intact, attached leaves proved to be a reliable, non-intrusive method for monitoring photosynthetic events and judging the physiological status of the plant (Kocheva *et al.*, 2004). The method is based on the registration of light emitted by PS II, and is

amongst the most perspective biophysical methods to evaluate the physiological state of the plant (Goltsev *et al.*, 2003). Among fluorescence methods, fluorescence induction is the most frequently used because it is sensitive, fast, easily measured, relatively cheap, and provides vast amounts of relevant information concerning photosynthesis (Susila *et al.*, 2004).

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CHAPTER 3

SOIL MICROBIAL ACTIVITY AS ASSESSMENT CRITERIA FOR REHABILITATION OF CO-DISPOSED DIAMOND TAILINGS BY ENZYMATIC ASSAYS AND SUBSTRATE INDUCED RESPIRATION.

ABSTRACT

Disposal of solid waste material as a result of mining activities can cause vast negative environmental impacts with regard to soil quality. South African legislation requires developers to rehabilitate these impacted areas in a sustainable manner. Rehabilitation of solid waste material at Finsch mine in the Northern Cape (De Beers) involves organic matter application, chemical amelioration and the use of different grass seed mixtures. Optimum amounts of chemical ameliorants to be applied during rehabilitation were determined by experimental plots at the mine. The aim of this study was to evaluate the rehabilitation experimental plots by the analysis of the tailings microbial communities as indicators of the status of rehabilitation. Soil quality can be evaluated in terms of physical, chemical and biological soil characteristics. Chemical analysis of the soil was done by 1:2 water extract, as well as ammonium acetate extraction. Potential enzymatic activity (dehydrogenase, β-glucosidase, alkaline and acid phosphatase, and urease) was determined as a measure of potential tailings microbial activity. Carbon dioxide produced by the active microbial biomass was measured by substrate induced respiration (SIR), and provides an indication of microbial activity in the tailings material. Both methods represent soil microbial functional diversity. In terms of overall enzymatic activity (dehydrogenase) application of 90 ton/ha organic material showed the best results. No statistically significant differences were noted among the experimental plots in terms of SIR.

Keywords: Soil quality, sustainability, rehabilitation, soil microbial communities, functional diversity, substrate-induced respiration.

3.1. INTRODUCTION

Soil is one of the earth's most important productive resources, functioning not only in the production of primary produce but also in the maintenance of local, regional, and global environmental quality. It is also the basis of agricultural and of natural plant communities (Doran and Zeiss, 2000). Soil has a direct influence on plant productivity, life support processes such as nutrient cycling, and is the source of microbial diversity which is mostly responsible for biogeochemical cycling of organic matter (Van Bruggen and Semenov, 2000). The processes of decomposition and synthesis of mineral and organic matter occur all the time and they are monitored and activated by a variety of enzymes. All these processes are part of soil metabolism, which is crucial for soil fertility maintenance and preservation (Nowak *et al.*, 2002). Most rehabilitation efforts in the past have focused on agricultural and vegetative production, and have failed to account for the soil component of the ecosystem (Mummey *et al.*, 2002). Land management is sustainable only when it maintains or improves resource quality, specifically the quality of soil (Garcia and Hernandez, 1997). There is of late much more interest and attention on land management strategies which aim at developing an ecosystem to such an extent that it is self reliant rather than depending on fertiliser and organic inputs (Bardgett and McAlister, 1999).

Co-disposed material produced by diamond mines in South Africa is discarded as waste material on slime dams and rock dumpsites (Chamber of Mines, 2003). Legislation in South Africa requires rehabilitation of these disturbed environments in a sustainable manner (South African Environmental Conservation Act 73:1989; National Environmental Management Act 107:1998). Soil quality can be defined as "The capacity of a soil to function within an ecosystem whether it is aggrading or degrading, and sustain biological productivity, maintain environmental quality, and promote plant, animal and human health" (Doran and Parkin, 1994). Soil characteristics can be divided into three categories namely physical, chemical and biological characteristics (Nortcliff, 2002), however the chemical and physical aspects of soil change slowly (6 months -1 year) and take too long to indicate significant change in soil quality. Soil biological properties are very accurate and sensitive indicators of environmental changes and can be used to assess changes in soil quality (Pascual *et al.*, 2000). Rehabilitation and application of amendments, as well as the development of plant cover play an important role in the restoration of the physical, chemical and biological properties of disturbed soils (de Mora *et al.*, 2005). The soil biological component consists mainly of the soil microbial biomass. Bacteria and fungi are the most important with regards to energy flow and nutrient transfer in ecosystems, as well as mineralisation and mobilisation of pollutants in the soil (Schloter *et al.*, 2003). Microbial activity is a general term and includes all metabolic reactions conducted by microorganisms in the soil. The importance of microbial activity in cycling organic matter and regulating active nutrient pools in soils suggests that the effect of disturbance on soil microorganisms is fundamentally related to ecosystem productivity (Brohon *et al.*, 2001). Although soil fertility depends on physical, chemical, and biological factors (Nortcliff, 2002), the

biological factors have not been widely used as indicators of soil quality. However, in the last few years there has been a growing interest in enzymology due to its ecological and biotechnological significance (Marcote *et al.*, 2001). Soil microbial activity can be measured in terms of soil enzyme activity because enzymes catalyse all biological reactions and are an integral part of nutrient cycling and biogeochemical transformations in the soil (Bandick and Dick, 1999). Soil enzymes are therefore commonly used as indicators for the assessment of effects of environmental changes on microbial activity and fertility status of soils (Pati and Sahu, 2003; Vepsalainen *et al.*, 2001). The data obtained from the response of microbial activity to environmental change can be used in modeling biogeochemical cycles (Li and Sarah, 2003). Changes in key functions and activities can also provide information about the progress of remediation operations or the sustainability of particular types of land management (Schloter *et al.*, 2003). Enzyme activity profiles therefore describe the functional diversity of the soil microbial communities (Vepsalainen *et al.*, 2001).

Enzymes in the soil are believed to be primarily of microbial origin but also originate from plants and animals (Bandick and Dick, 1999). A fraction of the soil enzyme activity is associated with living organisms (intracellular), the rest with abiotic components, which are then classified as extracellular (Li and Sarah, 2003). Although most enzymes function extracellularly, soil microorganisms are the main source of enzymes (Aon et al., 2001). The enzymes involved in the main biogeochemical cycles such as dehydrogenase, phosphomonoesterases namely acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1), urease (EC 3.5.1.5) and β-glucosidase (EC 3.2.1.21) were analysed in this study. These enzymes were chosen according to criteria of sensitivity to field management, importance in nutrient cycling, organic matter decomposition, as well as for simplicity of the assays (Bandick and Dick, 1999).

β-glucosidase regulates the supply of glucose as an energy source for microorganisms and breaks down cellulase chains into smaller glucose molecules, which can then be utilised by microbes (Turner *et al.*, 2002). Urease is involved in the N cycle (Bandick and Dick, 1999), and catalyses the hydrolysis of urea to CO_2 and NH_3 (Tabatabai, 1994). Dehydrogenase activity can be considered a direct measure of microbial activity (Trasar-Cepeda *et al.*, 2000; Li and Sarah, 2003) as it is involved in biological oxidation of organic compounds, which forms the basis of all dehydrogenation processes. Dehydrogenase in soil systems is involved in many different metabolic processes and form an integral part of the overall microorganism function in soil. Therefore the result of the assay of dehydrogenase activity would represent general activity of the active population (Tabatabai, 1994). Acid and alkaline phosphatase are involved in the P cycle, where it hydrolyses a variety of phosphomonoesters (Tabatabai, 1994), changing organic P into inorganic P (Li and Sarah, 2003). Arylsulfatase is also important in nutrient cycling because it releases plant available SO_4 (Dick, 1994) by the hydrolysis of organic S compounds to inorganic S (Li and Sarah, 2003).

Substrate induced respiration (SIR) is a measure of the respiration levels of the active microorganisms in soil. A substrate readily used by most microorganisms such as glucose is commonly used to stimulate the maximal respiratory response of the active soil microbial community (Dilly, 2003). Carbon dioxide produced by soil microbial respiration represents the active microbial biomass in soil (Lin and Brookes, 1999). Respective respiratory contributions of bacteria and fungi to the total CO_2 production are distinguished by application of antibiotics, specific to bacterial and fungal respiration, to inhibit either type of microorganism in turn (selective inhibition). In most soil ecosystems fungi is more prominent than bacteria, but the ratio varies from one environment to the next. In the active biological component of soil the fungal respiratory contribution is mostly much higher than that of bacteria in the same environment (Anderson and Domsch, 1975; 1980). Streptomycin specifically inhibits bacteria and cycloheximide specifically inhibits fungi (Atlas, 1997; Raubach *et al.*, 2003). There are many methods to estimate microbial biomass in soils, but of these methods, substrate-induced respiration (SIR) is one of the simplest and most rapid techniques (Cheng and Coleman, 1988). The rate at which fixed carbon substrates are oxidised to CO_2 in a soil sample is proportional to the quantities of organisms mediating the reaction (Tate, 2000), therefore SIR, according to Lin and Brookes (1999), seems to be a reliable method to represent the living component of the microbial biomass.

3.2. MATERIAL AND METHODS

3.2.1. Site description

The experiments conducted in these field trials are an extension of the research conducted at Finsch mine by Van Rensburg *et al.*, (2002) to determine the type and optimum concentration of chemical ameliorants to be applied for rehabilitation of the tailings dumps. This was accomplished by doing pot trials to reach an estimate of the optimum organic material concentrations and the percentage germination as well as the percentage plants that reach full maturity. The second aim of the referred paper was to validate the results from the pot trials by field trials to select the most appropriate grass species to use for rehabilitation, and was achieved by measuring the extent to which seeded species established (Van Rensburg *et al.*, 2002). The experimental plots were established at the mine on a platform with outer walls, which were constructed by using tailings and discard rock. Twenty-four VFPE geo-membrane liners measuring $4m^2$ in surface area and 1m deep were placed on the platform and filled with the co-disposed material. Tailings materials were used to fill the open spaces between the liner bags. The different plots were treated with organic material in the form of vermi-compost at rates of 60 and 90 tonnes per hectare. The plots also received fertiliser treatments of 3:1:5 (N: P: K, with K in the form of KNO_3) which was chlorine free, super-phosphate, $CaNO_3$ and $MgNO_3$ at rates of 625- (both 3:1:5 and super-phosphate), 25- and 12.5kg ha^{-1} respectively and the layout of the different treatment groups is summarised in table 1 (Van Rensburg *et al.*, 2002).

Table 1. Layout of the different treatment groups evaluated for the duration of the study.

Treatment	n	Organic material (tons ha^{-1})	Fertiliser type (A/B)*	Seed mix (1/2)#
Control 1	4	90	–	–
Control 2	4	60	–	–
A (090A1)	2	90	A	1
B (090B1)	2	90	B	1
C (090A2)	2	90	A	2
D (090B2)	2	90	B	2
E (060A1)	2	60	A	1
F (060B1)	2	60	B	1
G (060A2)	2	60	A	2
H (060B2)	2	60	B	2

*Fertiliser treatment A: KNO, 3:1:5 (N: P: K, Cl-free), and Super phosphate (625 kg ha^{-1}).

*Fertiliser treatment B: $CaNO_3$ (25 kg ha^{-1}) and $MgNO_3$ (12.5 kg ha^{-1})

#**Seed mix 1:** *Enneapogon cenchroides, Melinis repens, Chloris virgata, Urochloa brachyura, Eleusine coracana, Cenchrus ciliaris* var. Molopo, *Cenchrus cilliaris* var. Gayndah, *Eragrostis lehmanniana, Panicum maximum, Digitaria eriantha, Cynodon dactylon, Chloris gayana, Bothriochloa insculpta.*

Seed mix 2: *Enneapogon cenchroides, Melinis repens, Chloris virgata, Tragus berteronianus, Aristida congesta, Cenchrus cilliaris* var. Molopo, *Cenchrus cilliaris* var. Gayndah, *Eragrostis lehmanniana, Schmidtia pappophoroides, Fingerhuthia africana, Eragrostis echinochloidea, Cynodon dactylon, Chloris gayana.*

3.2.2. Sampling procedure

Tailings samples were collected (June 2004, 2005) using a random sampling design as suggested by Alef and Nannipieri (1995), where tailings material was removed from five random points in each plot, homogenised, sealed in plastic bags and kept at 4°C for the preservation of biological properties. The material was obtained from depth 0-15cm in the sites according to studies, which show that microbial population and activity decline with depth (Taylor *et al.*, 2002). Twenty-four homogenised samples were collected from the different sites and analysed separately.

3.2.3. Physical and chemical analysis of tailings material

Tailings samples were analysed and physical and chemical characterisation of the tailings material was done for each site by 1:2 water extraction procedure. This included determination of macro and microelements as well as other data such as %C, EC (electrical conductivity) and pH.

3.2.3. Determination of tailings dry mass

The weight of each tailings sample was determined in field moist state and also after drying at 105°C for 24hrs according the method of Alef and Nannipieri (1995). The weight after drying was used to calculate the percentage moisture content of the soil samples.

3.2.4. Measurement of potential soil enzyme activities

Tailings samples were collected and passed through a 2mm sieve. For the determination of dehydrogenase activity, tailings material was kept field moist, while air-dried samples were used for determination of β-glucosidase, urease, and acid (pH 6.5) and alkaline (pH 11) phosphatase. All analyses were carried out in triplicate (Alef and Nannipieri, 1995).

3.2.4.1. Dehydrogenase activity

Field moist tailings material (1.0g) was weighed in 50ml screw cap Erlenmeyer flasks and incubated in the dark for 2 h at 40°C with 1.5ml Tris (hydroxy-methyl)-aminomethane buffer and 2ml iodonitrotetrazolium chloride (INT). Controls were performed with sterilised material (1.0g samples, autoclaved at 121°C for 20min). The reaction was terminated by the addition of 10ml *N,N*-dimethylformamide/ethanol (1:1 v/v) extractant, shaking at 20min intervals for 1 h. The tailings suspension was filtered through Whatman no.2 filter paper and the absorbance of the filtrate was measured at 464nm. The dehydrogenase activity was expressed as μg INF g^{-1} dry weight $2h^{-1}$. Dry weight was determined for each sample as mentioned above and used to calculate true potential dehydrogenase activity.

3.2.4.2. β-Glucosidase and phosphatase activity

β-Glucosidase activity is based on p-nitrophenol release after cleavage of a synthetic substrate (p-nitrophenyl glucoside) (Dick *et al.*, 1996). 1.0g tailings (air-dried) was placed in 50ml screw cap Erlenmeyer flasks and incubated for 1 h at 37°C with 0.25ml toluene, 4ml modified universal buffer (pH 6.0) and 1ml p-nitrophenyl-β-D-glucosidase (PNG). The reaction was terminated by the addition of 1ml 0.5M calcium chloride ($CaCl_2$) and 4ml 0.1M Tris (hydroxy methyl)-aminomethane buffer (pH 12.0). Controls were performed by adding substrate immediately after incubation, before the addition of $CaCl_2$ and Tris-buffer. The tailings suspension was immediately filtered through Whatman no.2 filter paper and the absorbance of the filtrate was measured at 410nm. β-glucosidase activity was expressed as μg p-nitrophenol g^{-1} dry weight h^{-1}

3.2.4.4. Phosphomonoesterase activity

Acid and alkaline phosphatase activities were assayed using the method described by Alef and Nannipieri (1995). Phosphomonoesterase activity is based on p-nitrophenol release after cleavage of a synthetic substrate (p-nitrophenyl phosphate) (Dick *et al.*, 1996). Phosphatase activity was expressed as mg p-nitrophenol g^{-1} dry weight h^{-1}. 1.0g tailings (air-dried) was placed in 50ml screw cap Erlenmeyer flasks and incubated for 1 h at 37°C with 0.25ml toluene, 4ml

modified universal buffer (modified universal buffer pH 6.5 and pH 11.0 were used for acid and alkaline phosphomonoesterase, respectively.) and 1ml p-nitrophenyl-β-D-glucosidase (PNG). The reaction was terminated by the addition of 1ml 0.5M calcium chloride ($CaCl_2$) and 4ml 0.1M Tris (hydroxy methyl)-aminomethane buffer (pH 12.0). Controls were performed by adding substrate immediately after incubation, before the addition of $CaCl_2$ and Tris-buffer. The tailings suspension was immediately filtered through Whatman no.2 filter paper and the absorbance of the filtrate was measured at 410nm. Phosphomonoesterase activity was expressed as μg p-nitrophenol g^{-1} dry weight h^{-1}.

3.2.4.4. Urease activity

Urease was assayed using the procedure as described by Alef and Nannipieri (1995). Air-dried tailings material (5.0g) was incubated with 2.5ml urea solution at 37°C for 2 h. After incubation, 50ml of 1.0M potassium chloride (KCl) solution was added and the flasks shaken for 30min. The tailings suspensions were filtered through Whatman no.2 filter paper and the absorbance of the filtrate measured at 600nm. Controls were prepared with 2.5ml-distilled water and the urea solution was added at the end of the incubation, immediately before the addition of the KCl solution. Urease activity was expressed as μg NH_4-N g^{-1} dry weight $2h^{-1}$.

3.2.5. Measurement of substrate induced respiration (SIR)

The tailings material was sieved (<2mm) and then conditioned (optimum temperature and moisture conditions for optimal microbial activity) at 25°C for 7 days following the adjustment of soil moisture to 40% of the water holding capacity (WHC) (Water holding capacity refers to the maximum amount of moisture contained in a fixed amount of tailings material with no excess runoff). The conditioned soils are then stored at 4°C for not more than 14 days prior to analysis (Lin and Brookes, 1999). The substrate and antibiotics were added as a 2ml solution to 1g equivalent of dry weight of tailings material in McCartney bottles (27.7ml) to prevent moisture from being a limiting factor (West and Sparling, 1986) because most biogeochemical reactions occur in solution. After addition of the solutions, the bottles were sealed immediately and kept at 25°C for 2 h. The incubation time ensured detectable increases in CO_2 in the headspace of soils that responded slowly to substrate additions (Degens and Harris, 1997). 20ml samples of headspace gas was drawn from each sample bottle (vacuum pumping action) and analysed using an infrared gas analyser to measure the CO_2 efflux from the tailings microbial respiration. CO_2 in the headspace was analysed at 1 and 2 h respectively, using the substrate and antibiotic concentrations providing optimum SIR responses (Degens and Harris, 1997). Optimal rates of glucose and antibiotic application were determined and applied as follows. Three portions of twenty-four different samples, each consisting of 10g field moist tailings, amended with glucose solution- 6mg/g moist tailings, glucose solution + streptomycin- 6 mg/g moist tailings, and glucose solution + cycloheximide- 10 mg/g moist tailings.

3.2.6. Statistical analysis

Parametric and non-parametric statistical analyses were performed on all data obtained using STATISTICA 6 (StatSoft, Inc. 2001). The data was tested for normality using the Shapiro-Wilk's test. In the case of data being normally distributed (parametric) a breakdown and one-way ANOVA was performed and the Tukey's honest significant differences (HSD) test was used to determine statistical significance between the various samples. In the case of non-parametric data, non-parametric data analysis was performed and the Kruskal-Wallis ANOVA and Median test was used to determine statistically significant differences between samples. In all cases, there were no differences between the results obtained from the respective normality analyses. As a result, only the parametric analyses of the relevant statistics are discussed.

The relationship between tailings physical and chemical properties and the microbiological variables was investigated using multivariate ordination techniques. Principal Components Analysis (PCA) and Redundancy Analysis (RDA) was performed using CANOCO software (Canoco for Windows Version 4.0, GLW-CPRO ©). Principal Components Analyses were conducted on the tailings physical and chemical variables, as well as on the microbial enzymatic activities in order to determine how these variables were inter-correlated thereby to assess for multicolinearity between the variables. A Redundancy Analysis (RDA) was subsequently performed with the activities of the five enzymes assayed as species dependant variables, and the most significant soil variables as independent environmental factors. The most significant tailings 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 (Claassens *et al.*, 2005).

3.3. RESULTS AND DISCUSSION

3.3.1. Physical and chemical properties of experimental plots

The physical and chemical properties of the co-disposed tailings materials were ordinated with the different treatment regimes in the experimental plots by making use of a PCA. Figure 1 is an ordination diagram of the physical and chemical properties with the different grouping variables within the experimental plots. The eigenvalues for the first two ordination axes were 0.661 and 0.121, respectively. These two axes accounted for 78.2% of the total observed variance.

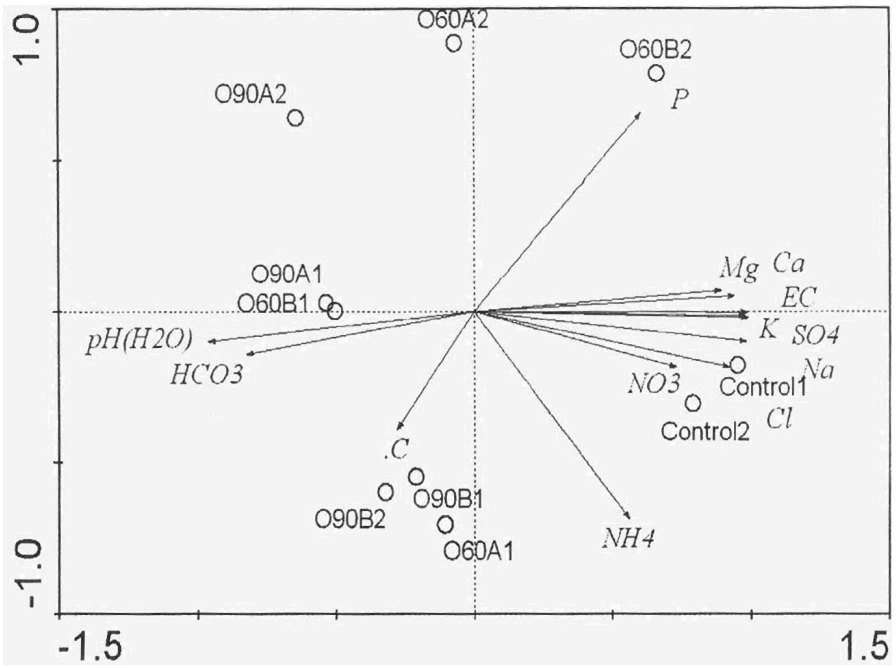

Figure 1. Principal Components Analysis (PCA) ordination diagram of the chemical properties of the various treatment sites.

The physical and chemical properties of the tailings material in the different experimental plots needed to be correlated with the different treatment regimes in order to explain potential enzymatic activities. Chemical components such as elemental C, P and N are of vital importance because the enzymes that were analysed in this experiment are directly involved in the cycling and modification of these elements so that they can be utilised by plants (Tabatabai 1994). Determination of the concentration of these different chemical components (Table 2) in the experimental plots can help to explain the associations that are found between potential enzymatic activity and the different organic and fertiliser applications in the field trial sites. The enzyme activities present in the tailings material represent the microbial community function of the active microbial biomass (Calderon *et al.*, 2000). From the PCA ordination diagram (Figure 1) it is evident that the two control sites and site 060B2 grouped to the right of the first ordination axis and were characterised by higher Mg, Ca, K, Cl, SO_4, NO_3, Na, and electrical conductivity (EC) than the other plots. All the other experimental plots, except site 060B2 grouped to the left of the first ordination axis. Sites 090B1, 090B2 and 060A1 were all characterised by higher levels of organic carbon content (%C), which can be expected in the plots treated with 90-ton/ha organic matter (vermi-compost).

Table 2. Summary of physical and chemical analyses of experimental plots

Treatments	Control1	Control2	O60A1	O60A2	O60B1	O60B2	O90A1	O90A2	O90B1	O90B2
Chemical Analysis										
Ca (mmol/L)	6.35 ± 2.27[a]	8.05 ± 2.34[a]	3.56 ± 3.34[a]	1.97 ± 1.52[a]	0.94 ± 0.68[a]	8.8 ± 1.3[a]	1.125 ± 0.92[a]	0.34 ± 0.11[a]	2.29 ± 2.05[a]	1.13 ± 0.39[a]
Mg (mmol/L)	2.05 ± 0.77[a]	2.33 ± 0.71[a]	1.09 ± 0.26[a]	0.79 ± 0.38[a]	0.63 ± 0.2[a]	2.61 ± 0.14[a]	0.95 ± 0.42[a]	0.58 ± 0.27[a]	1.43 ± 0.11[a]	0.35 ± 0.10[a]
K (mmol/L)	3.99 ± 0.90[a]	4.19 ± 0.78[a]	2.32 ± 1.95[a]	2.54 ± 1.17[a]	1.42 ± 1.00[a]	3.86 ± 0.26[a]	1.51 ± 0.84[a]	0.83 ± 0.37[a]	2.33 ± 1.81[a]	1.72 ± 0.08[a]
Na (mmol/L)	21.87 ± 6.36[a]	19.46 ± 3.80[a]	9.71 ± 9.20[a]	10.22 ± 5.99[a]	5.32 ± 4.60[a]	15.20 ± 0.60[a]	4.86 ± 4.35[a]	2.91 ± 2.14[a]	8.18 ± 6.93[a]	8.60 ± 0.38[a]
P (mmol/L)	0.01 ± 0.00[a]	0.01 ± 0.00[a]	0.014 ± 0.00[a]	0.01 ± 0.00[a]	0.02 ± 0.01[a]	0.01 ± 0.00[a]	0.03 ± 0.02[a]	0.01 ± 0.00[a]	0.02 ± 0.01[a]	0.014 ± 0.00[a]
SO_4 (mmol/L)	19.10 ± 5.79[a]	20.36 ± 4.64[a]	9.75 ± 9.42[a]	8.57 ± 5.60[a]	4.10 ± 3.57[a]	19.60 ± 1.85[a]	4.47 ± 3.48[a]	1.98 ± 1.37[a]	7.85 ± 6.32[a]	6.01 ± 0.66[a]
NO_3 (mmol/L)	1.15 ± 0.64[a]	0.37 ± 0.29[a]	0.23 ± 0.09[a]	0.04 ± 0.02[a]	0.058 ± 0.03[a]	0.14 ± 0.06[a]	0.059 ± 0.03[a]	0.06 ± 0.04[a]	0.05 ± 0.01[a]	0.03 ± 0.02[a]
NH_4 (mmol/L)	0.04 ± 0.01[a]	0.05 ± 0.01[a]	0.04 ± 0.01[a]	0.03 ± 0.00[a]	0.03 ± 0.00[a]	0.03 ± 0.01[a]	0.03 ± 0.00[a]	0.02 ± 0.00[a]	0.03 ± 0.01[a]	0.04 ± 0.01[a]
Cl (mmol/L)	2.13 ± 1.10[a]	1.97 ± 0.69[a]	0.38 ± 0.10[a]	0.15 ± 0.04[a]	0.25 ± 0.02[a]	1.05 ± 0.18[a]	0.11 ± 0.05[a]	0.16 ± 0.07[a]	0.63 ± 0.49[a]	0.35 ± 0.12[a]
HCO_3 (mmol/L)	0.86 ± 0.11[a]	1.09 ± 0.24[a]	1.25 ± 0.70[a]	1.03 ± 0.23[a]	1.48 ± 0.48[a]	0.83 ± 0.28[a]	1.48 ± 0.58[a]	1.45 ± 0.50[a]	1.30 ± 0.35[a]	1.05 ± 0.15[a]
EC (mS/cm)	4.24 ± 1.31[a]	4.42 ± 0.99[a]	2.15 ± 1.84[a]	1.84 ± 1.10[a]	1.00 ± 0.66[a]	4.12 ± 0.33[a]	1.07 ± 0.63[a]	0.57 ± 0.22[a]	1.78 ± 1.28[a]	1.35 ± 0.14[a]
pH (H_2O)	8.03 ± 0.37[a]	8.37 ± 0.17[a]	8.91 ± 0.58[a]	8.71 ± 0.21[a]	8.98 ± 0.28[a]	8.25 ± 0.07[a]	8.92 ± 0.27[a]	9.0 ± 0.19[a]	8.77 ± 0.43[a]	8.81 ± 0.00[a]
% Organic carbon	0.75 ± 0.17[a]	0.82 ± 0.18[a]	0.77 ± 0.04[a]	0.58 ± 0.18[a]	0.75 ± 0.06[a]	0.76 ± 0.07[a]	0.77 ± 0.28[a]	0.97 ± 0.06[a]	0.95 ± 0.01[a]	0.89 ± 0.33[a]

Values given are mean ± standard error

Sites with the same combination of superscript alphabetic letters indicate no significant differences among sites

Table 3. Summary of potential enzyme activity in experimental plots

Enzyme	Control1	Control2	O60A1	O60A2	O60B1	O60B2	O90A1	O90A2	O90B1	O90B2
Potential enzymatic activity 2004										
Dehydrogenase ($INFg^{-1}2h^{-1}$)	59.30 ± 38.42[a]	138.65 ± 41.95[a]	20.72 ± 1.89[a]	16.82 ± 7.20[a]	47.13 ± 15.42[a]	142.06 ± 44.99[a]	64.70 ± 33.78[a]	48.09 ± 3.72[a]	38.65 ± 30.10[a]	13.57 ± 0.95[a]
ß-Glucosidase ($PNPg^{-1}h^{-1}$)	2635122.17 ± 968316.36[a]	1776944.34 ± 932021.67[a]	2282801.13 ± 1374898.93[a]	3962052.07 ± 1786860.90[a]	1644553.02 ± 24076.67[a]	1764533.31 ± 322512.22[a]	2519346.39 ± 441124.09[a]	2156923.08 ± 498630.55[a]	2528027.45 ± 1339383.83[a]	2252287.64 ± 0.00[a]
Alkaline-phosphatase ($PNPg^{-1}h^{-1}$)	16303356.72 ± 1305098.56[a]	15618802.01 ± 95871.46[a]	18294871.95 ± 2976564.66[a]	18876382.66 ± 2928224.83[a]	13882054.99 ± 1921657.33[a]	18361810.92 ± 444899.71[a]	17021002.20 ± 1259723.51[a]	19964496.64 ± 3509236.49[a]	21606397.13 ± 2892206.42[a]	18882020.42 ± 1075588.99[a]
Acid-phosphatase ($PNPg^{-1}h^{-1}$)	5490815.28 ± 1069366.15[a]	5300934.45 ± 715602.31[a]	4074224.46 ± 123429.06[a]	4836899.43 ± 609511.34[a]	3617807.21 ± 671372.37[a]	4147797.92 ± 980845.62[a]	5119267.47 ± 1223546.75[a]	9970449.07 ± 3425245.56[a]	6828125.24 ± 2288285.16[a]	8287849.72 ± 2071801.31[a]
Urease ($NH4\text{-}Ng^{-1}2h^{-1}$)	2.33 ± 1.60[a]	3.40 ± 0.89[a]	3.24 ± 2.63[a]	10.57 ± 5.73[a]	3.88 ± 2.89[a]	1.76 ± 1.52[a]	8.62 ± 6.42[a]	6.76 ± 2.25[a]	2.65 ± 0.98[a]	8.63 ± 0.50[a]
Potential enzyme activity 2005										
Dehydrogenase ($INFg^{-1}2h^{-1}$)	37.66 ± 3.29[a]	34.48 ± 7.01[a]	7.50 ± 0.94[a]	41.89 ± 7.41[a]	42.49 ± 1.76[a]	33.69 ± 15.96[a]	28.76 ± 21.15[a]	55.32 ± 18.17[a]	61.60 ± 16.76[a]	16.52 ± 7.30[a]
ß-Glucosidase ($PNPg^{-1}h^{-1}$)	155503.40 ± 21280.44[a]	108363.75 ± 31188.58[a]	77517.20 ± 16970.44[a]	73892.88 ± 10509.47[a]	25164.78 ± 7292.76[a]	147202.58 ± 43599.81[a]	114021.82 ± 38522.03[a]	147842.70 ± 72146.03[a]	192290.94 ± 133254.19[a]	110992.56 ± 19992.18[a]
Alkaline-phosphatase ($PNPg^{-1}h^{-1}$)	418436.90 ± 70213.94[a]	485504.61 ± 79664.85[a]	380878.45 ± 172677.31[a]	602744.50 ± 164026.50[a]	365407.35 ± 169755.55[a]	196007.69 ± 39807.41[a]	299800.75 ± 2993.69[a]	418656.83 ± 158203.95[a]	404684.16 ± 42195.14[a]	511711.52 ± 73225.02[a]
Acid-phosphatase ($PNPg^{-1}h^{-1}$)	733188.70 ± 124906.47[a]	402824.08 ± 116314.27[a]	272173.10 ± 142433.86[a]	348596.65 ± 214298.06[a]	487664.20 ± 221212.73[a]	509575.86 ± 72162.94[a]	526357.73 ±306548.84[a]	532942.93 ± 140190.54[a]	717070.69 ± 349027.61[a]	160896.62 ± 6632.82[a]
Urease ($NH4\text{-}Ng^{-1}2h^{-1}$)	5.14 ± 0.99[a]	5.12 ± 0.39[a]	4.17 ± 0.84[a]	2.49 ± 0.14[a]	2.62 ± 2.05[a]	5.63 ± 0.71[a]	5.33 ± 0.72[a]	4.75 ± 1.60[a]	4.99 ± 1.54[a]	3.72 ± 1.16[a]

Values given are mean ± standard error

Sites with the same combination of superscript alphabetic letters indicate no significant differences among sites

3.3.2. Enzymatic activity

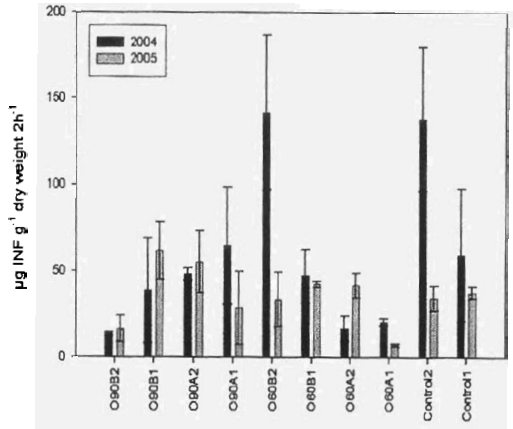

Figure 2. Dehydrogenase activity

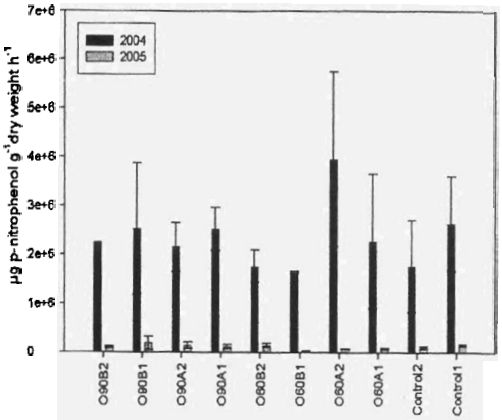

Figure 3. β-glucosidase activity

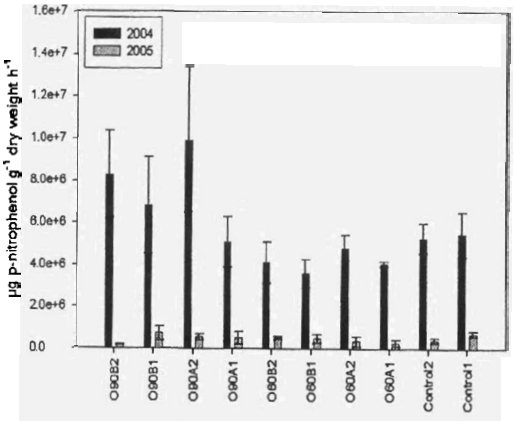

Figure 4. Acid phosphatase activity

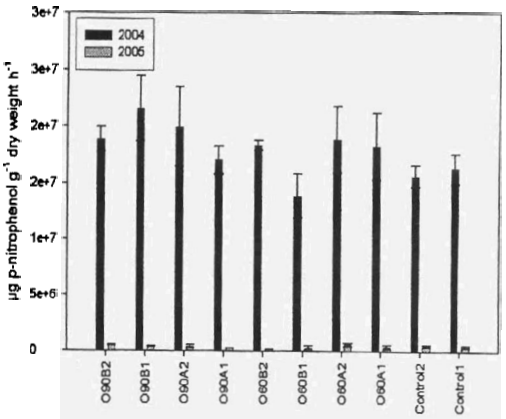

Figure 5. Alkaline phosphatase activity

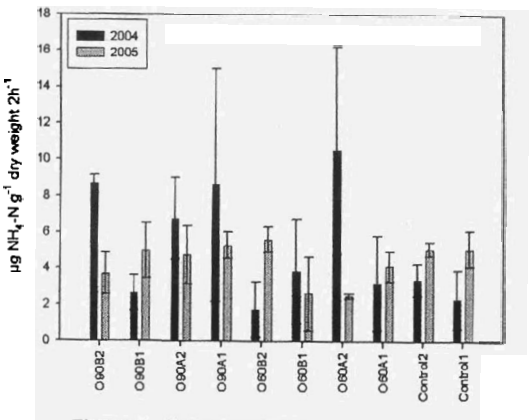

Figure 6. Urease activity

The highest dehydrogenase activity (Table 3, Figure 2) for 2004 was found in site 060B2. Higher dehydrogenase activity was also found in the experimental plot Control2 (60 tons/ha vermi-compost application). These two treatments sites had higher levels of dehydrogenase

activity than all the other experimental plots. Dehydrogenase activity (Table 3, Figure 2) for 2005 seemed to be more evenly distributed between the different sites. There were statistically significant differences ($p<0.05$) in dehydrogenase activity although higher general dehydrogenase activity was found in experimental plots 090B1 and 090A2 possibly indicating higher overall microbial activity in experimental plots treated with 90-ton/ha vermi-compost.

The highest β-glucosidase activity for 2004 was found in site 060A2 (Table 3, Figure 3). Overall, the experimental plots treated with 90-tons/ha vermi-compost applications showed higher β-glucosidase activity than the sites treated with 60-tons/ha vermi-compost. The controls that received no inorganic fertilisers showed no significant difference ($p>0.05$) in β-glucosidase activity compared to the rest of the experimental plots. Similar activity for β-glucosidase (2005) was found in all sites. Overall β-glucosidase activity was much lower in 2005 than in 2004. The 90-tons/ha vermi-compost application field trials in 2005 showed higher β-glucosidase activity than the 60-tons/ha vermi-compost experimental plots. Despite general decreasing β-glucosidase activity in 2005, β-glucosidase activity seems to be similarly present in all the sites, possibly indicating stable biogeochemical cycles. Increased β-glucosidase activity was found in experimental plots 090B1 and 090A2 for 2005. The control sites also had slightly higher β-glucosidase activity than the other sites.

For 2004, experimental plot 090A2 showed the highest acid phosphatase (9970449.07μg p-nitrophenol g^{-1} dry weight h^{-1}) activity in the tailings material (Table 3, figure 4). A general trend can be seen, where sites, which were treated with 90-tons/ha vermi-compost showed higher levels of acid phosphatase activity compared to the experimental plots treated with 60-tons/ha vermi-compost. The control sites both displayed similar enzymatic activity for acid phosphatase even though they received different amounts of organic material (control1 received 90ton/ha, control2 received 60ton/ha vermi-compost). The highest acid phosphatase activity for 2005 (733188.70 μg p-nitrophenol g^{-1} dry weight h^{-1}) was found in experimental plots control 1, which was treated with 90 ton/ha vermi-compost, however site 090B2 showed the lowest acid phosphatase activity (160896.62 μg p-nitrophenol g^{-1} dry weight h^{-1}) for all the experimental plots. General acid phosphatase activity was much less in 2005 than in 2004, and showed no statistically significant difference between the experimental plots ($p>0.05$).

Alkaline phosphatase activity (2004) (Table 3, Figure 5) showed no statistically significant differences ($p>0.05$) between the experimental plots, which might suggest that the enzyme alkaline phosphatase is abundant in the tailings material at this stage. The soil pH is also favourable for existence of alkaline phosphatase (pH 7.8) in the tailings material. For 2005, alkaline phosphatase activity was generally similar in all the trial sites but lower than in 2004 (Table 3, Figure 5). Alkaline phosphatase enzyme activity in the control sites showed no

significant difference compared to the other experimental plots, which were treated with inorganic fertilisers.

The field enzyme urease (2004) showed the highest activity in treatment site 060A2 (10.57 μg NH_4-N g^{-1} dry weight $2h^{-1}$). The experimental plots that received 90-tons/ha vermi-compost all had higher urease activity compared to the experimental plots treated with 60-tons/ha vermi-compost. Both the control sites displayed lower urease activity than the other plots even though control 1 had vermi-compost application of 90 tons/ha. In 2005 there was increased urease activity (Table 2, Figure 6) in most of the experimental plots compared to 2004, including the control sites. Experimental plots such as 090B1, 060B2 and control 1 that showed the lowest urease activity in 2004, showed increased urease activity in 2005. Overall urease activity seemed to increase in 2005 and all the experimental plots showed similar amounts of activity.

An RDA ordination (Figure 7) is used to show the relationship between the chemical and physical properties of the tailings material and potential enzymatic activity for the different experimental plots in 2004. Eigenvalues (Figure 7) for the first two axes were 0.466 and 0.227, respectively and the total observed variance for the first two axes was 69.3%. The first axis associated with %C (r^2 = -0.298) and the second axis with NH_4 (r^2= 0.279). The effect of the specific environmental variables on the enzymatic activities was not statistically significant (p = 0.708).

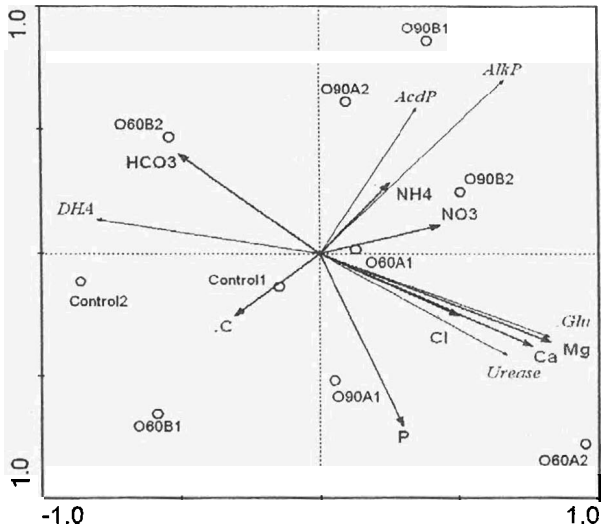

Figure 7. Redundancy Analysis (RDA) ordination diagram illustrating the relationship between the physical and chemical properties of tailings material and potential enzymatic activities for all the field trials. Representative enzymes are dehydrogenase (DHA), acid phosphatase (AcdP), alkaline phosphatase (AlkP), ß-glucosidase (Glu), and urease.

A redundancy analysis (RDA) ordination (Figure 8) displaying the relationship between the chemical and physical properties of the tailings material and potential enzymatic activity for the different experimental plots in 2005. Eigenvalues (Figure 8) for the first two axes were 0.546 and 0.225, respectively and the total observed variance for the first two axes was 77.1%. The first axis associated with NH_4 (r^2= 0.249), and the second axis with %C (r^2= -0.161). The effect of the specific environmental variables on the enzymatic activities was not statistically significant (p = 0.202).

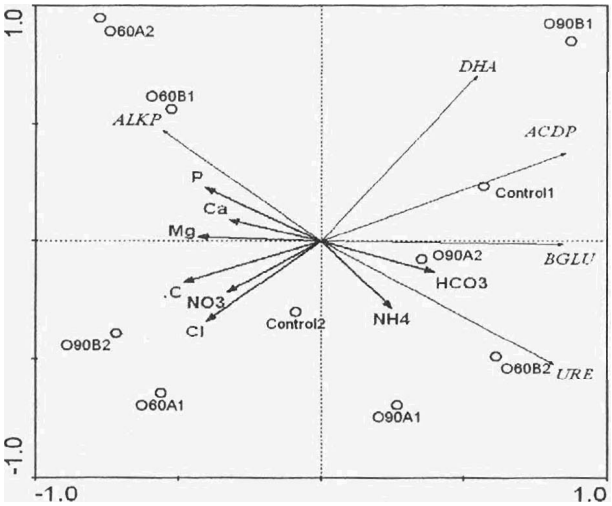

Figure 8. Redundancy Analysis (RDA) ordination diagram illustrating the relationship between the physical and chemical properties of tailings material and potential enzymatic activities for all experimental plots in 2005. Representative enzymes are dehydrogenase (DHA), acid phosphatase (AcdP), alkaline phosphatase (AlkP), ß-glucosidase (Glu), and urease

Experimental plots 060B2 and control2 (60-ton/ha vermi-compost) were characterised by higher dehydrogenase activity compared to the other plots (Figure 7). Experimental plots 060B1 and control 1 also showed higher dehydrogenase activity than the rest of the sites but not as much as sites 060B2 and control 2. The higher dehydrogenase activity in the 60-ton/ha vermi-compost experimental plots could mean that there was higher overall microbial activity in these sites than in the other experimental plots because dehydrogenase is representative of microbial activity as a whole (Li and Sarah, 2003). An association is also visible between dehydrogenase activity and organic C content in experimental plots 060B2, 060B1, control 1 and control 2, which can be expected since carbon is critical for microbial growth and proliferation. Positive correlation between acid and alkaline phosphatase activity in experimental plots 090A2 and 090B2 can be observed (Figure 7). These treatments sites were also characterised by higher NO_3 levels than in the other experimental plots. Generally, β-glucosidase activity is directly related to the carbon cycle and energy production for

microorganisms (Turner *et al.*, 2002). Urease activity and organic P association characterised experimental plots 090A1 and 060A2 (Figure 7).

In 2005, experimental plots 090B1 and control 1 were both characterised by higher levels of dehydrogenase activity compared to the other sites (Figure 8, Table 3), which is possibly due to the higher organic amendments in these sites (90 tons/ha) leading to more extensive development of biogeochemical cycles. Experimental plot control 1 was also characterised by high dehydrogenase activity in 2004 (Figure 7, Table 3). Dehydrogenase activity, which lies to the right of the first ordination axis, had no positive association with any of the environmental variables lying to the left of the first axis (Figure 8), except for NH_4 and HCO_3, which showed very weak association with dehydrogenase activity. The lower presence of dehydrogenase activity is a possible indication that microbial activity was generally lower in all the experimental plots.

There was strong association between alkaline phosphatase activity and organic P in experimental plots 060A2 and 060B1 (Figure 8). The association between organic P levels and alkaline phosphatase activity could be expected seeing that the pH of the co-disposed material ranges from pH 7.7 – pH 9.2 (Table 2) providing an ideal environment for optimal utilisation of phosphate substrates by alkaline phosphatase. Alkaline phosphatase activity lies to the left of the first ordination axis, positively correlated with most of the environmental variables, whilst all the other enzymatic activities were grouped to the right of the axis. Higher alkaline phosphatase activity could be expected in the plots treated with superphosphates. The most acid phosphatase activity was found in plot control 1 (Figure 8, Table 3) and slightly less acid phosphatase activity in plot 090B1. ß-glucosidase activity correlated strongly with organic C content which characterised experimental plots 090A2 and control 1, possibly due to the higher organic material application in these sites (90-tons/ha vermi-compost). ß-glucosidase is directly involved in the carbon cycle (Turner *et al.*, 2002) and higher activity levels are usually indicative of higher substrate levels in the soil. Experimental plots 090A1, 060B2 and 090A2 were all characterised by a positive association between NH_4 levels and urease activity in the tailings material. Urease is involved in the N-cycle and transforms urea into plant available nitrates (Tabatabai, 1994). Experimental plots 090B2 and 060A1 were characterised by a positive association between percentage C and NO_3, but showed no statistically significant differences in enzymatic activity.

3.3.3. Substrate-induced respiration measurements

The different treatments of the experimental plots were compared to each other in terms of soil microbial respiration. CO_2 measurements were used as representative of microbial C in the soil to determine the active microbial component, and selective inhibition was applied to determine fungal and bacterial contributions to soil respiration respectively.

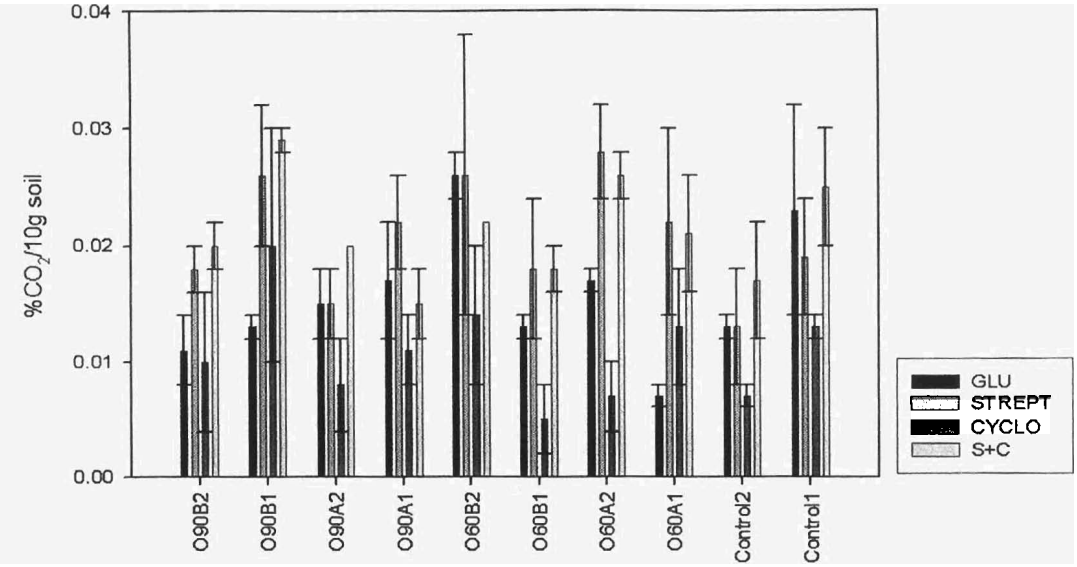

Figure 9. Substrate induced respiration and selective inhibition measurements

However, statistical analyses indicated no significant differences in the data ($P>0.05$) (Figure 9). Bacterial and fungal contributions to soil respiration, respectively, could not be clearly differentiated. There was almost no inhibition of CO_2 production when soils were treated with streptomycin alone. However, cycloheximide application slightly inhibited microbial respiration implying that the fungal communities in the tailings material may be more abundant than bacterial communities and contribute the larger part of basal respiration occurring in the tailings material as was found in the case study of Anderson and Domsch (1980). Both the antibiotics and the glucose were added in solution because the tailings from the experimental plots tended to have low water content. For efficient distribution of the antibiotics and glucose in the soil samples, addition in solution produced better results. Respiratory activity also increases with enough moisture present (West and Sparling, 1986).

3.4. CONCLUSION

Soil consists of complex interactions between many natural processes and integrates physical, chemical and biological properties into a functional unit (Nortcliff, 2002). The soil biological component is vital to soil quality, and microorganisms mediate most biogeochemical transformations in soil such as organic matter degradation and decomposition (Brohon *et al.*, 2001). Many soil biological parameters could be used as indicators of soil quality, however no single parameter could represent soil quality as a whole (Schloter *et al.*, 2003). All soil characteristics need to be considered and integrated to form a broader measure of soil status. Potential microbial activity is responsible for nutrient cycling and availability for plant uptake (C, N, P and S cycles) (Pati and Sahu, 2003), therefore

measurement of microbial activity (enzymatic activity and SIR) in the tailings material will reflect the status of the ecosystem (Schloter *et al.*, 2003).

Enzyme activity in this study was clearly linked to tailings physical and chemical properties such as % organic C, and organic P and N levels. It is therefore advisable for land management strategies to firstly rectify chemical imbalances in the soil to form the basis for development of healthy microbial populations. It is evident from the results that potential enzyme activity was greater in field trials treated with more organic matter (90 tons/ha vermi-compost) than in sites treated with 60-tons/ha vermi-compost. Although potential enzyme activity in general decreased with time, biogeochemical cycles seemed to be well established because of evenly distributed enzymatic activity in all the treatment sites. Potential microbial enzymatic activity could be used as one of many assessment criteria's combined to assess the rehabilitation of tailings material. However, due to lack of significant statistical differences in microbial activity among the different experimental plots, land management recommendations cannot yet be made from the study. Substrate induced respiration measurements also showed no statistically significant differences between experimental plots and therefore cannot be used to make any comparison between different rehabilitation strategies for this experiment.

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CHAPTER 4

SOIL MICROBIAL COMMUNITY STRUCTURE AS ASSESSMENT CRITERIA FOR REHABILITATION OF CO-DISPOSED DIAMOND TAILINGS BY PHOSPHOLIPID EXTRACTION, FRACTIONATION AND ANALYSIS

ABSTRACT

Disposal of solid waste material as a result of mining activities can cause vast negative environmental impacts with regard to soil ecosystems. South African legislation requires developers to rehabilitate these impacted areas in a sustainable manner. Rehabilitation of solid waste material at Finsch mine (De Beers) in the Northern Cape involves application of vermi-compost and inorganic fertilisers, as well as seeding with different grass seed mixtures. Field trials were conducted at the mine to determine the optimum amounts of organic and inorganic material to be applied during the rehabilitation of the solid waste material. The aim of this study was to assess rehabilitation by the analysis of soil microbial community structure using phospholipid fatty acids (PLFAs) as signature lipid biomarkers for different microbial groups. The tailings material was evaluated in terms of physical, chemical and biological properties. Chemical analysis of the soil was done by the 1:2 water extract technique, as well as the ammonium acetate extraction Procedure. Overall, the experimental plots that recieved 60-ton/ha vermi-compost showed higher estimated viable microbial biomass than the plots treated with 90-ton/ha vermi-compost. Fungal to bacterial ratios, which were initially lower in 2004, seemed to increase in 2005.

Keywords: lipid biomarkers, microbial community structure, rehabilitation, soil microbial communities, soil quality.

4.1. INTRODUCTION

Soil is the source of microbial diversity which is mostly responsible for biogeochemical cycling of organic matter and has a direct influence on plant productivity and life support processes such as nutrient cycling (van Bruggen and Semenov, 2000). It is also one of the earth's most important productive resources, functioning not only in the production of primary produce but also in the maintenance of environmental and ecosystem quality and forms the basis of agricultural and natural plant communities (Doran and Zeiss, 2000). Co-disposed material produced by diamond mines in South Africa is discarded as waste material on tailing dams and rock dumps. Legislation in South Africa requires rehabilitation of these disturbed environments in a sustainable manner (South African Environmental conservation Act 73:1989; National Environmental Management Act (NEMA) 107:1998) and mining closure is granted according to accepted measures of ecological rehabilitation of the relevant sites.

Most rehabilitation efforts in the past have made use of different environmental indicators, which reflect the state of soil quality (Pankhurst *et al.*, 1995). Doran and Parkin (1994) defined soil quality as "the capacity of a soil to function within an ecosystem and sustain biological productivity, maintain environmental quality and promote plant, animal and human health". Nortcliff (2002) divides soil properties into three categories namely physical, chemical and biological characteristics. However, the chemical and physical aspects of soil change too slowly to show significant change in soil quality. Soil biological properties are very accurate and sensitive indicators to environmental changes and can be used to assess changes in soil quality (Pascual *et al.*, 2000). The rehabilitation and application of amendments, as well as the development of plant cover, play an important role in the restoration of the physical, chemical and biological properties of disturbed soils (De Mora *et al.*, 2005). The soil biological component consists mainly of the soil microbial biomass. Bacteria and fungi are the most important with regards to energy flow and nutrient transfer in ecosystems. Fungi and bacteria generally dominate in the soil microbial biomass and are responsible for the liberation of nutrients available for plants but also to the mineralisation and mobilisation of pollutants in the soil (Schloter *et al.*, 2003). An important indicator of reestablishment of soil microbial community stability is the relative proportions of bacterial and fungal biomass. The estimation of fungal and bacterial contributions to the total microbial biomass remains important to understanding processes of plant residue decomposition and nutrient cycling (Bardgett and McAlister, 1999).

Microbial activity is a general term and includes all metabolic reactions conducted in the soil. The importance of microbial activity in cycling organic matter and regulating active nutrient pools in soils suggests that the effect of disturbance on soil microorganisms is fundamentally related to ecosystem productivity (Brohon *et al.*, 2001). Although soil fertility depends on physical, chemical, and biological factors of the soil environment (Nortcliff, 2002), the biological factors have never been widely used as indicators of soil quality (Marcote *et al.*, 2001). All intact cells

contain polar lipids, and polar lipids in microbes are primarily phospholipids. Analysis of phospholipid fatty acids (PLFAs) provides a quantitative measure of the microbial biomass that contains intact cellular membranes, and is thus representative of the active microbial population (White *et al.*, 1996). PLFAs are rapidly turned over upon cell death and thus represent the current living community, both qualitatively and quantitatively. Fatty acid profiling is used to describe microbial communities and to differentiate among environmental samples by their fatty acid "fingerprint" (Carpenter-Boggs *et al.*, 1998). These PLFA profiles are also good indicators of environmental changes or disturbances (Frostegard *et al.*, 1996) because such disturbances cause rapid changes in soil microbial community structures (Widmer *et al.*, 2001). The changes that occur after soil disturbances are usually quite complex in these microbial community structures (Calderon *et al.*, 2000).

Phospholipids, the major components of cell membranes, have been used to estimate the biomass of microorganisms and several phospholipid fatty acids (PLFAs) have been found to be indicative of specific microorganisms. Bacterial PLFAs characteristically consist of the following fatty acids: 18:1ω7, i17: 1ω7, 17:0cyc, 19:0cyc, 17:0, i15:0, a15:0 and i16:0. The PLFA 19:0cyc is mainly found in actinomycetes and 18:2ω6 is indicative of fungi although small quantities of 18:2ω6 also occur in some bacteria (Arao, 1999). The analysis of PLFAs 16:1ω7c, 18:1ω7, 16:1ω5, cy17:0 and cy19:0 are indicative of gram-negative bacteria (Soderberg *et al.*, 2004). Phospholipids fatty acids (PLFAs) maintain cell fluidity, enabling the transport of nutrients into the cell and the elimination of metabolic products. Soil bacteria can be divided into two groups based on high content of either branched-chain fatty acids or monoenic and cyclopropane fatty acids. The former would indicate Gram-positive bacteria and the latter Gram-negative ones. Measuring the ratio of cis/trans fatty acids can also monitor the nutritional status of some bacteria (Ponder and Tadros, 2002). The PLFA method might be a reliable way for measuring the total microbial biomass and additionally, it gives information of the different microbial groups (e.g. Gram-positive, Gram-negative, anaerobes, eukaryotes). Phospholipids constitute an important part of all cell membranes, and the composition of PLFAs can be taken to provide a fingerprint of the microbial community structure. The identification of specific PLFAs also allows for the estimation of fungal and bacterial biomass (Griffiths *et al.*, 1999). Different groups of bacteria are characterised by specific PLFAs; therefore a change in phospholipid profiles in soil would indicate a change in the microbial community of that soil (Ibekwe and Kennedy, 1998). Lipids are also one of the most useful biochemical measures of in situ interactions between microbial species and their environments because lipid compositions can indicate temperature-, redox-, stress-, or nutritional conditions (Zhang *et al.*, 2004). It is, however, difficult to relate changes in PLFA patterns to certain groups of microorganisms because dominating PLFAs exist in a wide range of microbial species. But by making use of multivariate statistical methods such as Principal Component Analysis (PCA) and Redundancy Analysis (RDA), it is possible to separate soil microorganisms on the basis of their fatty acid profiles (Frostegard *et al.*, 1996).

4.2. MATERIALS AND METHODS

4.2.1. Site details

The comparative study of the experimental field trials is a continuation of a project conducted by Van Rensburg *et al.*, (2002) to determine the type and optimum concentration of chemical ameliorants to be applied for rehabilitation of the tailings material experimental plots. Pot trials were used to get an estimate of the optimum organic material concentrations and the percentage germination and the percentage of plants (mostly grass species) that reach full maturity. The previous study then proceeded to validate the results from the pot trials by field trials to select the most appropriate grass species to use for rehabilitation, which would be achieved by analysis of the success by which seeded species established during the field trials (Van Rensburg *et al.*, 2002). Experimental plots were established at the mine on a platform with outer walls, which were constructed by using tailings and discard rocks. Twenty-four VFPE geo-membrane liners measuring $4m^2$ in surface area and 1m deep were placed on the platform and filled with the co-disposed tailings material. Tailings material was used to fill the open spaces in between the liner bags. The different plots were treated with organic material in the form of vermi-compost at rates of 60 and 90 tonnes per hectare.

Table 1. Layout of the different experimental plots evaluated for the duration of the study.

Treatment	n	Vermi-compost (tons ha^{-1})	Chemical amelioration (A/B)*	Seed mix (1/2)#
Control 1	**4**	**90**	–	–
Control 2	**4**	**60**	–	–
A (090A1)	**2**	**90**	**A**	**1**
B (090B1)	2	90	B	1
C (090A2)	2	90	A	2
D (090B2)	2	90	B	2
E (060A1)	2	60	A	1
F (060B1)	2	60	B	1
G (060A2)	2	60	A	2
H (060B2)	**2**	**60**	**B**	**2**

*Fertiliser treatment A: KNO, 3:1:5 (N: P: K, Cl-free), and Super phosphate (625 kg ha^{-1}).

*Fertiliser treatment B: $CaNO_3$ (25 kg ha^{-1}) and $MgNO_3$ (12.5 kg ha^{-1})

#**Seed mix 1:** *Enneapogon cenchroides, Melinis repens, Chloris virgata, Urochloa brachyura, Eleusine coracana, Cenchrus ciliaris* var. Molopo, *Cenchrus ciliaris* var. Gayndah, *Eragrostis lehmanniana, Panicum maximum, Digitaria eriantha, Cynodon dactylon, Chloris gayana, Bothriochloa insculpta.*

Seed mix 2: *Enneapogon cenchroides, Melinis repens, Chloris virgata, Tragus berteronianus, Aristida congesta, Cenchrus ciliaris* var. Molopo, *Cenchrus ciliaris* var. Gayndah, *Eragrostis lehmanniana, Schmidtia pappophoroides, Fingerhuthia africana, Eragrostis echinochloidea, Cynodon dactylon, Chloris gayana*

The plots also received fertiliser treatments of 3:1:5 (N: P: K, with K in the form of KNO_3) which was chlorine free, super-phosphate, $CaNO_3$ and $MgNO_3$ at rates of 625- (both 3:1:5 and super-phosphate), 25- and 12.5kg ha^{-1} respectively and the layout of the different treatment groups is summarised in table 1 (Van Rensburg *et al.*, 2002). No further addition on organic or inorganic fertilisers followed the initial experimental plot treatments, and the current study was incorporated to evaluate the outcome of the different rehabilitation treatments and the quality status of the tailings material.

4.2.2. Sampling procedure

Soil samples were collected using a random sampling design as suggested by Alef and Nannipieri (1995). Five random samples were taken from each plot, homogenised, sealed in plastic bags and kept at 4°C for the preservation of biological properties. The soil was obtained from depth 0-15cm in the tailings material. Twenty-four homogenised samples were collected from the experimental plots and analysed separately.

4.2.3. Physical and chemical analysis of tailings material

Tailings samples were analysed and physical and chemical characterisation of the tailings material was done for each site by the 1:2 water extraction technique. This included determination of macro and microelements as well as other data such as %C, EC (electrical conductivity) and pH (Alef and Nannipieri, 1995).

4.2.4. Determination of dry mass

The weight of each sample was determined in field moist state and also after drying at 105°C for 24hrs according the method of Alef and Nannipieri (1995). The weight after drying was used to calculate the percentage moisture of the soil samples.

4.2.5. Phospholipid extraction, fractionation, and analysis

Total lipids were extracted from 5g lyophilised soils according to a modified Bligh and Dyer procedure (White and 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 trans-esterified 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 60m SPB-1 column (0.25mm I.D., 0.250 μm film thickness). Definitive identification of peaks was undertaken using gas chromatography-mass spectrometry of selected samples using a Hewlett-Packard 6890 series II chromatograph interphased with a Hewlett-Packard 5973 mass selective detector. Methyl nonadecanonate (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 and Kennedy, 1998).

4.2.6. Statistical analysis

All samples were analysed in triplicate. PLFA profiles were analysed with STATISTICA 6 (Statsoft, Inc ©). Results of the signature lipid biomarker analyses were interpreted as described by Ringelberg et al., (1998). A three-tiered statistical approach was used where hypothesis testing was first made using analysis of variance (ANOVA) followed by the application of multivariant analysis such as Principal Components Analysis (PCA) for the determination of sample similarity. Redundancy Analysis (RDA) was subsequently performed with the PLFA group-data as species dependant variables and the most significant tailings variables as independent environmental factors. The most significant tailings 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. Factor analysis was also used as a data reduction method and in conjunction with hierarchical cluster analysis to assess changes in community structure. An ANOVA was performed on the factor scores and Tukey's Honest Significant Difference test (HSD) was used to identify significant differences between sites, with the within-experiment family-wise error rate set at $p = 0.05$. Hierarchical cluster analyses were performed on the means from the transformed mol percent (mol%) PLFA using Ward's method. Factor analyses of biomass and arcsine square root transformed PLFA data were performed using both iterative and non-iterative extraction techniques (Peacock et al., 2001). Factor analysis was also used as a data reduction method and in conjunction with hierarchical cluster analyses to assess changes in community structure (Claassens *et al.*, 2005).

4.3. RESULTS AND DISCUSSION

4.3.1. Physical and chemical characterisation of the rehabilitation sites

Principal Component Analysis (PCA) was used to indicate possible associations between the physical and chemical properties of the tailings and the different treatment regimes in the experimental plots. A PCA ordination diagram (Figure 7) showed the variation between the different experimental plots based on physical and chemical properties of the tailings material. The eigenvalues for the first two ordination axes were 0.661 and 0.121, respectively. These two axes accounted for 78.2% of the total observed variance.

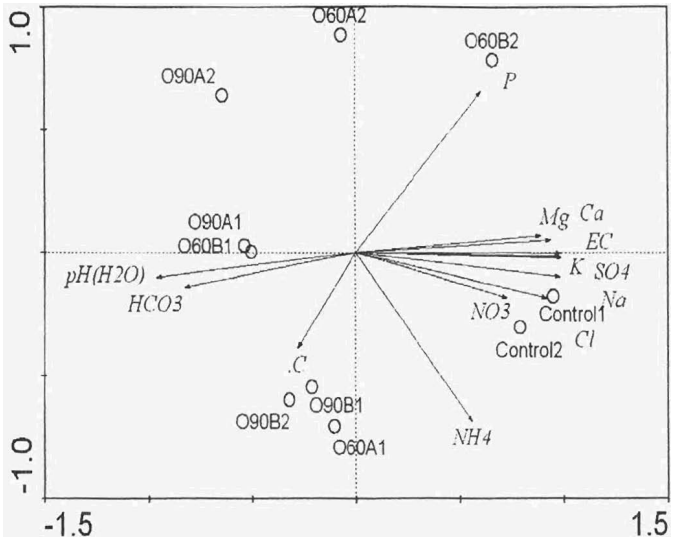

Figure 1. Principal Components Analysis ordination diagram of the physical and chemical properties of the experimental plots.

Chemical components such as elemental C, P and N are of vital importance because they are biogeochemically transformed by microorganisms for utilisation by plants (Tabatabai 1994). Determination of the levels of these different chemical components in the experimental plots can help to explain the associations that are found between the PLFA groups and the different environmental variables, which characterise the different experimental plots. The different PLFA groups represent the microbial community structure since all microbial membranes contain certain signature PLFA's (Calderon *et al.*, 2000).

From the PCA ordination diagram (Figure 1) it is evident that the two control sites grouped to the right of the first ordination axis and were characterised by higher concentrations of Mg, Ca, K, SO_4, NO_3, Na, and higher electrical conductivity (EC). All the other experimental plots, except site 060B2 grouped to the left of the first ordination axis. According to the ordination, treatment site 060B2 was characterised by higher P concentrations in the soil. Treatments sites 090B1 and 090B2 and 060A1 were characterised by high organic carbon content (%C), which can be expected in the case of 090B2 because of the high levels of organic material that was applied in this site.

Table 2. Summary of results for physical and chemical analyses of tailings material

Treatments	Control1	Control2	O60A1	O60A2	O60B1	O60B2	O90A1	O90A2	O90B1	O90B2
Chemical Analysis										
Ca (mmol/L)	6.35 ± 2.27[a]	8.05 ± 2.34[a]	3.56 ± 3.34[a]	1.97 ± 1.52[a]	0.94 ± 0.68[a]	8.8 ± 1.3[a]	1.125 ± 0.92[a]	0.34 ± 0.11[a]	2.29 ± 2.05[a]	1.13 ± 0.39[a]
Mg (mmol/L)	2.05 ± 0.77[a]	2.33 ± 0.71[a]	1.09 ± 0.26[a]	0.79 ± 0.38[a]	0.63 ± 0.2[a]	2.61 ± 0.14[a]	0.95 ± 0.42[a]	0.58 ± 0.27[a]	1.43 ± 0.11[a]	0.35 ± 0.10[a]
K (mmol/L)	3.99 ± 0.90[a]	4.19 ± 0.78[a]	2.32 ± 1.95[a]	2.54 ± 1.17[a]	1.42 ± 1.00[a]	3.86 ± 0.26[a]	1.51 ± 0.84[a]	0.83 ± 0.37[a]	2.33 ± 1.81[a]	1.72 ± 0.08[a]
Na (mmol/L)	21.87 ± 6.36[a]	19.46 ± 3.80[a]	9.71 ± 9.20[a]	10.22 ± 5.99[a]	5.32 ± 4.60[a]	15.20 ± 0.60[a]	4.86 ± 4.35[a]	2.91 ± 2.14[a]	8.18 ± 6.93[a]	8.60 ± 0.38[a]
P (mmol/L)	0.01 ± 0.00[a]	0.01 ± 0.00[a]	0.014 ± 0.00[a]	0.01 ± 0.00[a]	0.02 ± 0.01[a]	0.01 ± 0.00[a]	0.03 ± 0.02[a]	0.01 ± 0.00[a]	0.02 ± 0.01[a]	0.014 ± 0.00[a]
SO_4 (mmol/L)	19.10 ± 5.79[a]	20.36 ± 4.64[a]	9.75 ± 9.42[a]	8.57 ± 5.60[a]	4.10 ± 3.57[a]	19.60 ± 1.85[a]	4.47 ± 3.48[a]	1.98 ± 1.37[a]	7.85 ± 6.32[a]	6.01 ± 0.66[a]
NO_3 (mmol/L)	1.15 ± 0.64[a]	0.37 ± 0.29[a]	0.23 ± 0.09[a]	0.04 ± 0.02[a]	0.058 ± 0.03[a]	0.14 ± 0.06[a]	0.059 ± 0.03[a]	0.06 ± 0.04[a]	0.05 ± 0.01[a]	0.03 ± 0.02[a]
NH_4 (mmol/L)	0.04 ± 0.01[a]	0.05 ± 0.01[a]	0.04 ± 0.01[a]	0.03 ± 0.00[a]	0.03 ± 0.00[a]	0.03 ± 0.01[a]	0.03 ± 0.00[a]	0.02 ± 0.00[a]	0.03 ± 0.01[a]	0.04 ± 0.01[a]
Cl (mmol/L)	2.13 ± 1.10[a]	1.97 ± 0.69[a]	0.38 ± 0.10[a]	0.15 ± 0.04[a]	0.25 ± 0.02[a]	1.05 ± 0.18[a]	0.11 ± 0.05[a]	0.16 ± 0.07[a]	0.63 ± 0.49[a]	0.35 ± 0.12[a]
HCO_3 (mmol/L)	0.86 ± 0.11[a]	1.09 ± 0.24[a]	1.25 ± 0.70[a]	1.03 ± 0.23[a]	1.48 ± 0.48[a]	0.83 ± 0.28[a]	1.48 ± 0.58[a]	1.45 ± 0.50[a]	1.30 ± 0.35[a]	1.05 ± 0.15[a]
EC (mS/cm)	4.24 ± 1.31[a]	4.42 ± 0.99[a]	2.15 ± 1.84[a]	1.84 ± 1.10[a]	1.00 ± 0.66[a]	4.12 ± 0.33[a]	1.07 ± 0.63[a]	0.57 ± 0.22[a]	1.78 ± 1.28[a]	1.35 ± 0.14[a]
pH (H_2O)	8.03 ± 0.37[a]	8.37 ± 0.17[a]	8.91 ± 0.58[a]	8.71 ± 0.21[a]	8.98 ± 0.28[a]	8.25 ± 0.07[a]	8.92 ± 0.27[a]	9.0 ± 0.19[a]	8.77 ± 0.43[a]	8.81 ± 0.00[a]
% Organic carbon	0.75 ± 0.17[a]	0.82 ± 0.18[a]	0.77 ± 0.04[a]	0.58 ± 0.18[a]	0.75 ± 0.06[a]	0.76 ± 0.07[a]	0.77 ± 0.28[a]	0.97 ± 0.06[a]	0.95 ± 0.01[a]	0.89 ± 0.33[a]

Values given are mean ± standard error

Sites with the same combination of superscript alphabetic letters indicate no significant differences among sites

Table 3. Percentage fractions of PLFA groups for experimental plots

Treatments	Control1	Control2	O60A1	O60A2	O60B1	O60B2	O90A1	O90A2	O90B1	O90B2
Lipid Analysis 2004										
Estimated Biomass (Pmol/g dry weight)	11919.77 ± 5108.86 [a]	14077.02 ± 11606.57 [a]	5992.06 ± 1819.87 [a]	10703.55 ± 6423.34 [a]	14520.48 ± 7370.05 [a]	8653.12 ± 5575.07 [a]	2799.67 ± 1169.54 [a]	4446.04 ± 1386.18 [a]	6531.84 ± 217.94 [a]	4764.95 ± 468.00 [a]
Total Nsats	28.25 ± 2.94 [a]	26.93 ± 1.71 [a]	27.56 ± 1.84 [a]	36.77 ± 11.76 [a]	48.31 ± 0.20 [a]	24.82 ± 2.09 [a]	29.12 ± 0.48 [a]	27.77 ± 0.59 [a]	34.92 ± 11.50 [a]	22.57 ± 1.62 [a]
Total MBSats	1.36 ± 0.39 [a]	1.71 ± 0.75 [a]	2.01 ± 0.66 [a]	1.77 ± 0.40 [a]	1.33 ± 0.22 [a]	2.08 ± 0.78 [a]	2.61 ± 0.03 [a]	2.15 ± 0.52 [a]	1.67 ± 0.00 [a]	1.92 ± 0.24 [a]
Total TBSats	15.54 ± 3.93 [a]	15.97 ± 6.59 [a]	18.28 ± 0.59 [a]	19.32 ± 3.73 [a]	16.90 ± 3.45 [a]	20.28 ± 0.41 [a]	20.20 ± 0.81 [a]	22.38 ± 1.12 [a]	18.45 ± 0.47 [a]	18.71 ± 7.16 [a]
Total Monos	47.63 ± 8.98 [a]	51.40 ± 7.43 [a]	44.52 ± 0.66 [a]	36.59 ± 7.26 [a]	26.81 ± 0.38 [a]	45.39 ± 0.04 [a]	41.64 ± 0.74 [a]	43.29 ± 1.21 [a]	35.46 ± 7.01 [a]	42.99 ± 2.19 [a]
Total Polys	7.22 ± 5.06 [a]	3.87 ± 2.06 [a]	7.63 ± 0.07 [a]	4.79 ± 1.14 [a]	6.38 ± 3.98 [a]	7.43 ± 2.51 [a]	6.43 ± 0.52 [a]	4.41 ± 1.01 [a]	9.50 ± 4.96 [a]	13.51 ± 11.52 [a]
Lipid Analysis 2005										
Estimated Biomass	5347.23 ± 1808.70 [a]	4786.06 ± 1079.34 [a]	7342.63 ± 4173.70 [a]	6453.36 ± 836.09 [a]	5399.92 ± 0.00 [a]	7599.90 ± 2360.11 [a]	4425.29 ± 107.23 [a]	6329.49 ± 4177.45 [a]	6592.45 ± 966.02 [a]	4861.41 ± 485.28 [a]
Total Nsats	29.65 ± 3.96 [a]	20.76 ± 0.98 [a]	21.40 ± 0.70 [a]	22.22 ± 1.81 [a]	20.69 ± 0.00 [a]	20.32 ± 0.70 [a]	21.26 ± 0.93 [a]	19.97 ± 1.29 [a]	20.90 ± 0.38 [a]	24.79 ± 3.85 [a]
Total MBSats	1.23 ± 0.49 [a]	2.07 ± 0.44 [a]	2.46 ± 0.84 [a]	1.39 ± 0.13 [a]	2.57 ± 0.00 [a]	1.62 ± 0.74 [a]	2.49 ± 0.22 [a]	1.92 ± 0.16 [a]	2.60 ± 0.67 [a]	5.29 ± 3.70 [a]
Total TBSats	15.36 ± 2.75 [a]	17.36 ± 3.13 [a]	19.49 ± 1.06 [a]	17.34 ± 0.32 [a]	17.47 ± 0.00 [a]	20.59 ± 1.78 [a]	22.02 ± 0.28 [a]	16.11 ± 1.28 [a]	21.61 ± 0.77 [a]	26.78 ± 6.77 [a]
Total Monos	40.04 ± 3.00 [a]	46.87 ± 0.76 [a]	45.12 ± 1.12 [a]	41.30 ± 3.96 [a]	47.83 ± 0.00 [a]	46.68 ± 0.88 [a]	46.78 ± 1.09 [a]	51.51 ± 0.98 [a]	46.01 ± 1.67 [a]	38.51 ± 12.71 [a]
Total Polys	12.63 ± 2.85 [a]	11.95 ± 4.71 [a]	9.55 ± 2.72 [a]	17.12 ± 6.22 [a]	10.88 ± 0.00 [a]	9.86 ± 2.36 [a]	6.68 ± 0.68 [a]	9.56 ± 1.49 [a]	7.45 ± 0.53 [a]	3.72 ± 1.76 [a]

Values given are mean ± standard error

Sites with the same combination of superscript alphabetic letters indicate no significant differences among sites

4.3.2. Phospholipid fatty acid analysis

The microbial community structure can be inferred from the composition of major PLFA groups present in the soil ecosystem. These major PLFA groups include the following: terminally branched saturated fatty acids (TBsats), indicative of the Gram-positive bacteria in the microbial population; monounsaturated fatty acids (Monos), indicative of the Gram-negative bacteria; polyunsaturated fatty acids (polys), which indicate micro-eukaryotes, especially fungi; normal saturated fatty acids (Nsats), general biomarkers for prokaryotic and eukaryotic organisms; and mid-chain branched fatty acids (MBsats) representative of Actinomycetes and sulphate-reducing bacteria (White *et al*, 1996).

Redundancy Analysis (RDA) was used to illustrate the relationship between the soil chemical properties and major PLFA groups in the experimental plots for 2004. Eigenvalues for the first two axes were 0.499 and 0.261, respectively and the total observed variance of the first two axes was 76.1%. The first axis associated with NH_4 (r^2 = 0.6910) and the second axis with %C (r^2= 0.4559). The effect of the specific environmental variables on the community structure was not statistically significant (p = 1.000).

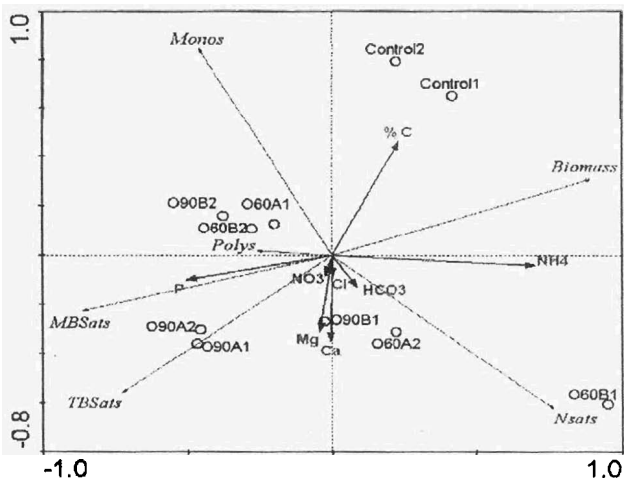

Figure 2. Redundancy Analysis (RDA) ordination diagram illustrating the relationship between the dominant environmental variables and major PLFA groups for experimental plots.

Experimental plots 090B2, 060A1, and 060B2 were characterised by the highest abundance of polyunsaturated fatty acids, indicative of fungi (Figure 2, Table 3). These sites were also characterised by higher P concentrations in the tailings material. The experimental plots control 1 and control 2 had the highest estimated viable biomass and were positively associated with higher %C compared to the other plots. Experimental plots 090A1 and 090A2 had the highest occurrence of Gram-positive bacteria and Actinomycetes and was

characterised by lower organic C content compared to the other plots. Experimental plot 060B1 had the highest abundance of normal saturated fatty acids (Table 3).

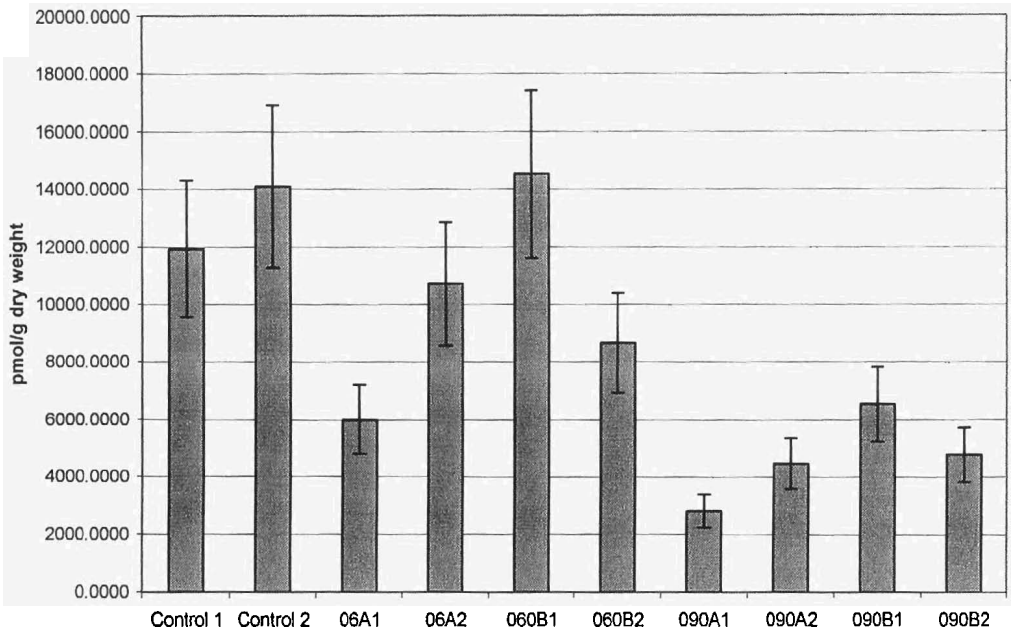

Figure 3. Estimated viable biomass of soil microbial population

The highest estimated viable biomass (Figure 3, Table 3) was found in experimental plots 060A2 and Control2, which both received 60-ton/ha vermi-compost. Control 1 also had higher estimated viable biomass compared to all the other plots but overall higher biomass was recorded in the 60-ton/ha (vermi-compost) plots rather than in the 90-ton/ha (vermi-compost) experimental plots. According to Villar *et al.*, (2004) there should be more microbial biomass and activity where more organic material is applied. However in the duration of this study, plots treated with 60-tons/ha vermi-compost seemed to show higher estimated viable biomass compared to the plots treated with 90-ton/ha.

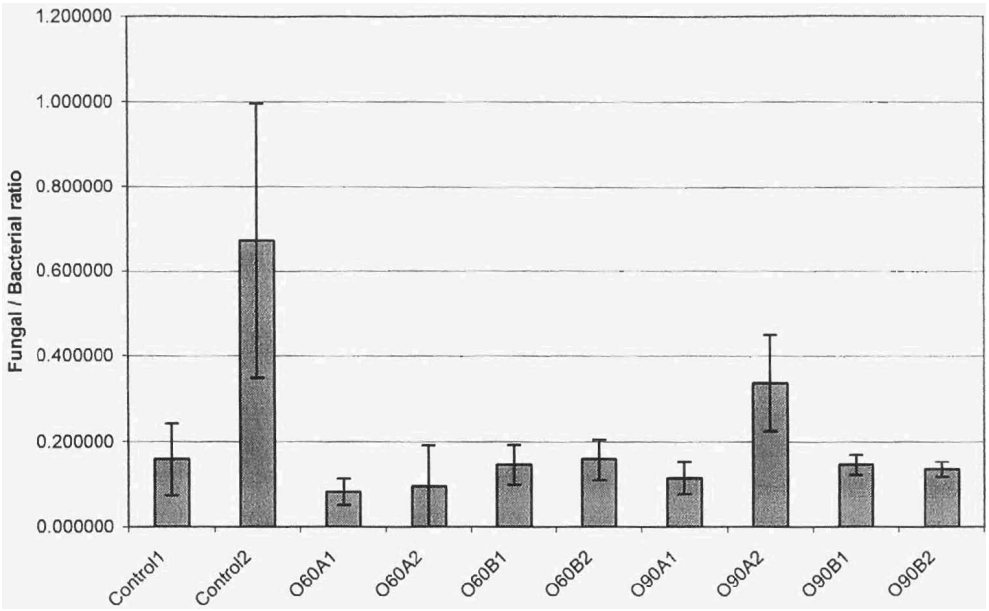

Figure 4. Fungal to bacterial ratios for all experimental plots

According to figure 4, the highest ratio of fungi to bacteria for 2004 was found in the control 2 plot (1.0). Experimental plot 090A2 had a higher fungal to bacterial ratio compared to the other plots (0.3). All the experimental plots except for plot control2 and 090A2 have fungal to bacterial ratios of less than 0.2. According to Anderson and Domsch (1975, 1980), undisturbed healthy soils have a fungal to bacterial ratio of greater than 1. However, bacterial to fungal ratios (Figure 4) in the experimental plots are considerably less than 1, possibly indicating a disturbance of the microbial population by tailings rectification procedures. Apart from control 2, the 90-ton/ha vermi-compost sites seemed to show higher fungal to bacterial ratios compared to the 60-ton/ha vermi-compost sites. From the results obtained (Table 2) it is evident that the tailings material is alkaline by nature with a pH of above 8.0, which could explain the low fungal to bacterial ratios in most of the experimental plots since fungal communities prefer acidic conditions for optimal respiratory activity (Evgenia *et al.*, 1998).

An RDA ordination was used to illustrate the relationship between the soil chemical properties and major PLFA groups for the experimental plots in 2005. Eigenvalues for the first two axes were 0.533 and 0.232, respectively and the total observed variance of the first two axes was 76.5%. The first axis associated with NH_4 (r^2 = 0.278) and the second axis with %C (r^2= 0.01). The effect of the specific environmental variables on the community structure was statistically significant (p = 0.022).

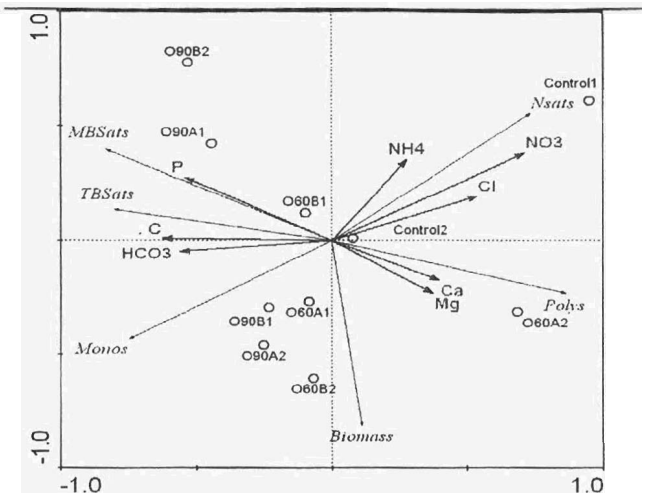

Figure 5. Redundancy Analysis (RDA) ordination diagram illustrating the relationship between the dominant environmental variables and soil microbial community structure for all treatments.

Plot 060A2 was characterised by positive association between organic C and P concentrations, and bacterial PLFA groups. Organic C (r^2 = 0.047) and organic P (r^2 = 0.27) showed high correlation with the second axis. All the experimental plots except for the control sites and plot 060A2 were characterised by a high mole percentage of terminally branched saturated fatty acids (TBSats), mono unsaturated fatty acids (Monos) and mid-chain branched saturated fatty acids (MBSats). These groups of PLFAs are representative of gram-positive bacteria, gram-negative bacteria and sulfate reducing bacteria respectively. The dominance of bacterial communities in the tailings material is evident in most of the experimental plots, which associate strongly with bacterial PLFA groups (Figure 5). Experimental plots 090B1, 060A1, 090A2, 060B2 and 060A2 were all characterised by higher estimated viable biomass (Table 3) and high mole percentage of monounsaturated fatty acids and polyunsaturated fatty acids (indicative of fungal populations) showing the co-existence of bacterial and fungal communities in the plots. Experimental plot 060A2 was characterised by the highest mole percentage polyunsaturated fatty acids indicative of microeukaryotes especially fungi (17.12 ± 6.22) as well as high correlation between environmental variables Ca and Mg (r^2 = 0.96) and the fungal PLFA group (Polys). Both the control sites were characterised by a strong correlation between normal saturated fatty acids (Nsats) and NH_4 concentrations in the tailings material (Figure 5). The PLFA group called normal saturated fatty acids is a general biomarker for prokaryotic and eukaryotic microorganisms.

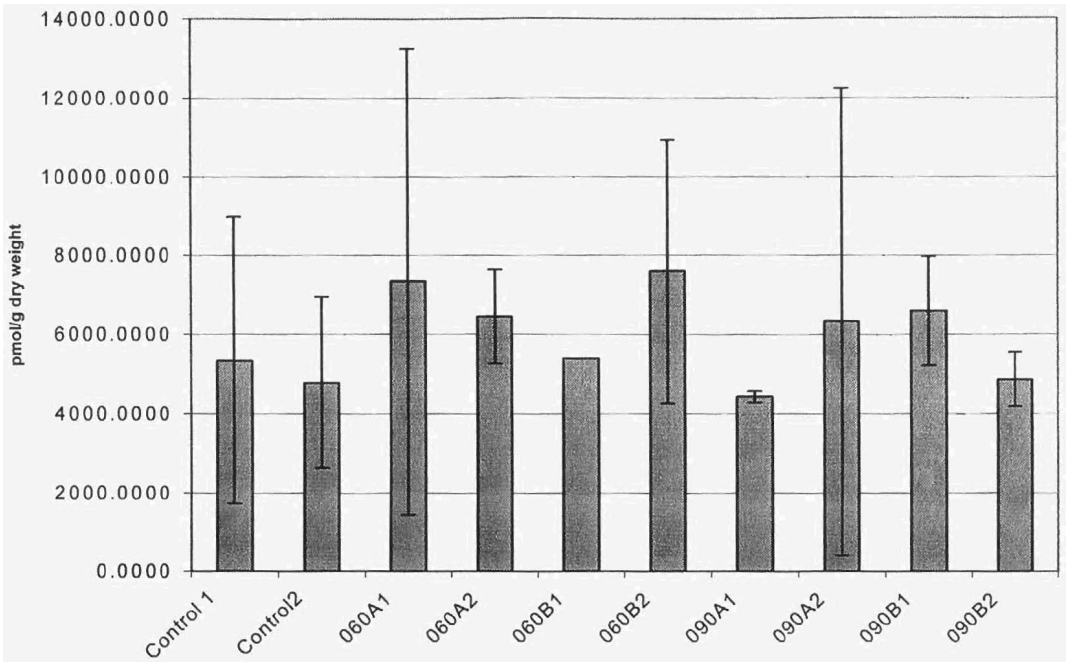

Figure 6. Estimated viable biomass of soil microbial population

The highest estimated viable biomass (7600 pmol/g dry weight) was found in experimental plot 060B2 closely followed by plot 060A1 (7342 pmol/g dry weight)(Table 3). Once again there seemed to be higher estimated viable biomass in the experimental plots treated with 60-ton/ha vermi-compost than the plots treated with 90-ton/ha vermi-compost, although the difference in biomass between the two treatments is less than in 2004 (Figure 6). The control plots had lower estimated viable biomass values than most of the experimental plots as opposed to 2004 where the control plots had among the highest biomass values (Table 3). The lowest estimated biomass value was found in plot 090A1, followed by plot 090B2. Overall there was a smaller difference for estimated viable biomass values among all the experimental plots and less variation than in 2004.

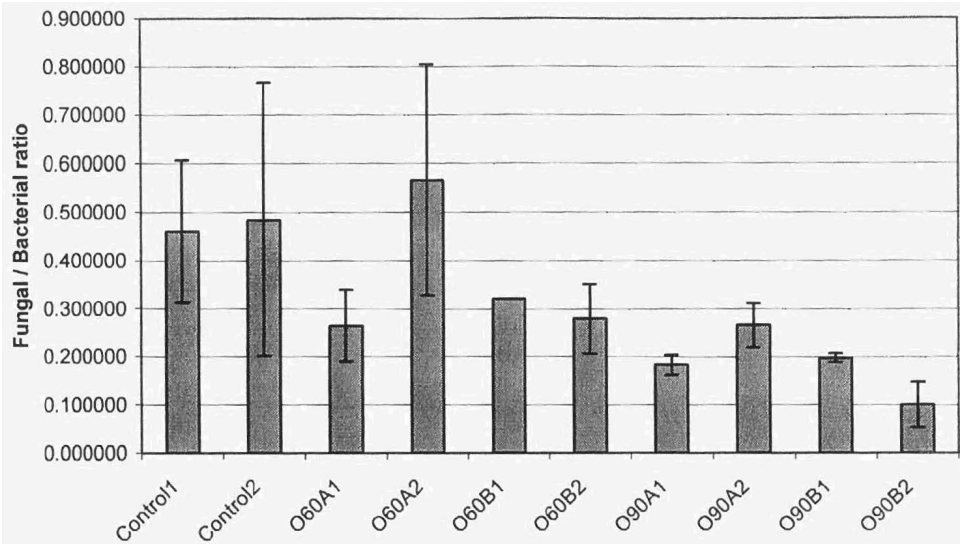

Figure 7. Fungal to bacterial ratios for all the experimental plots

The highest fungal to bacterial ratio (2005) was found in experimental plot 060A2 (0.8), followed by both control plots which showed an increased fungal to bacterial ratio compared to 2004 (Figure 7). The fungal to bacterial ratio seemed to increase in most of the plots showing increased fungal presence and aggrading ecosystems in the tailings material (Anderson and Domsch 1980). Both the control sites had higher fungal to bacterial ratios compared to all the other experimental plots except for plot 060A2. Application of inorganic fertilisers, which increases nutrient availability, usually favours the bacterial community thereby lowering the fungal to bacterial ratio (Grayston *et al.*, 2001) Overall the fungal to bacterial ratio was higher in the plots treated with 60-ton/ha vermi-compost compared to those treated with 90-ton/ha vermi-compost possibly because fungal dominance is favoured by low soil fertility, as is the case in the tailings material (Grayston *et al.*, 2001).

4.4. CONCLUSION

Soil ecosystems form the basis for all forms of life and primary production by plants. Microbial populations in soil are responsible for the regulation of all biogeochemical transformations and the cycling of plant available nutrients necessary for plant growth and ecosystem productivity (Van Bruggen and Semenov, 2000). Therefore, productive and healthy ecosystems will only be sustainable if stable microbial communities and nutrient cycles are developed and established in the tailings material. The PLFA analyses in this study showed varied differences in microbial community structure among the experimental plots which were mostly characterised by high mole percentages of mid-chain branched saturated fatty acids, terminally branched saturated fatty acids and mono unsaturated fatty acid PLFA groups all representing bacterial communities. Microbial communities seemed to have developed increased fungal (polyunsaturated fatty acids) to bacterial ratios over time indicating positive

progression of rehabilitation and sustainability in the experimental plots. Estimated viable biomass was higher in the experimental plots treated with 60-tons/ha vermi-compost compared to the plots treated with 90-tons/ha vermi-compost indicating a possible optimal level of organic matter application for microbial growth and activity. Microbial community structure is therefore a useful tool for the assessment of soil quality and ecosystem sustainability.

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CHAPTER 5

VEGETATIVE RESPONSE TO REHABILITATION TECHNIQUES MEASURED BY PHOTOSYNTHESIS, SPECIES FREQUENCY AND ESTABLISHMENT, AND PLANT BIOMASS

Abstract

Solid waste material generated by mining activities can cause vast negative environmental impacts with regard to soil quality and sustainable vegetation establishment. The physical, chemical and biological components of solid waste material (co-disposed diamond tailings) are usually unfavorable for growth and establishment of vegetation compared to topsoil found in the natural environment. South African legislation requires developers to rehabilitate these impacted areas in a sustainable manner. Rehabilitation of solid waste material at Finsch mine in the Northern Cape (De Beers) involves organic matter application, chemical amelioration and different grass seed mixtures. Optimum amounts of chemical ameliorants to be applied during rehabilitation were determined by field trials at the mine. The aim of this study was to evaluate the rehabilitation trials by the analysis of vegetation characteristics as indicators of sustainability in response to different soil treatments. Soil quality can be evaluated in terms of physical, chemical and biological soil characteristics. Chemical analysis of the soil was done by 1:2 water extract technique, as well as ammonium acetate extraction procedure. Plant growth response to rehabilitation was evaluated by chloropyll *a* fluorescence, subjective measurements of vegetation species frequency and density, and plant biomass. The application of 60 ton/ha vermi-compost showed better results than 90-ton/ha in terms of plant biomass. General plant vitality was also higher in the 60-ton/ha experimental plots.

Keywords: Rehabilitation, soil quality, species frequency, plant biomass, chlorophyll a fluorescence, plant vitality.

5.1. Introduction

Solid waste material produced by mining activities can have vast negative environmental impacts such as significant chemical imbalances, which could become toxic and highly unfavourable to vegetation (Adriano *et al.*, 1980). Co-disposed material produced by diamond mines in South Africa is discarded as waste material on slime dams and rock dumpsites (Chamber of Mines, 2003), and have vast elemental ratio imbalances (Van Rensburg *et al.*, 2002). Physical factors, such as compaction, of these stockpiles reduce seedling establishment and growth. Compaction often causes N to become the most limiting factor in such an anaerobic environment (Jordan *et al.*, 2003).

Legislation in South Africa requires rehabilitation of these disturbed environments in a n acceptable and sustainable manner (South African Environmental Conservation Act 73:1989; National Environmental Management Act (NEMA) 107:1998). Soil quality involves many aspects (Filip, 2002) but can be divided into three categories namely physical, chemical and biological characteristics (Nortcliff, 2002), all of which need to be actively integrated in a correctly functioning soil (Gil-Sotres *et al.*, 2004). Rehabilitation and application of amendments, as well as the development of plant cover, play an important role in the restoration of the physical, chemical and biological properties of disturbed soils (de Mora *et al.*, 2005). Criteria for judging reclamation success of these disturbed soil systems largely encompasses only visually distinguishable aboveground indicators such as vegetation coverage, but also need to account for the health and composition of the soil microbial populations, which forms the basis for all terrestrial ecosystems (Mummey *et al.*, 2002). Land management is sustainable only when it maintains or improves resource quality, specifically the quality of soil (Garcia and Hernandez, 1997). There is of late much more interest and attention on land management strategies which aim to develop an ecosystem to such an extent that it is self reliant rather than depending on fertiliser and organic inputs (Bardgett and McAlister, 1999).

Soil serves as an important biogeochemical regulator for the flow of substances into, through, and out of an ecosystem. The disturbance of soils can lead to critical changes in the biosphere, which could negatively impact certain forms of life (Snakin *et al.*, 1996). Soil is a critically important component of the earth's biosphere, functioning not only in the production of food and fiber (Karlen *et al.*, 2003), but also forming the basis of agricultural and natural plant communities (Doran and Zeiss, 2000). Soil has a direct influence on plant productivity, and soil functions include life support processes like plant anchorage and nutrient supply, water retention and conductivity, support of food webs, and environmental regulatory functions, such as nutrient cycling serving as a source of microbial diversity and remediation of environmental perturbations (Van Bruggen and Semenov, 2000). During their development, plants are subjected to various environmental stresses (Parvanova *et al.*, 2004), and because most plants

are immobile and fixed to specific locations and conditions, it is important for them to respond and adapt in order to survive (Taylor *et al.*, 2003). These environmental influences affect photosynthetic activity in plants. One of the most important environmental factors that inhibit photosynthesis is water stress, and studies have shown how water stress could result in damage to the oxygen-evolving complex of photosystem II (PSII) in plants (Lu and Zhang, 1998). Other stress factors such as excess light exposure (photoinhibition) and continual unfavorable temperatures also negatively impact plant metabolism (Adams *et al.*, 1994). Salinity in soils is another major stress factor for plants, especially in arid and semi-arid regions, and can severely limit plant productivity (Shanon, 1998). The negative effects of salinity on plant growth are associated with water stress (low osmotic potential) in plants, nutritional imbalance, specific ion effect, or a combination of these factors (Ashraff, 1994).

The solid waste material produced by kimberlite diamond mines in South Africa also known as co-disposed diamond tailings, is sodic as well as saline by nature (Van Rensburg *et al.*, 2002). The major adverse effect of soil salinity is the reduction in availability of soil water to plants. This is due to the presence of salt in water and increases the amount of energy required by the plant to extract water from the soil solution (Schachtman and Liu, 1999). Plants under salinity stress reduce their solute potential by accumulating organic and inorganic solutes to maintain continuous water absorption at low soil water potential (Morant-Monceau, 2004). Many plants develop mechanisms either to exclude salt from their cells or to tolerate its presence within the cells (Parida and Das, 2005). These osmotic adjustments are often made in plants to tolerate drought or salinity stress (Souza, 2004), but occur at the expense of plant growth and biomass production (Lee *et al.*, 2004) because of the energy requirements for this process (Ashraf, 1994). Potassium uptake is vital for plant growth, but in saline soils, Na competes with K for uptake across the plasma membrane of plant cells, which result in high Na:K ratios. These high ratios reduce plant growth and eventually become toxic to the plant (Schachtman and Liu, 1999). Morant-Monceau (2004) found that K uptake declines in the presence of excess Na, and that a lack of adequate Ca can enhance substitution of Na for K in plants.

Chlorophyll a fluorescence has often been used as a screening tool for stress tolerance and provides valuable information about the response of the photochemical reaction to stress (Strasser *et al.*, 2000). The ability of the chlorophyll molecule not only to absorb light and to carry out the primary photochemical reaction, but also to dissipate part of this energy as fluorescence, makes fluorescence a very convenient probe for the state of photosynthetic apparatus. The method is based on the registration of light emitted by photosystem II (PS II), and is amongst the most accurate biophysical methods to evaluate the physiological state of the plant (Goltsev *et al*, 2003). All photosynthetic material such as chlorophyll a, exhibit a polyphasic rise of the chlorophyll fluorescence transient during the first exposure to illumination. These phases are labeled as O, J, I, P (Strasser *et al.*, 1995). The use of chlorophyll fluorescence from intact leaves proved to be a reliable, nonintrusive method for monitoring

photosynthetic events and judging the physiological status of the plant (Kocheva *et al*, 2004). Every environmental change forces the photosynthetic system to adapt by changing its physiological state. This is reflected in the shape of the fast polyphasic fluorescence transient, which has been shown to change under altered environmental conditions, such as light intensity, temperature, drought, or chemical influences (Strasser *et al.*, 2000). Modifications in PSII photochemistry of water-stressed plants can therefore be evaluated by the measuring of certain parameters in the polyphasic rise of fluorescence transients (Lu and Zhang, 1998).

5.2. Material and methods

5.2.1. Site details

This study is a continuation of the rehabilitation project conducted by Van Rensburg *et al.* (2002) to determine the type and optimum concentration of chemical ameliorants to be applied for rehabilitation of co-disposed tailings material. Pot trials were performed to estimate the optimum organic material concentrations, percentage germination and the percentage of plants that reached full maturity. The initial pot trial experiments were then followed by field trials to select the most appropriate grass species to use for rehabilitation, which was determined by the level of success with which seeded grass species established (Van Rensburg *et al.*, 2002). The aim of this study then follows to determine which experimental plot displayed the highest plant vitality, plant growth, species diversity and establishment. The experimental plots were established at the mine on a platform with outer walls, which were constructed by using tailings and discarded rocks. Twenty-four VFPE geo-membrane liners measuring $4m^2$ in surface area and 1m in depth were placed on the platform and filled with the co-disposed material. Tailings were used to fill the open spaces in between the liner bags. The different plots were treated with organic material in the form of vermi-compost at rates of 60 and 90-ton/ha. The plots also received fertiliser treatments of 3:1:5 (N: P: K, with K in the form of KNO_3) which were chlorine free, super-phosphate, $CaNO_3$ and $MgNO_3$ at rates of 625- (both 3:1:5 and super-phosphate), 25- and 12.5kg ha^{-1} respectively. The layout of the different treatment groups is summarised in table 1 (Van Rensburg *et al.*, 2002).

Table 1. Layout of the different treatment groups evaluated for the duration of the study.

Treatment	n	Organic material (tons ha^{-1})	Chemical amelioration (A/B)*	Seed mix (1/2)#
Control 1	**4**	**90**	–	–
Control 2	**4**	**60**	–	–
A (090A1)	2	90	A	1
B (090B1)	2	90	B	1
C (090A2)	2	90	A	2
D (090B2)	2	90	B	2
E (060A1)	2	60	A	1
F (060B1)	2	60	B	1
G (060A2)	2	60	A	2
H (060B2)	**2**	**60**	**B**	**2**

*Fertiliser treatment A: KNO, 3:1:5 (N: P: K, Cl-free), and Super phosphate (625 kg ha^{-1}).

*Fertiliser treatment B: $CaNO_3$ (25 kg ha^{-1}) and $MgNO_3$ (12.5 kg ha^{-1})

#**Seed mix 1:** *Enneapogon cenchroides, Melinis repens, Chloris virgata, Urochloa brachyura, Eleusine coracana, Cenchrus ciliaris* var. Molopo, *Cenchrus cilliaris* var. Gayndah, *Eragrostis lehmanniana, Panicum maximum, Digitaria eriantha, Cynodon dactylon, Chloris gayana, Bothriochloa insculpta.*

Seed mix 2: *Enneapogon cenchroides, Melinis repens, Chloris virgata, Tragus berteronianus, Aristida congesta, Cenchrus ciliaris* var. Molopo, *Cenchrus cilliaris* var. Gayndah, *Eragrostis lehmanniana, Schmidtia pappophoroides, Fingerhuthia africana, Eragrostis echinochloidea, Cynodon dactylon, Chloris gayana*

5.2.2. Physical and chemical analysis of tailings material

Tailings samples were analysed and physical and chemical characterisation of the tailings material was done for each site by using a 1:2 water extraction technique. This included determination of macro and microelements as well as other data such as %C, EC (electrical conductivity) and pH.

5.2.3. Biomass production - clippings of the herbaceous component

Each experimental plot (e.g. A1-A6) was divided into quarters and subsequently the quarter containing the highest vegetation cover (quadrant with most visible biomass) was selected. All the plant material (dead and live material) within this specific quarter was cut to a height of 5-10cm above ground level. The clippings contained only herbaceous material such as forbs and grasses. The plant material was placed in paper bags and labeled according to different treatments. The material was dried at 60°C for 48 hours and weighed (Kent And Koker, 1992).

5.2.4. Fluorescence measurements (JIP test)

Plant vitality can be assessed by analysis of the photosynthetic apparatus of plants namely photosystem II (PSII). By following the behaviour of the photosynthetic apparatus, stress and adaptations of plants can be monitored. The fast phase fluorescence transients (graphical representation of energy fluxes in response to light) were quantified by means of the JIP test. The JIP test parameter used to quantify the effect of rehabilitation on the vitality of the grass species was the so-called Performance Index (PI_{ABS}). This is a multi-parametric expression that takes into account independent factors that affect photosynthesis, namely absorption (RC/ABS), the quantum efficiency of trapping [$\varphi_{PO}/(1-\varphi_{PO})$], and efficiency of conversion of trapped excitation energy to electron transport [$\psi_0/(1-\psi_0)$] (Strasser *et al.*, 2001). The index PI_{ABS} is defined as follows:

$$PI_{ABS} = \frac{RC}{ABS} \cdot \frac{\varphi_{PO}}{1-\varphi_{PO}} \cdot \frac{\psi_O}{1-\psi_O}$$

Photosynthesis in this case was analysed in terms of the Performance Index (PI_{ABS}), which can be defined as the total driving force for photosynthesis of the observed system, created by summing the partial driving forces for each of the several energy fluxes (onset of fluorescence rise O-J-I-P) (Van Heerden *et al*, 2004). All fluorescence measurements were performed on dark-adapted leaves of the species *Cenchrus ciliaris*. The fluorescence induction curves were measured with a plant efficiency analyser (PEA; Hansatech Instruments Ltd, King's Lynn, Norfolk, UK) and recorded for 1 s (Strasser *et al.*, 2000). The fluorescence transients were induced by a red light (peak at 650nm), which provides sufficient excitation energy to ensure the closure of all reaction centres of PSII. This is provided by the PEA through an array of six light-emitting diodes. Each Chlorophyll *a* fluorescence induction curve was analysed according to the JIP-test (Strasser and Strasser, 1995).

From the stored data, the following values were selected and used by the JIP-test for the calculation of several expressions leading to the dynamic description of a photosynthetic sample at a given physiological state:

- The maximal measured fluorescence intensity F_m
- The fluorescence intensity at 50μs, considered being F_0 , i.e. the intensity when all reaction centres are open.
- The fluorescence intensity at 150μs, at 300μs, at 2ms (denoted as F_J) and at 60ms (denoted as F_I) (Strasser and Strasser, 1995).

5.2.5. Grass species frequency and establishment

Estimated ratings of species composition were made of every treatment according to the method of Kent and Coker (1992). Species frequency analysis was done only for grass species in the various treatments. Twenty random sampling sites were selected in each trial plot, and

the number of times a certain grass species was recorded closest to the point of analysis was converted to percentage by multiplication of factor 5 (n = 20, x5 = 100%). This result represented percentage frequency of a certain species in a trial plot.

5.3. Results and discussion

5.3.1. Physical and chemical characterisation of rehabilitation sites

The chemical properties of the soil were ordinated with the different treatment regimes in the experimental plots by making use of a principle component analysis (PCA). Figure 1 shows the ordination of the physical and chemical properties with the different experimental plots. The eigenvalues for the first two ordination axes were 0.661 (axis 1) and 0.121 (axis 2), respectively. These two axes accounted for 78.2% of the total observed variance.

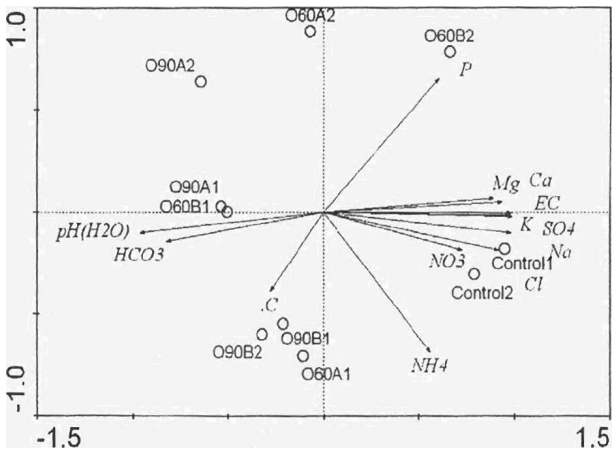

Figure 1. Principal Components Analysis (PCA) ordination diagram of the physical and chemical properties of the experimental plots.

The physical and chemical properties of the tailings in the experimental plots are correlated with the different treatment regimes so that plant responses to different treatments in the experimental plots can be explained on this basis. Chemical components of the tailings such as elemental C, P and N are of vital importance because they are the basic components of protein and carbohydrate synthesis in plants (Tabatabai 1994). Determination of the levels of these different chemical components in the experimental sites can help to explain the associations between environmental variables such as plant photosynthetic activity, plant biomass and chemical properties of the tailings, and how they characterise the different experimental plots. From the PCA ordination diagram (Figure 1) it is evident that the two control sites grouped to the right of the first ordination axis and were characterised by higher Mg, Ca, K, Cl, SO_4, NO_3, Na, and electrical conductivity (EC) than the other plots. All the other treatment sites, except plot 060B2 grouped to the left of the first ordination axis (X-axis). Experimental sites 090B1 and

090B2 and 060A1 were strongly associated with organic carbon content (%C), which can be expected since high levels of organic material were applied in these sites.

Table 2. Summary of results for physical and chemical analysis

Treatments	Control1	Control2	O60A1	O60A2	O60B1	O60B2	O90A1	O90A2	O90B1	O90B2
Chemical Analysis										
Ca (mmol/L)	6.35 ± 2.27[a]	8.05 ± 2.34[a]	3.56 ± 3.34[a]	1.97 ± 1.52[a]	0.94 ± 0.68[a]	8.8 ± 1.3[a]	1.125 ± 0.92[a]	0.34 ± 0.11[a]	2.29 ± 2.05[a]	1.13 ± 0.39[a]
Mg (mmol/L)	2.05 ± 0.77[a]	2.33 ± 0.71[a]	1.09 ± 0.26[a]	0.79 ± 0.38[a]	0.63 ± 0.2[a]	2.61 ± 0.14[a]	0.95 ± 0.42[a]	0.58 ± 0.27[a]	1.43 ± 0.11[a]	0.35 ± 0.10[a]
K (mmol/L)	3.99 ± 0.90[a]	4.19 ± 0.78[a]	2.32 ± 1.95[a]	2.54 ± 1.17[a]	1.42 ± 1.00[a]	3.86 ± 0.26[a]	1.51 ± 0.84[a]	0.83 ± 0.37[a]	2.33 ± 1.81[a]	1.72 ± 0.08[a]
Na (mmol/L)	21.87 ± 6.36[a]	19.46 ± 3.80[a]	9.71 ± 9.20[a]	10.22 ± 5.99[a]	5.32 ± 4.60[a]	15.20 ± 0.60[a]	4.86 ± 4.35[a]	2.91 ± 2.14[a]	8.18 ± 6.93[a]	8.60 ± 0.38[a]
P (mmol/L)	0.01 ± 0.00[a]	0.01 ± 0.00[a]	0.014 ± 0.00[a]	0.01 ± 0.00[a]	0.02 ± 0.01[a]	0.01 ± 0.00[a]	0.03 ± 0.02[a]	0.01 ± 0.00[a]	0.02 ± 0.01[a]	0.014 ± 0.00[a]
SO_4 (mmol/L)	19.10 ± 5.79[a]	20.36 ± 4.64[a]	9.75 ± 9.42[a]	8.57 ± 5.60[a]	4.10 ± 3.57[a]	19.60 ± 1.85[a]	4.47 ± 3.48[a]	1.98 ± 1.37[a]	7.85 ± 6.32[a]	6.01 ± 0.66[a]
NO_3 (mmol/L)	1.15 ± 0.64[a]	0.37 ± 0.29[a]	0.23 ± 0.09[a]	0.04 ± 0.02[a]	0.058 ± 0.03[a]	0.14 ± 0.06[a]	0.059 ± 0.03[a]	0.06 ± 0.04[a]	0.05 ± 0.01[a]	0.03 ± 0.02[a]
NH_4 (mmol/L)	0.04 ± 0.01[a]	0.05 ± 0.01[a]	0.04 ± 0.01[a]	0.03 ± 0.00[a]	0.03 ± 0.00[a]	0.03 ± 0.01[a]	0.03 ± 0.00[a]	0.02 ± 0.00[a]	0.03 ± 0.01[a]	0.04 ± 0.01[a]
Cl (mmol/L)	2.13 ± 1.10[a]	1.97 ± 0.69[a]	0.38 ± 0.10[a]	0.15 ± 0.04[a]	0.25 ± 0.02[a]	1.05 ± 0.18[a]	0.11 ± 0.05[a]	0.16 ± 0.07[a]	0.63 ± 0.49[a]	0.35 ± 0.12[a]
HCO_3 (mmol/L)	0.86 ± 0.11[a]	1.09 ± 0.24[a]	1.25 ± 0.70[a]	1.03 ± 0.23[a]	1.48 ± 0.48[a]	0.83 ± 0.28[a]	1.48 ± 0.58[a]	1.45 ± 0.50[a]	1.30 ± 0.35[a]	1.05 ± 0.15[a]
EC (mS/cm)	4.24 ± 1.31[a]	4.42 ± 0.99[a]	2.15 ± 1.84[a]	1.84 ± 1.10[a]	1.00 ± 0.66[a]	4.12 ± 0.33[a]	1.07 ± 0.63[a]	0.57 ± 0.22[a]	1.78 ± 1.28[a]	1.35 ± 0.14[a]
pH (H_2O)	8.03 ± 0.37[a]	8.37 ± 0.17[a]	8.91 ± 0.58[a]	8.71 ± 0.21[a]	8.98 ± 0.28[a]	8.25 ± 0.07[a]	8.92 ± 0.27[a]	9.0 ± 0.19[a]	8.77 ± 0.43[a]	8.81 ± 0.00[a]
% Organic carbon	0.75 ± 0.17[a]	0.82 ± 0.18[a]	0.77 ± 0.04[a]	0.58 ± 0.18[a]	0.75 ± 0.06[a]	0.76 ± 0.07[a]	0.77 ± 0.28[a]	0.97 ± 0.06[a]	0.95 ± 0.01[a]	0.89 ± 0.33[a]

Values given are mean ± standard error

Sites with the same combination of superscript alphabetic letters indicate no significant differences among sites

5.3.2. Grass species frequency and establishment

2004 **2005**

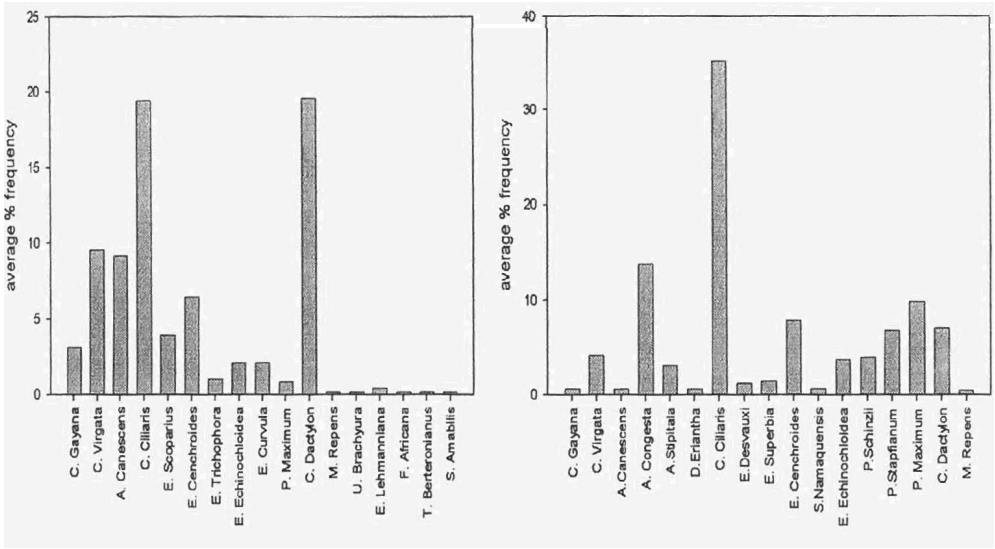

Figure 2a. **Figure 2b.**

Average frequency % for all grass species on the treatment sites

The average species frequency for all the field trial plots are presented in figure 2a and 2b. The species *C.ciliaris* (perennial) and *C.dactylon* (perennial, creeping grass) were most dominantly found to occur in the experimental plots for 2004 (Figure 2a). Most of these species were in the reproductive stage, which indicates that seed production is taking place and that the grass species could occur in higher densities in these sites in future (higher germination rates and establishment). Numerous seeds were visible in-between the plants on the soil surface, captured within this protective environment where the plants will be able to establish, should enough moisture be available and the extent of competition not be too high between the different species within the different successional stage groups (pioneer, sub-climax and climax species). The high occurrence of small seedlings of species such as *C.virgata* (Figure 2a) in many of the sites indicated an increase in establishment of young seedlings. All three the phenological stages (seedlings, vegetative and reproductive) of growth were present in the sites, indicating establishment of sown-in species and the possibility of succession in future partly indicating successful rehabilitation.

The perennial grass *C.ciliaris* (Blue Buffalo grass) was the dominating species in most of the experimental plots (Figure 2b) in 2005 and showed higher average percentage frequency than the previous year (35% compared to 18%). *A.congesta* showed a strong presence in most of the treatment sites (14%) and seemed to have established in the place of *A.canescens*, which had rapidly diminished from the previous year (Figure 2b). Most of the *A.congesta* species were at seedling stage indicating recent germination in the sites, and

although this species was present in the original seeding mixtures, it did not appear to have established until this particular season. *P.maximum* also increased in frequency from the previous year, whilst *C.dactylon* seemed to have decreased in frequency. *P.schinzii* and *P.stapfianum* both seemed to have been established by natural processes since they were not in the original seeding mixtures for the sites. Vegetative and reproductive phases of grass growth were also clearly evident and indicated the possibility of sustained grass cover in all the sites (Figure 2b).

5.3.3. Plant biomass

Figure 3 shows the biomass measurements for all vegetation on the different treatment sites after two seasons.

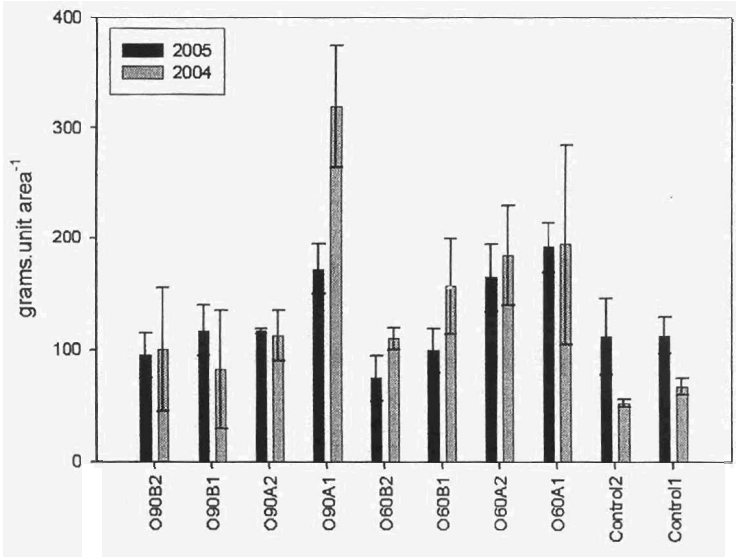

Figure 3. Plant biomass measurements for the treatment sites in grams per unit area

The weighed biomass of all the plots consisted of any vegetation in a specific quarter of the plots. The highest plant biomass in 2004 was found in site 090A1, but on average, higher biomass was found among the plots treated with 60ton/ha organic material (vermi-compost). The three highest values for plant biomass were found in the experimental plots treated with fertiliser type A (Super-phosphate). The two control sites had the lowest biomass values compared to all the other experimental plots in 2005, which could be as a result of no subsequent seeding or fertiliser application. However, the control sites had similar species diversity and species frequencies to the other sites, indicating that the fertiliser application used, in this case could be the reason for higher biomass. Treatment sites 060B2, Control1 and Control2 all had high levels of Na and other chemicals in the soil according to the data presented in table 2 and figure 1, which could be a possible explanation for low plant biomass in all three these plots. High levels of Na in soil reduce plant growth and development since Na competes with K for uptake in plant roots (Schachtman and Liu, 1999). Plant growth is

also retarded by salinity stress because of the energy requirements of osmotic adjustments that plants make in response to this stress (Lee *et al.*, 2004).

The highest plant biomass in 2005 was found in experimental plot 060A1, but general plant biomass seemed to be similar in all the sites. Plant biomass in the control plots increased from the previous season and no statistically significant differences in plant biomass between plots Control1 and Control2 were evident. However, plant biomass in both years was generally higher in experimental plots 090A1, 090A2, 060A1 and 060A2 which all received fertiliser type A (superphosphate). Thus fertliser type A seemed to show better results in terms of plant biomass than fertiliser type B (Figure 3).

5.3.4. Fluorescence measurements (JIP-test)

Not all the trial sites offered suitable material for fluorescence measurements during the 2004 trial. Measurements were done in a relatively dry period at the onset of winter. Most of the treatments sites however did provide some photosynthetic activity but not enough to determine any important physiological differences. Values for the performance index were considerably low (< 2) as a result of the winter period where most of the grasses finished an annual cycle. After these cycles photosynthetic material in leaves dry up and abscise in some cases. The low performance index values could be a result of water stress in the extensively dry region of the Northern Cape. However, water stress significantly reduces carbon dioxide assimilation rate and leaf stomatal conductance and not photosynthesis directly (Lu and Zang, 1998). Osmotic stress also causes rapid dehydration but the photo systems retain their efficiency, and this kind of stress also causes severe injury to the plant cell membranes (Kocheva *et al*, 2004). Thus, very small differences made it difficult to draw any conclusions. The following season, however, showed better results in terms of photosynthetic activity and performance index measurements were significantly higher than in 2004.

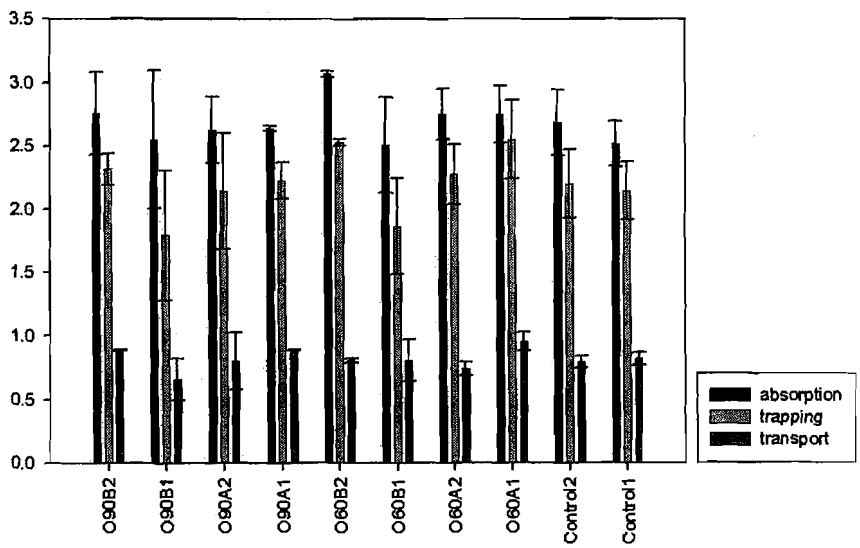

Figure 4. The three components of performance index

By quantification of the fluorescence transients, the average energy fluxes of absorbance, trapping and conversion of excitation energy to electron transport were determined for both years (Figure 4). The energy ratios of these three factors were calculated and the values were used to determine the performance index (the product of the three factors namely light absorption, electron trapping and electron transport) (Strasser *et al.*, 2001). Treatment sites 060B1 and 090B1 both had relatively low values for light energy absorption and trapping compared to the other sites; however these differences were not statistically significant. The efficiency of energy conversion to electron transport (ψ_0/ (1-ψ_0)) seemed to be similar in all the treatment sites with no large differences.

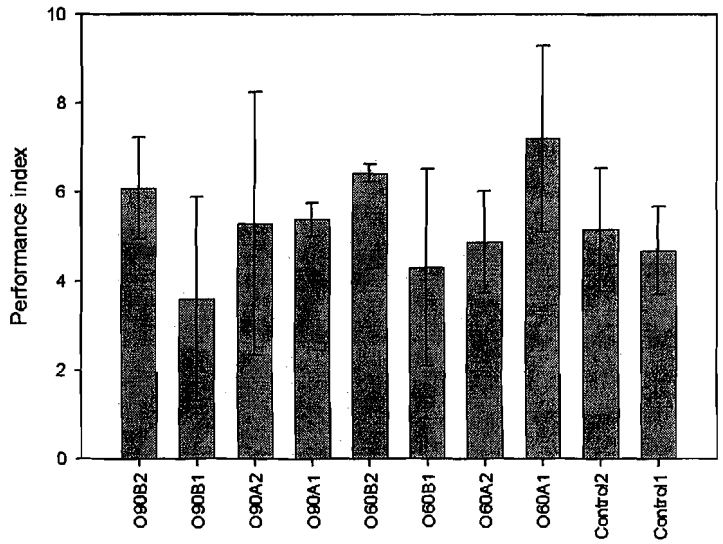

Figure 5. Fluorescence measurements for all the treatment sites (Performance Index)

Plant vitality is determined by performance index (PI_{abs}) of the photosynthetic apparatus of grasses in the different experimental plots and is influenced by energy absorption, trapping and conversion into the electron transport chain as mentioned above. Experimental plot 060A1 showed the highest plant vitality (highest value for PI_{abs}) followed by plot 060B2 (Figure 5). On average, the sites treated with 60ton/ha vermi-compost seemed to show higher PI values than those treated with 90ton/ha vermi-compost. The experimental plots 090B1 and 060B1 showed the least plant vitality (lowest values for PI_{abs}), which is a result of less efficient absorption and trapping of light energy (figure 4) in these plants. This is indicative that these sites could be experiencing a higher level of environmental stresses than the other experimental plots, which negatively impacts their vitality and photosynthetic performance. Performance indices for the control sites showed no significant statistical difference to the experimental plots that received inorganic fertilisers, although plot Control2 (60-ton/ha vermi-compost application) had a slightly higher PI value than plot Control1 (90-ton/ha).

5.4. Conclusion

Rehabilitation processes need to take into account vegetation and soil aspects of the environment (Mummey *et al.*, 2002) and must ensure sustainability of plant growth as well as aggrading soil quality (Garcia and Hernandez, 1997). Performance index as a measure of plant vitality is an effective tool for assessing soil quality and rehabilitation progress, since plants respond rapidly to environmental disturbances in terms of growth and photosynthetic activity (Van Heerden *et al.*, 2004). The experimental plots treated with 60-ton/ha vermi-compost seemed to have higher plant vitality than those treated with 90-ton/ha possibly indicating an optimum organic matter application of not more than 60-ton/ha. The few experimental plots that had higher PI_{abs} values compared to the rest could be indicative of environmental stresses influencing photosynthetic activity, and as a result plant vitality, in most of the plots. In terms of grass species frequency and establishment, dominating species such as *C.ciliaris* and *C.dactylon* seemed to remain strongly established in most of the experimental plots whilst other less prominent species seemed to have decreased in occurrence frequency. New grass species are continuously occurring in most of the treatment sites. However, all the phenological stages (seedlings, vegetative and reproductive) of growth were present in the sites, indicating successful establishment of sown-in species and the notion towards successful rehabilitation and establishment of grass species. Plant biomass seemed to be relatively similar and evenly distributed in all the experimental plots indicating widespread plant growth, productivity and distribution in all the trial plots. In general, treatment sites with 60-tons/ha application of organic material had more plant biomass and higher performance index values than the sites that received 90-ton/ha vermi-compost. Plant vitality in terms of species establishment, plant biomass and performance index could summarise vegetation responses to different rehabilitation treatments and need to be considered for quantifying sustainability of ecosystems in future.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

1.1. Background

Mining activities are a major cause of environmental disturbances especially to soil ecosystems by the production of vast amounts of solid waste material in the form of waste rock dumpsites or slime dams with gold, platinum and diamond tailings (Chamber of Mines, 2003). Mining is one of the major cornerstones of the global and South African economy but its adverse effects on the environment can affect sustainability of ecosystems in the future. Although legislation in South Africa and many other countries requires the mining company to restore and rehabilitate the impacted areas to a satisfactory level (DEAT, 2003), it is almost impossible to restore the perturbed environment to its original nature. Chemically imbalanced solid waste material usually requires many years of organic and inorganic inputs before sustained vegetation can be established on the material. Many past rehabilitation efforts have focused on agricultural productivity alone and failed to account for sustainability of plant vitality, growth and production (Mummey *et al.*, 2002), which is usually dependant on soil quality. Soil forms the basis of all environmental ecosystems and its functions include hosting biogeochemical cycles of nutrients by microorganisms as well as primary plant production (Van Bruggen and Semenov, 2000).

1.2. General discussion

The physical, chemical and biological properties of soil all interact in a complex manner to sustain stable vegetation and healthy biogeochemical cycles (Karlen *et al.*, 2003). Physical and chemical properties of soil change over long periods of time, however, biological soil properties such as microbial populations and plant life, are extremely sensitive to disturbances of soil ecosystems thereby providing immediate and accurate information on changes in soil quality (Pascual *et al.*, 2000; Gil-Sotres *et al.*, 2004). The use of microbiological and biochemical soil parameters, such as enzymatic activity, phospholipid fatty acid analyses (PLFA), photosynthetic performance index, and substrate induced respiration are often used as indicators of certain environmental stresses during rehabilitation processes. Good status of the soil microorganisms (high quantity, activity and diversity) is a prerequisite of good soil quality, soil fertility, and tolerance of the soil to stress factors (Hofman *et al.*, 2002).

The co-disposed diamond tailings produced by diamond mines in South Africa are characterised by diverse chemical and physical properties. The tailings material has extremely high levels of Na and lower levels of Mg and Ca than what is necessary for

sustained plant growth (Van Rensburg *et al.*, 2002). Prior to this study, experimental rehabilitation plots were set up at Finsch mine in the Northern Cape to determine the effect of different organic and inorganic matter applications on soil quality and plant vitality. During the current study, the relationship between the soil physical and chemical characteristics, microbiological properties such as enzymatic activity and analysis of PLFAs, and plant vitality parameters such performance index (PI_{abs}) as well as subjective species frequency analyses, was investigated using multivariate statistical analysis which includes Principal Components Analysis (PCA) and Redundancy Analysis (RDA). According to the one-way ANOVA, none of the environmental variables showed any statistically significant differences between the experimental plots at a probability level of 0.05 or less. The tailings material in the twenty-four experimental plots were analysed by 1:2 water extraction for physical and chemical properties. Organic carbon content (C%), phosphorus (P), chlorine (Cl), calcium (Ca), magnesium (Mg), nitrate (NO_3), and ammonium (NH_4) all had the greatest statistical effect on microbial function and activity (Figure 7, Chapter 3), community structure (Figure 2, Chapter 4) and plant vitality (Figure 2, Chapter 5).

The dehydrogenase activity in the experimental plots seemed to be positively associated with increased organic matter application (vermi-compost) and with organic carbon concentrations. Although alkaline phosphatase activity decreased in the experimental plots over time, there were no statistically significant differences among the sites in 2005 possibly indicating well-established phosphatase cycles in the tailings material for this period. Alkaline phosphatase activity was positively associated with nitrate and ammonium concentrations as well as with phosphorus concentrations, which could be expected. Acid phosphatase activity also decreased considerably over time but was positively associated with higher vermi-compost applications, and with nitrate and ammonium concentrations in the tailings material. Overall ß-glucosidase activity decreased slightly over time although there were no statistically significant differences among the sites in 2005 possibly indicating stabilisation of biogeochemical cycles. ß-glucosidase activity was positively associated with higher vermi-compost application and with organic carbon content in the experimental plots. Urease activity was higher in tailings material treated with 90-ton/ha vermi-compost. Unlike all the other enzymatic assays, overall urease activity increased in the experimental plots showing higher activity in 2005. Together with the increase in urease activity, no statistically significant differences were evident among the plots. Urease activity was positively associated with ammonium concentrations in the experimental plots.

Substrate-induced respiration together with selective inhibition was used to measure the respiratory contributions of fungi and bacteria respectively to overall basal respiration (CO_2 production). However, statistical analyses indicated no significant differences in the data

(Figure 9, Chapter 3). Bacterial and fungal contributions to soil respiration respectively could not be clearly differentiated. There was almost no inhibition of CO_2 production when soils were treated with streptomycin alone. However, cycloheximide application slightly inhibited microbial respiration implying that the fungal communities in the tailings material may contribute the larger part of basal respiration occurring in the tailings material.

Redundancy analysis (RDA) was also used to determine the relationship between microbial community structure and the physical and chemical properties of the tailings material in the experimental plots (Figure 2, Chapter 4). Phospholipid fatty acids were analysed to characterise the microbial communities occurring in the different experimental plots since these signature lipid biomarkers represent different groups of microbial populations (White et al., 1996). Most of the experimental plots were characterised by high levels of monounsaturated fatty acids, indicative of Gram-negative bacteria, mid-chain branched saturated fatty acids, indicative of sulphate-reducing bacteria, and terminally branched saturated fatty acids, indicative of Gram-positive bacteria showing an overall strong presence of bacterial communities in the plots. The presence of polyunsaturated fatty acids, indicative of fungal communities, seemed to decrease in 2005 among the experimental plots. Most of the experimental plots except for the control sites were characterised by positive association between phosphorus concentrations and bacterial PLFA groups. Estimated viable biomass in terms of PLFA analyses was higher in experimental plots treated with 60-ton/ha vermi-compost as opposed to those treated with 90-ton/ha. Fungal to bacterial ratios increased from 2004 to 2005 but were still less than 1, indicating dominance of bacterial communities in the tailings material of the experimental plots.

Vegetation analyses in terms of species frequency showed that *Cenchrus ciliaris* and *Cynodon dactylon* were the most prevalent species in most of the experimental plots and overall establishment of grass species was present in the experimental plots, indicating successful establishment of sown-in species and the possibility of successful rehabilitation. Plant biomass was slightly higher in experimental plots treated with inorganic fertiliser type A (superphosphates) and the control sites showed increased plant biomass for 2005. Plant vitality as determined by performance index (PI_{abs}) of the photosynthetic apparatus of grasses in the different experimental plots is a multiparametric expression and a product of light energy absorption, light energy trapping and energy conversion into the electron transport chain as mentioned above. In general, the sites treated with 60ton/ha vermi-compost seemed to show higher PI_{abs} values than those treated with 90ton/ha vermi-compost. Certain experimental plots had lower PI_{abs} values than the other plots due to low levels of light energy absorption and trapping in the photosystems of the grasses.

1.3. General conclusions

The complex and integrated properties of soil make it necessary for as many biochemical environmental parameters as possible to be included when attempting to assess soil quality. No sole indicator can accurately portray all the crucial processes that simultaneously occur in the soil environment. However, the environmental parameters used in this study do represent the interaction between the major biogeochemical cycles in soil environments and plant growth and productivity. It is evident that enzymatic activity increased with increased organic matter application possibly because of the microbial community being stimulated to produce more enzymes by the abundance of organic substrates to be degraded in the tailings material, however microbial biomass was found to increase with lower amounts of organic matter application possibly indicating an optimum concentration of organic matter for the reproduction and establishment of microbial communities irrespective of excess organic matter application beyond the optimum level. Bacterial communities were more abundant than fungal communities in the tailings material (fungal to bacterial ratio of less than 1), which could be expected since disturbed or cultivated soils usually show bacterial dominance whereas undisturbed healthy soils usually show fungal dominance (Anderson and Domsch 1980). Plant vitality also increased with lower amounts of organic matter application, which could mean that excess organic matter above an optimum level negatively impacts photosynthetic activity in plants by forcing plants to decrease their osmotic potential to tolerate the excess nutrients in the tailings environment. This environmental stress causes an energy deficiency and lowers plant vitality. Plant biomass was positively associated with inorganic fertilizer type A (superphosphate) application in the experimental plots, which could indicate that plant nutrition is more important than any environmental stresses. Superphosphates stimulate plant growth and development whereas fertilizer type B was used to lower the pH in the tailings material. It is evident from the study that microbial enzymatic activity and microbial respiration as well as microbial community structure and plant vitality and biomass production could serve as effective tools in the assessment of rehabilitation processes in disturbed soil environments especially in the mining industry.

1.4. Recommendations

- For future studies of similar nature it would be suitable for the comparative study to include reference sites which receive no artificial amendments at all to see what difference these chemical adjustments make in terms of soil quality
- Regular seasonal application of inorganic and organic fertilisers should continue at any specific rehabilitation trial until a particular level of sustainability is reached in the microbial community and plant growth and establishment
- Specific attention should be paid to grass species that have established themselves on the tailings material and must be considered for future rehabilitation projects
- From this experimental study it is evident that most of the environmental parameters used indicated more positive results in the experimental plots that received less vermi-compost and should be considered in the future
- In terms of vegetation coverage and establishment, application of superphosphates showed better results for plant biomass and photosynthetic activity therefore it would be better to use fertiliser that provide nutritional support rather than using specific fertilisers to adjust the soil pH
- Physical environmental factors such as slope, wind and rain erosion and infiltration vs runoff, specifically characteristic of rock dumps, should be considered because these factors greatly influence soil ecosystems
- Regular monitoring of environmental parameters could be implemented to recognise any trends in tailings properties throughout the duration of the rehabilitation whether negative or positive so that the necessary management plans can be incorporated

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