THE ASSESSMENT OF SOIL MICROBIAL AND PLANT PHYSIOLOGICAL CHANGES DURING THE TREATMENT OF SOIL CONTAINING BROMACIL, TEBUTHIURON AND ETHIDIMURON

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I dedicate this work to my parents. Whom I love deeply and have the greatest respect for. The example you have shown me, teaching me to be ambitious, passionate and to be curious of all things. But without the opportunities you have made possible I would not be where I am. Thank you for your support during my search. I love you.

DECLARATION

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

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Language and style used in this dissertation are in accordance with the requirements of the journal *Water, Air and Soil Pollution*.

This dissertation represents a compilation of manuscripts, where each chapter is an individual entity and some repetition between the chapters has been unavoidable.

Summary

Increased amounts of pesticide production and application of pesticides for agriculture, plant protection and animal health has resulted in soil, water and air pollution, consequently relating a serious risk to the environment and also to human health. Pesticides include several groups of compounds, herbicides, insecticides, rodenticides and fumigants consisting of several hundred individual chemicals. Herbicides are an integral part of modern agriculture and for industries requiring total vegetation control. Most herbicides are soil applied and more and more concern is raised that herbicides not only affect target organisms but also the microbial community present in soil. The ESKOM sub-station Zeus, in Mpumalanga (South Africa) used to apply an industrial weed control program for the eradication of vegetation, which led to the contamination of soil by several herbicides. These herbicides consisted of Bromacil, Tebuthiuron and Ethidimuron which are all photosynthesis inhibitors, more specifically, they disrupt the plastoquinone protein during electron transport at photosystem II (PSII). In this study the effect of bio-stimulation and bio-augmentation of a specific bioremediation agent (B350) as prescribed by ESKOM, on residual herbicides, Bromacil, Tebuthiuron and Ethidimuron was evaluated by monitoring the soil physical and chemical properties, microbial attributes, including potential microbial activity and community structure, as well as the physiological effect experienced by plants (*Cynodon dactylon* and *Zea mays).* Results from soil physical and chemical analyses were correlated with results obtained for the functional and structural diversity of microbial communities. All results were investigated through statistical and multivariate analysis and the most prominent soil physical and chemical parameters that influence the biological and biochemical properties of the soil were identified. Results obtained from this study indicated that there were no significant difference ($p < 0.05$) between the treatments, with bioremediation agent, irradiated agent and without the agent based on results obtained from soil microbial properties and plant physiology. Before the trial started the uncontaminated soil showed an active microbial function, characterised by dehydrogenase, urease and arylsulphatase activity, but community structure was not very diverse. The contaminated soil, irradiated contaminated soil and silica sand showed less enzymatic function and was characterised by phospholipid fatty acid groups, mid-branched saturated fatty acids, terminally branched saturated fatty acids, normal saturated fatty acids and monosaturated fatty acids which are indicative of microorganisms that survive better in harsh environments. Three weeks after

the addition of the specific bioremediation took place, the uncontaminated soil showed an increase in β-glucosidase activity and percentage organic carbon (%C), which could be a result of the presence of available plant material. Furthermore, an increase in major PLFA groups were seen, suggesting that an increase in diversity within the soil community occurred. The contaminated soil, irradiated contaminated soil and silica sand once again was characterised by a low microbial function and diversity, showing no improvement. Fluorescence data clearly show a decline in PS II function that result in the decline of the rate of photosynthesis, which was seen from CO_2 gas exchange rates. Furthermore, the decrease in photosynthetic activity after three weeks was too severe to supply additional information about the mechanism within photosynthesis or the photoprotective mechanisms. A detailed study was conducted in which a 3:1 dilution of contaminated soil with silica sand, was also monitored for changes within plant physiology. Results revealed that inhibition of PS II function already takes place within a few days time and the decline in photosynthesis is as a result of electron transport that does not supply adenosine triphosphate (ATP) and β-nicotinamide adenine dinucleotide (NADPH) to the Calvin cycle (or Reductive Pentose Phosphate pathway). It does not appear that rubulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) is affected within the Calvin cycle. As a result of PS II function failure, reaction centres are damaged by the production of harmful singlet oxygen and photoprotective mechanisms (xanthophyll cycle) can not be activated. Thus, except for dealing with ineffective electron transport, additional damage is caused to physiological functions. After six weeks a decrease in the estimated viable biomass for all growth mediums was found. Results of the of *trans-* to *cis-* monoenoic fatty acids and cyclopropyl fatty acids to their monoenoic precursors ratios indicated that the soil microbial community for the contaminated growth mediums, all experienced nutritional stress throughout this trail. The specific bioremediation agent (B350) used, seemed to have no effect on the microbial function and community structure within soil and as agent had no effect on the residual herbicides or the plant physiology which experienced an extreme decline in major metabolic functions.

Keywords: Bioremediation, herbicides, microbial community structure, plant physiology, soil enzymatic activity, soil quality

Opsomming

Verhoogde vlakke van plaagdoderproduksie en toediening van plaagdoder vir landbou, plantbeskerming en gesondheid van diere het gelei tot besoedeling van grond, water en lug met die gevolg van 'n verhoogde risiko in die omgewing asook vir die gesondheid van die mens. Plaagdoders sluit verskeie groepe verbindings in: plantdoders, insekdoders, knaagdierdoders en berokingsmiddels wat bestaan uit honderde verskillende individuele chemiese verbindings. Plantdoders vorm 'n belangrike komponent van moderne landbou en industriële maatskapye wat dit gebruik vir totale plantegroei beheer. Meeste plantdoders is grond gerig en toenemende gevaar bestaan dat dit nie net die teikenorganismes bereik nie, maar ook die mikrobiese gemeenskap wat in die grond teenwoordig is. Die ESKOM-substasie Zeus in Mpumulanga (Suid-Afrika) het 'n industriële plantdoderbeheerprogram gebruik vir die totale beheer van plantegroei, wat gelei het tot die besmetting van grond deur verskeie plantdoders. Hierdie plantdoders sluit in Bromacil, Tebuthiuron en Ethidimuron, wat almal fotosintese-inhibeerders is. Meer spesifiek is dit verantwoordelik vir die ontkoppeling van die plastoquinoonproteïen gedurende elektrontransport in fotosisteem II (PS II). Met die studie is die effek van biostimulasie en bioaugmentasie van 'n bioremediëringsmiddel (B350) soos voorgeskryf deur ESKOM, op Bromacil, Tebuthiuron en Ethidimuron ondersoek. Die effek is deur die bestudering van die fisiese-, chemiese- en mikrobiologiese grondeienskappe ondersoek, wat ingesluit het, die potensiële mikrobiese ensiemaktiwiteit en die analise van mikrobiese gemeenskapstruktuur asook die fisiologiese effek op plante (*Cynodon dactylon* en *Zea mays*). 'n Korrelasie tussen die resultate van die fisiese en chemiese analise is getref met die funksionele en strukturele diversiteit. Alle resultate is met behulp van statistiese en meervoudigevariansie-analises ondersoek. Die mees dominante fisiese en chemiese grond parameters wat die biologiese en biochemiese grondeienskappe die meeste beïnvloed is geïdentifiseer. Resultate verkry vanuit hierdie studie het getoon dat daar geen statisties betekenisvolle verskille ($p < 0.05$) tussen verskillende behandelings met bioremedïeringsmiddel, bestraalde middel en sonder die toediening van die spesifieke middel, was nie. Voor die studie (voor die toediening van die bioremediëringsmiddel) het resultate gewys dat nie-besoedelde grond 'n aktiewe mikrobiese funksie gehad het, wat gekarakteriseer is deur die dehidrogenase, urease en arielsulfatase aktiwiteit, maar die gemeenskapstruktuur nie baie divers nie was. Die besoedelde grond, bestraalde besoelde grond en silika sand het lae mikrobiese aktiwiteit getoon wat gekarakteriseer is deur

fosfolipied-vetsuur (PLFA) groepe wat aanduidend is van mikroörganismes wat in uiterste omgewings kan oorleef. Drie weke na die toedienning van die spesifieke bioremediëringsmiddel het die nie-besoedelde grond 'n toename getoon in β-glukosidase aktiwiteit en die persentasie organiese koolstof (%C), wat toegeskryf kan word aan die teenwoordigheid van beskikbare plantmateriaal. Verder is 'n toename in hoof PLFA groepe gevind wat impliseer dat daar 'n toename in die diversiteit in die grondgemeenskap plaasgevind het. Die besoedelde-, bestraalde besoedelde- en silika sand het laer ensiemaktiwiteit getoon. Fluoresensie data wys dat daar 'n afname in die funksie van PS II plaasgevind het wat lei tot die afname in fotosintese soos waargeneem in die CO_2 gaswisseling tempo's wat bepaal is. Die afname in fotosintese aktiwiteit was te groot na drie weke om inligting te verskaf aangaande die werking van fotosintese en fotobeskermingmeganismes. 'n Opvolgstudie is gedoen waar 'n 3:1 verdunning van die besoedelde grond gemaak is met silika sand. Die resultate het getoon dat die inhibisie van die funksie van PS II binne 'n tydperk van 'n paar dae plaasvind en dat die afname in fotosintese 'n gevolg van die blokkering van elektrontransport is, wat nie meer adenosien trifosfaat (ATP) en β-nikotienamied adenien difosfaat (NADPH) aan die Kalvin siklus voorsien nie. Dit wil voorkom dat ribulosebisfosfaat karboksilase (Rubisco) nie tydens die Kalvin siklus afgebreek word nie. As gevolg van PS II meganisme wat nie funksioneer nie, word die reaksiesentrums beskadig deur die vorming van vrye radikale en fotobeskermingmeganismes (xantofil siklus) wat nie geaktiveer kan word nie. Daarom, buiten die onaktiewe elektrontransport word addisionele beskadiging aan die plantfisiologie veroorsaak. Na ses weke word 'n afname in die lewensvatbare mikrobiese biomassa vir al die groeimediums waargeneem. Die ander mediums het geen mikrobiese aktiwiteit of teenwoordigheid van hoof PLFA groepe getoon nie, wat bewys dat daar 'n afname in die gemeenskap diversiteit was. Die spesifieke bioremediëringsmiddel (B350) wat gebruik is, het skynbaar geen effek op die mikrobiese funksie of gemeenskapstruktuur gehad nie, en as gevolg dat die bioremediëringsmiddel geen effek op die heersende plantdoder gehad het nie, is daar drastiese inhibisies van die fisiologie (metabolisme van die plant) waargeneem.

Sleutelwoorde: Bioremediëring, grondensimatieseaktiwiteit, grondkwaliteit, mikrobiese gemeenskapstruktuur, plantdoders, plantfisiologie.

LIST OF ABBREVIATIONS

Φ	Yield
A	CO_2 assimilation rate at atmospheric CO_2 concentration
Amax	Maximum CO_2 assimilation rate at saturating CO_2 concentration
ABS/CS	Phenomenological energy flux (per excited cross section) for absorption
ABS/RC	Specific energy flux (per PS II reaction centre) for absorption
ANOVA	analysis of variance
Bmonos	branched monosaturated fatty acids
Bsat	base saturation
C	carbon
C_a	Atmospheric CO_2 concentration
C_i	Intercellular CO_2 concentration
$CaCl_2$	Calcium chloride
CE	Carboxylation efficiency
CEC	cation exchange capacity
CO_2	carbon dioxide
CS	Cross-section
DNA	deoxyribonucleic acid
DI_0/CS_M	The phenomological energy flux (per excited cross section) for dissipation
DI_0/RC	The specific energy flux (per PS II reaction center) for dissipation
$\delta A/\delta C_i$	Initial slope of the demand function of the A:Ci response
EC	electrical conductivity
EDTA	Ethylenediamine-tetraacetic acid
ET_0/CS_M	Phenomenological energy flux (per excited cross section) for electron transport
ET_0/RC	Specific energy flux (per PS II reaction centre) for electron transport
F:B	fungal/bacterial ratio
G_l	Total conductance, i.e. boundary layer and stomatal
G_s	Stomatal conductance
HCO_3^-	bicarbonate ion

H_2S	hydrogen sulphur
INF	iodonitrotetrazolium chloride formazan
INT	iodonitrotetrazolium chloride
k_F	Fluorescence emission
k_H	Heat dissipation
k_N	Nonphotochemical rate constant
k_P	Photochemical rate constant
k_X	Energy migration to PS II
KCl	potassium chloride
l	Relative stomatal limitation of photosynthesis
MBSats	mid-chain branched fatty acids
Monos	monosaturated fatty acids
N	nitrogen
N_2	molecular nitrogen
NADPH	β-Nicotinamide adenine dinucleotide
NH_3	ammonia
NH_4	ammonium
N_2O	dinitrous oxide
NO_2	nitrite
NO_3	nitrate
NSats	normal saturated fatty acids
O_2	oxygen
P	phosphorous
PEA	Plant Efficiency Analyser
PEG	Polyethylene glycol
PEP	Phosphoenol pyruvate
PLFA	phospholipid fatty acid
PMSF	Phenylmethylsulfonyl fluoride
PNG	p-nitrophenyl-β-D-glucosidase
PNP	para-nitrophenol
PO_4^-	phosphate

Polys	polyunsaturated fatty acids
PSI	Photosystem one
PSII	Photosystem two
PVPP	Polyvinylpolypyrrolidone
Φ_{Do}	Quantum yield of energy dissipation
Φ_{Eo}	Quantum yield of electron transport
Φ_{Po}	Maximum yield of primary photochemistry
Ψ_0	Efficiency with which a trapped excitation can move an electron further than Q_A^- into the electron transport chain
Ψ_L	Leaf water potential
Ψ_S	Osmotic potential
Γ	CO_2 compensation point
Q_A	Primary quinone acceptor
RC	Reaction centers
RC/ABS	Density of active PS II reaction centers per total absorption
RC/CS_M	Density of active PS II reaction centers per excited cross section
RDA	Redundancy Analysis
Rubisco	Rubilose-1, 5-bisphosphate carboxylase-oxygenase
S	Sulphur
S_2	sulphide
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SO_4^-	sulphate
SOM	soil organic matter
TBSats	terminally branched saturated fatty acids
TR_0/CS_M	Phenomenological energy flux (per excited cross section) for trapping
TR_0/RC	Specific energy flux (per PS II reaction center) for trapping
Tukey HSD	Tukey Honest Significant Difference

Chapter 1

1.1 Introduction

The development and modernisation of the world resulted in an increase of agricultural practices due to alarming growth of the world population (Dikshith, 1991). Parallel to the modernisation of the world, our knowledge of plant and animal physiology and biochemistry has increased and several chemicals are now available to control undesired plants and animal species in agriculture. These contaminants (pesticides, which include herbicides) are chemically synthesised, they degrade very slowly due to the lack of degradative enzymes in natural microflora and as a result they accumulate in the environment and exert their toxic effects (Parsek et al., 1995) Increased production and application of pesticides for agriculture as well as for plant protection and animal health has caused the pollution of soil, ground and surface water. This involves a serious risk to the environment and also to human health due to direct exposure or through residues in food and drinking water (Gonzales-Pradas et al., 1987; Kouras et al., 1998). The usefulness of pesticides are not doubted, they quickly kill a considerable portion of a pest population and at times provide the only practical method for preventing economic loss. However, the application of pesticides should be considered with care and a clear understanding of the pest life cycle is important (Liu, et al., 2005), to minimise the negative effects on the environment.

Herbicides are an integral part of modern agriculture and for industries requiring total vegetation control. Herbicide sales in South Africa amounted to 47% of the total pesticide sales in 1997, thereby its importance is clear (Goszszynska, 2001). The majority of herbicides are soil applied, especially in the control of annual and broad-leaved weeds under industrial conditions. Thus, by applying soil-acting pre-emergence herbicides, total weed control can be achieved (Vermeulen et al., 1996).

For many years ESKOM (South Africa) has used herbicides for industrial weed control in and around the power stations, under power lines, servitude's and at substations. Treatments were usually applied to the strip between security fences and consisted of Bromacil, Ethidimuron and Tebuthiuron. The herbicides belong to the uracil and

phenylurea families, respectively. The urea family is one of the most important herbicide classes used worldwide and can be used selectively for germinating grass and broad leaved weeds in many crops, especially cereals or non-selectively for total weed control as part of the maintenance of roads, railways, parks, etc. Bromacil a substituted uracil, is a highly effective herbicide which is a potent inhibitor of the photosynthesis processes. These three herbicides are all photosynthesis inhibitors, more specifically, they disrupt the b-quinone protein during electron transport at photosystem II (PS II) (Camillieri et al., 1987). Other processes also occur that are most commonly responsible for plant death (Herbicide Handbook, 1994). These processes include the formation of singlet oxygen and triplet chlorophyll molecules that produces a lipid radical and starts a cascade of lipid peroxidation. Lipids and proteins are attacked and oxidised, therefore, chlorophyll and carotenoids are lost because of membranes that leak and allow cells and cell organelles to dry and disintegrate (Frank et al., 1999; Frank et al., 2000; Herbicide Handbook, 1994). The herbicides are designed to be stable in soil and are not prone to chemical, hydrolytic or photolytic degradation. Therefore, they are ideal for use as encroachment control agents, since they will persist in soil for long periods of time (Goszszynska, 2001).

Soil persistence is the length of time a herbicide remains active in soil and herbicides vary in their potential to persist in soil. Several factors determine the length of time herbicides persist, including soil factors (soil composition, soil chemistry and microbial activity), climatic conditions (moisture, temperature and sunlight) and herbicidal properties (water solubility, vapor pressure and the compound's susceptibility to chemical and microbial breakdown) (Curran, 1998).

There is an increasing concern that herbicides not only affect the target organisms (weeds) but also the microbial community present in soil. The non-target effect may degrade the performance of important soil functions which include organic matter degradation, the nitrogen cycle and methane oxidation (Brussaard, 1997). El Fantroussi et al., (1999) showed that the long-term application of the herbicides Linuron and Diuron had a negative effect on the bacterial group *Acidobacterium*. Recently, the ability of microorganisms to metabolise some pesticides have received much attention, due to the

environmental persistence and toxicity of these chemicals. However, in some cases microbial metabolism of contaminants may produce toxic metabolites. A variety of microorganisms are known to utilise organic pesticides as their sole carbon or energy source. They include *Pseudomonas pickettii, Alcaligenes eutrophus, Desulfomonile tiedje* and *Phanerochaete chrysosporium* (Gonzales-Pradas et al., 1997; Kouras et al., 1998). Microorganisms require certain environmental conditions for optimal growth and utilisation of any contaminant. Factors that affect microbial activity are moisture, temperature, pH, oxygen and mineral nutrient status (Curran, 1998).

Microbial communities are critical components of soil and may probably be the earliest predictors of soil quality changes (Scow et al., 1998). They largely determine biogeochemical cycles, turnover processes of organic matter and the fertility and quality of soil (Zelles, 1999). Soil quality is defined as the 'continued capacity of soil to function as a vital living system, within ecosystem and land use boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal and human health' (Doran and Safley, 1997). According to Schloter et al., (2003) since soil microorganisms can respond quickly, they reflect a hazardous environment and are thus considered when monitoring the soil status. Doran and Safley (1997) considered physical (texture, rooting depth, infiltration rate, bulk density, water retention capacity); chemical (pH, total carbon, electrical conductivity, nutrient status); and biological (carbon and nitrogen microbial biomass, potentially minerisable nitrogen, soil respiration) parameters as criteria for the minimum evaluation of soil quality. Biological and biochemical parameters are sensitive to slight modifications that can take place in the presence of any degrading contaminant. Therefore, when monitoring the total sustainability of soil natural functions and its different uses the biological and biochemical parameters must be evaluated (Filip, 2002; Nannipieri et al., 1990). Microbial consortiums possesses the ability to accommodate environmental obstacles by adjusting the activity rates, biomass and community structure of microorganisms (Schloter et al., 2003). Soil microbial biomass is the living component of soil organic matter, excluding soil animals and plant roots, and is responsible for mineralisation and turnover of organic materials (Henderson, 1995; Nambiar, 1997).

Microbial activity can be defined as the large variety of activities carried out by microorganisms in soil, but should not be confused with biological activity which include activities of other organisms as well as plant roots (Nannipieri, 1990; Nannipieri, 2002). Soil microbial activity contributes to the liberation of nutrients available for plants but also to the mineralisation and mobilisation of xenobiotics. Nutrient cycling in soils involves biochemical, chemical and physiochemical reactions, with biochemical processes being mediated by microorganisms, plant roots and soil animals. All biochemical reactions are catalysed by enzymes, which cause chemical reactions to proceed at faster rates (Kandeler et al., 1996). According to Schloter et al. (2003), microbial activity measurements include enzymatic assays that catalyse substrate-specific transformations and can be used to help ascertain effects of soil management, land use and specific environmental conditions.

The microbial populations of soil includes an enormous diversity of bacteria, algae, fungi, protozoa, viruses and actinomycetes (Torsvik et al., 1990). The composition and activity of soil microbial communities are responsible for determining biogeochemical cycles, the turnover processes of organic material and the fertility and quality of soils. The activity and biomass of soil microorganisms respond to soil management, organic material and the abiotic environment and are influenced by plant litter and rhizosphere effects (Calderon et al., 2000). Recent studies have shown the relationships between soil microbial community composition and biotic and abiotic environmental conditions (Steenwerth et al., 2003). Phospholipid fatty acid (PLFA) analysis uses the cell membrane lipids within microorganisms as biomarkers for specific groups of organisms. They create a profile of the microbial community and certain biomarkers are used to indicate microbial stress (Guckert et al., 1986).

Plants play an important role in our lives and the environment has a significant role in plant growth and development. Environmental stresses encompass a wide range of physical conditions, which can significantly alter plant metabolism, growth and development, leading at their extremes to plant death. These stresses include drought, high salinity, temperature extreme, metal toxicity, ultraviolet radiation, nutrient

deprivation, high light stress and hypoxia (Dat et al., 2000). Plants by their very nature, are embedded in the soil and unable to escape exposure to these environmental extremes and therefore must respond to survive (Strasser et al., 1995).

Nie et al. (1992) found that a decrease in photosynthetic competence is combined with changes in the photosynthetic apparatus which include a reduction in pigment content, lower activities of several enzymes of photosynthetic carbon metabolism (Stamp, 1984; Stamp, 1987), modifications in thylakoid membranes, depression in the composition of photochemical activities of photosystem I (PS I) and PS II (Robertson et al., 1993) as well as a decrease in the rate of CO_2 assimilation when young maize leaves are exposed to stress conditions like sub-optimal temperatures (Nie et al., 1992).

The environment has a unique capability to minimise pollution and remediate itself. Several processes may occur in the contaminated place itself and naturally attenuate the pollutants accumulated through modern lifestyles (Alexander, 1999). Natural attenuation includes chemical, physical and biological processes such as dispersion, sorption, volatilasation, abiotic oxidation, hydrolysis and biodegradation or intrinsic bioremediation (Suthersan, 1999). Several strategies have been devised to remediate and restore polluted environments, including physical, chemical and biological approaches requiring the involvement of biological agents (Iwanmoto and Nasu, 2001).

Recent years have witnessed an enormous growth in the controlled, practical use of microorganisms for the degradation of chemical pollutants (Sawyer et al., 1994). These technologies rely on the biodegradative activities of microorganisms and focus on enhancing present but slow biodegradation processes in nature or technologies that bring chemicals into contact with microorganisms in some type of reactor that allows for rapid transformation (Bellon-Maurel et al., 2001). In many instances, the focus of attention is on existing sites of pollution and such technologies are encompassed by the term "bioremediation" (Alexander, 1999). The goal of bioremediation is to degrade organic pollutants to concentrations that are either undetectable or if detectable, to concentrations below the limits established as safe or acceptable by regulatory bodies (Alexander, 1999).

Bioremediation allows natural processes to degrade harmful compounds in the environment. It uses microorganisms, such as bacteria, protozoa and fungi to degrade compounds by optimising conditions for biodegradation through aeration, addition of nutrients and control of pH and temperature (Atlas and Bartha, 1992; Morgan and Watkinson, 1989; Norris, 1994). The most important aspect of bioremediation is the fact that it involves the management of a contaminated site using biologically based technologies to prevent, minimise or mitigate damage to human health or the environment. There are three basic components of any bioremediation processes: microorganisms, a potentially biodegradable compound and a bioreactor in which the process can take place (Pankhurst et al., 1995).

1.2 Problem statement

ESKOM implemented a total weed control program which almost completely eradicated vegetation at Zeus, a substation in the Mpumalanga province. The bare soil was exposed to the destructive effects of rain, wind and temperature. These resulted in progressive erosion and consequently in certain areas the topsoil layer was absent. In the past, ESKOM's primary concern was the rehabilitation of eroded areas and in 1998, the severely eroded Zeus substation was successfully revegetated. They introduced plants to this area but plants developed symptoms typically associated with that of urea herbicides exposure. The conclusion was that the soil still contained herbicides at unacceptably high concentrations. In such a situation, it is difficult to reclaim contaminated areas back to natural vegetation or croplands. One option is to physically remove polluted soil but this is a costly operation. Another option is the use of plants and microorganisms to remove contaminants from soils without excavation, in other words the soil remains in place (*in situ*) during the treatment.

Results obtained from a previous study undertaken by the Plant Protection Research Institute (PPRI), Agricultural Research Council, Roodeplaat Dam, demonstrated that despite a 30% reduction in the residual herbicides concentration (Bromacil, Tebuthiuron and Ethidimuron), the plants investigated (*Cynodon dactylon*) exhibited similar

phytotoxic symptoms as well as growth patterns to the untreated control samples and eventually died. In their study, the herbicide contaminated soil was obtained from the Zeus sub-station of ESKOM, and the addition of various ameliorants (fine sorb, kraal manure and compost potting mixture) as well as bioremediation agents (bacterial strains E30, B350 and LT1) were tested. Based on the results obtained, the PPRI researchers concluded that a decrease in herbicide concentration alone was insufficient to prevent plant death.

1.3 Objectives

The objectives of the present investigation were to determine whether premature plant death was due to the persistence of herbicides by applying a specific bioremediation agent (B350), as prescribed by ESKOM, to quantify the effect of biostimulation and bioaugmentation on the functional and structural diversity of the microbial communities in the relevant soil. Furthermore, to identify the site of action in the physiological metabolism of the plant and to evaluate the plant stress physiology.

1.3.1 Specific Outcomes

- Quantification of the effect of biostimulation (irradiated inoculation of the bioremediation agent B350) and bioaugmentation (inoculation of unirradiated bioremediation agent B350) on the functional and structural diversity of the microbial communities in soil.
    - Functional diversity was quantified by assay of microbial enzymatic activity.
    - Structural diversity was quantified by analysis of signature lipid biomarkers, specifically the phospholipid fatty acids.
- Quantification of phytotoxicity on *Cynodon dactylon* and *Zea mays*.
    - Plant function was evaluated by determining concentrations of plant pigments (zeaxanthin, violaxanthin, antheraxanthin, chlorophyll a and b).

- Identification of the phytotoxicity effect based on the analysis of chlorophyll fluorescence.
    - This approach was facilitated in the identification of the mode of action and specific metabolic site of action of the herbicides.
- Quantification of plantphysiology
    - CO_2 gas exchange was determined to confirm chlorophyll fluorescence results.

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Chapter 2: Literature Review

2.1 Industrialisation of Resources and Agriculture

Humans have always inhabited two worlds: One is the natural world of plants, animals, soils, air and water that preceded us by billions of years and of which we are part. And the other being the world of social institutions and artifacts that we create for ourselves using science, technology and political organisation. Both worlds are essential to our lives, but integrating them successfully causes enduring tensions. The human population has grown at an alarming rate during this century. Nearly 6 billion people occupy the earth and we are adding about 100 million more each year (Cunningham and Saigo, 1997). With this alarming growth we have to consider our available resources for the present and future.

Mankind is dependent on air, water and soil our principal components, which are vulnerable to degradation and pollution (Wackermagel and Rees, 1996). The world population has multiplied more than-three fold since 1900, the world economy has expanded twenty times, fossil fuel consumption has grown by a factor of thirty and industrial production has increased fifty times (Glasby, 1995). To achieve desirable economic yields that meet the increasing demand for more food worldwide, the use of pesticides (herbicides, fungicides, insecticides, etc.) is a critical component of the current production system (Van Vuuren and Bouwman, 2004).

Since the beginning of the industrial revolution, an enormous number of organic compounds have been chemically synthesised (Sabate et al., 2003). Direct application of these synthetic chemicals over a long period and indirect application through generation of chemical waste from every major economic sector, including agriculture, the oil industry, the textile and paper industries and the defence and aerospace industries, have resulted in environmental contamination (Ashman and Puri, 2002). Unlike naturally occurring organic compounds that are readily degraded upon introduction into the environment, some of these synthetic chemicals are extremely resistant to biodegradation by native flora. Therefore, hazardous wastes and chemicals have become one of the major problems of modern society worldwide. With increased sophistication of chemical, technological and engineering science, many new substances have been developed that are capable of causing unpredictable secondary effects on the environment and living organisms (Chaudhry, 1994). Hazardous wastes and toxic chemicals pose complex

environmental problems by directly affecting the air, water, soil and sediment while indirectly and unpredictably affecting living organisms that use these resources (Gadd, 2004).

2.2 Pesticides

The development and modernisation of the world resulted in an advanced increase of agricultural practices due to alarming growth of the world population (Dikshith, 1991). Parallel to the modernisation of the world, our knowledge of plant and animal physiology and biochemistry has increased and several chemicals are now available to control undesired plant and animal species in agriculture. Increased levels of pesticide production and application of pesticides for agriculture, plant protection and animal health has resulted in soil, water and air pollution and consequently relating a serious risk to the environment and also to human health (Gonzalez-Pradas, 2004). The health hazard involved is a result of the entry of contaminants into the food chain of humans and animals which can accumulate in one organism and have destructive effects. These contaminants (pesticides, herbicides, etc.) are chemically synthesised, they degrade very slowly due to the lack of degradative enzymes in natural microflora and as a result they accumulate in the environment and exert their toxic effects (Parsek et al., 1995). Alarming levels of pesticides in air, water and soil have been reported, some of which are persistent, toxic, mutagenic, carcinogenic and tumorogenic (Shelton et al., 1997).

The usefulness of pesticides are not doubted, they quickly kill a considerable portion of a pest population and at times provide the only practical method for preventing economic loss. The application of pesticides should be considered with care and a clear understanding of the pest life cycle is important. Thereby, pesticide application can occur when the pest is at its most vulnerable, limiting negative environmental effects and achieving maximum effects at minimum levels of pesticide (Liu et al., 2004).

Several pest control strategies are available to farmers by combining biological, cultural, physical and chemical tools in a way to reduce economic, health and environmental risks (Liu et al., 2004). Pesticides include herbicides, fungicides and insecticides which target plants, fungi and insects respectively. The largest part of pesticide application is used by farmers for several agricultural practices but these applications can cause severe contamination of the soil and groundwater (Ritter et al., 1994).

It is extremely difficult to decide on one method for pesticide disposal that can be universally applied, since such a wide variety of pesticides is in use worldwide. Therefore, several methods for removal and disposal of these chemicals are needed to resolve this problem (Gonzalez-Pradas et al., 1987). The ability of microorganisms to metabolise some pesticides have received much attention over recent years. However, the metabolisation of these chemicals, might produce toxic metabolites and a variety of microorganisms are known to utilise organic pesticides as the sole carbon or energy source, adding to the pollution problem (Kouras et al., 1998). Only a few soil-applied herbicides have been shown to be susceptible to mineralisation by pure cultures of microorganisms. The lack of mineralisation may be due to the structural diversity of herbicides, which contains several structural groups requiring multiple catabolic enzymes that are not all found in a single organism (Shelton et al., 1997). In soil it is more than likely that the degradation of herbicides starts with an enzymatic attack by relatively non-specific oxidases like the peroxidases produced by fungi and actinomycetes. Hydrolases and/or ring cleavage enzymes are responsible for futher metabolisation which results in products that are mineralised by means of catabolic pathways (Esposito et al., 1998).

The efficiency and environmental impact of pesticides are influenced by their persistence and ability to move through the soil profile. Pesticides which are not readily degraded or adsorbed by the soil colloids sometimes leach through the soil profile, thereby contributing to the contamination of groundwater. Groundwater contamination could be severe, not only in nature but for the general public as well (Alva and Singh, 1991).

2.2.1 Herbicides

Different herbicides have different uses, the application for agricultural purposes requires selective action against weeds, but crop plants should tolerate the treatment without injury. On the other hand, non-selective herbicides are used for complete plant eradication in industrial areas, railroads, highways or airfields. Some herbicides are photosynthetic inhibitors of submergent and emergent vegetation (Forsyth et al., 1997).

In the environment only a small fraction of herbicides dispersed reaches its target position where it exerts its role as a herbicide, the rest enters the soil (Tixier et al., 2002). When a herbicide enters the soil, it can possibly be biodegraded but it may also be susceptible to photochemical reactions induced by sunlight (Tomlin, 2000). Most soil-applied

herbicides are sprayed on the soil surface and consequent rainfall or irrigation alters the distribution of the herbicide in the environment (Nishimoto and Rahman, 1985). The distribution of herbicides in the environment may have several negative impacts, including adverse affects on the growth and survival of benthic diatom biofilms in aquatic systems. These biofilms are responsible for the stabilisation of sediments in aquatic systems through their mechanisms to produce extracellular polymeric substances (EPS) (Patterson, 1995; Smith and Underwood, 1998). Other free-living components of soil biota that are affected by the presence of herbicides in the soil environment, include bacteria, fungi, microalgae and invertebrates (Vroumsia et al., 1996).

The ability to predict herbicide activity for different soils over a variety of climatic conditions would allow researchers some guidelines for developing more accurate herbicide recommendations (Nishimoto and Rahman, 1985). Important properties of herbicides include water solubility, soil adsorption, chemical and photodecomposition, microbial breakdown and volatility (U.S. Department of Agriculture, 1984). The concern that herbicides not only affect the target organisms, but also other functions which include organic matter degradation, the nitrogen cycle and methane oxidation, are increasing. These soil functions are all critical to soil health and maintenance of a balanced soil ecosystem (Brussaard, 1997).

Herbicide effectivity may also be influenced by placement of the herbicide and should be considered before application. Some herbicides are more toxic through shoot absorption where others might be more effective by root zone absorption (Nishimoto and Warren, 1971).

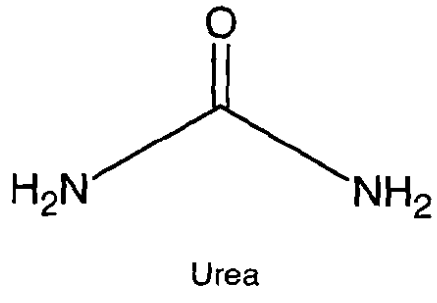
2.2.1.1 Urea Herbicides

Urea herbicides are one of the most important classes of herbicides used worldwide and can be used selectively for germinating grass and broad leaved weeds in many crops, especially cereals, or non-selectively for total weed control as part of the maintenance of roads, railways, parks, etc. These are broad-spectrum herbicides for the control of herbaceous and woody plants (Anon, 2004).

The urea herbicides are applied on soil, can persist in soil for several months and are directly or indirectly toxic to a wide variety of organisms (Tomlin, 1997). They generally have high water solubilities and low tendencies to adsorb to soil, therefore they are mobile in soil. Several studies have shown that the ability for urea herbicides to undergo natural degradation with respect to the phenyl structure are slow (Berger, 1997; Kristensen et al., 2001). Urea herbicides affect photosynthesis of the plant upon entering the plant via the roots (McFarlane et al., 1987a).

There exist limited information on the effect of urea herbicides on soil microbial communities due to the fact that conventional methods based on cultivation of microbial communities and on their metabolic activities does not allow the reality that 90% of all microorganisms living and growing in nature are noncompliant to selective enrichment cultures (Wardle and Parkinson, 1990). Microbial processes are mainly responsible for the degradation of xenobiotics in soil (Cox et al., 1996). The urea structure is slowly mineralised in various soils and subsurface environments by processes of natural attenuation (Berger, 1999).

The herbicides Tebuthiuron and Ethidimuron are known as substituted ureas because of their chemical structures. By substituting three of the hydrogen atoms of urea (Figure 1), a general fertiliser, with other chemical groups, this group of herbicides is produced (Klingman and Ashton, 1975). Common substitutions include a phenyl, methyl and/or methoxy group (Ashton and Monaco, 1991).



Urea

Figure 1: The chemical structure formula of the urea group.

Substituted ureas were discovered and investigated for their herbicidal properties. Over the decades several studies were conducted that enlighted the world about the biological effects of urea derivatives (Herbicide Handbook, 1994). Bucha and Todd (1951) revealed that annual and perennial grasses had a toxic effect towards Monuron [3-(p-chlorophenyl)-1,1-dimethylurea]. The use of 1,1-dialkyl-3-(halophenyl) ureas were granted in 1953 by U.S. patents for the first time. Thereby initiating Du Pont to develop Fenuron [1,1-dimethyl-3-phenylurea], Diuron [3-(p-chlorophenyl)-1,1-dimethylurea] and Neburon [1-butyl-3-(3,4-dichlorophenyl)-1-methylurea] for commercial herbicidal use which was the starting point for the practical use of ureas as herbicides (Geissbühler et al., 1975).

The mode of action of urea herbicides in plants are dependent on the capacity of plants to translocate compounds from their environment into various plant organs and tissues (Geissbühler et al., 1975). The apoplastic xylem system is responsible for absorption and translocation to upper plant parts (Bayer and Yamaguchi, 1965; Geissbühler et al., 1963; Haun and Peterson, 1954). If urea herbicides are applied to the leaves, apoplastic translocation also occurs (Geissbühler et al., 1963) and it should be noted that specific urea formulations determine the amount absorbed and translocated from root to shoot (Smith and Sheets, 1967). It is generally accepted that ureas are taken up from nutrient soil and soil solutions by roots and translocated into stems and leaves by the transpiration stream. Soil application has the most phytotoxic effect for grown plants, whereas foliar application is less effective (Steinert and Stritzke, 1977).

Shortly after the discovery of phenylureas their powerful effect on the photosynthetic mechanism was discovered (Cooke, 1956). The movement of oxygen in the presence of living chloroplasts and a suitable hydrogen acceptor or else known as the Hill reaction (Camillieri et al., 1987) is usually the primary site of action (photosystem II) of urea herbicides. The formation of ATP and $NADPH_2$ is prevented by this inhibition and results in the lack of required energy for carbon dioxide fixation. Other processes also occur that are most commonly responsible for plant death (Herbicide Handbook, 1994). These processes include the formation of singlet oxygen and triplet chlorophyll molecules that produces a lipid radical and starts a cascade of lipid peroxidation. Lipids and proteins are attacked and oxidised, therefore chlorophyll and carotenoids are lost because of

membranes that leak and allow cells and cell organelles to dry and disintegrate (Frank et al., 1999; Frank et al., 2000; Herbicide Handbook, 1994).

Phytotoxic symptoms occur either as acute or chronic effects and are primarily observed in the leaves (Ashton and Crafts, 1981). Acute symptoms appear within a few days and are dependent on high concentrations of applied herbicide, whereas chronic injury is a result of lower concentrations and develop later (Coffman et al., 1993). Visual effects include appearance of light green areas, water-soaked appearance, wilting of leaves, the appearance of silver and/or grey blotches and yellowing (Vermeulen et al., 1996).

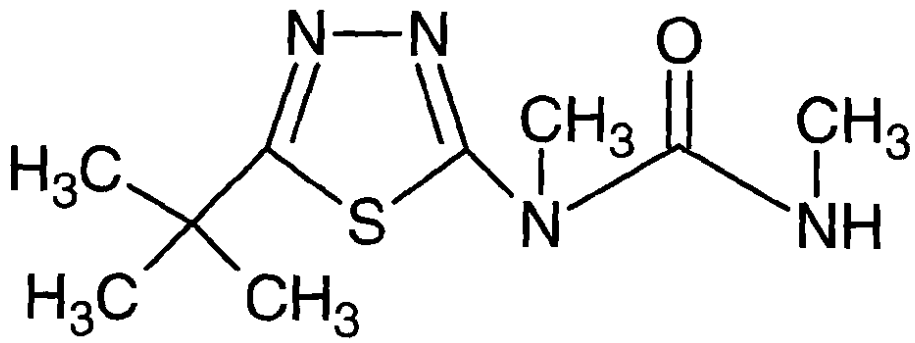
The chemical and physical properties of the biological environment exerts a direct impact on the metabolism and mode of action of substituted ureas and should not be ignored. For example, water plays an important role in determining the persistence of Tebuthiuron in soil due to the fact that it is more soluble than other herbicides (Bovey et al., 1978). The transportation of soil-active herbicides Tebuthiuron (Figure 2) and Ethidimuron (Figure 3) to the root zone are dependent on precipitation (Whisenant and Clary, 1987). Pesticide leaching alters with a change in use patterns, soil texture, total organic carbon in soil, half-life and depth of water (Domagalski and Dubrovsky, 1992). Therefore, Tebuthiuron phytotoxicity is influenced by soil characteristics (Coffman et al., 1993). Clay and organic matter content exerts a direct effect on the toxicity of Tebuthiuron with an increase in toxicity as clay and organic matter increases. The same applies for the adsorption of Tebuthiuron to soils, with the highest in organic soils, intermediate in clay soils and lowest in sandy soils (Geissbühler et al., 1975). A study conducted by Duncan and Scifres (1983) revealed that the phytotoxicity of Tebuthiuron is inversely related to clay and organic matter content.

2.2.1.1.1 Tebuthiuron

Three tebuthiuron formulations (Figure 2) are registered in South Africa (Vermeulen et al., 1996). Grazer GG (20% macro granules), Tebusan GG (75.2% macro granules) and Reclaim WP (75.2% wettable powder) which is used to control undesirable woody plants in pastures and rangelands. In Table 1 the chemical and physical properties of tebuthiuron are shown.

Table 1: Chemical and physical properties of Tebuthiuron as indicated by Herbicide Handbook (1994), Tomlin (1997), Vermeulen et al., (1996), Worthing (1993) and Zweig (1980).

Properties	**Tebuthiuron**
Empirical formula	$C_9H_{16}N_4OS$
Structural formula	See Figure 2
Common name	Tebuthiuron
Chemical name (IUPAC)	1-(5-tert-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea
Trade names	Grazer GG, Tebusan GG, Reclaim WP, Grazer SC, Tebusan SC
Physical state	Odourless, colourless solid (technical grade)
Molecular mass	228.3
Melting point	161.5-164°C
Degradation	Thermally decomposes close to melting point
Vapour pressure	0.27mPa at 25°C or 2×10^{-6}mm Hg at 25°C
Solubility	2.5g L^{-1}
Half-life in soil	15 months at 200-300mm annual rainfall, depending on organic and clay content present



Tebuthiuron

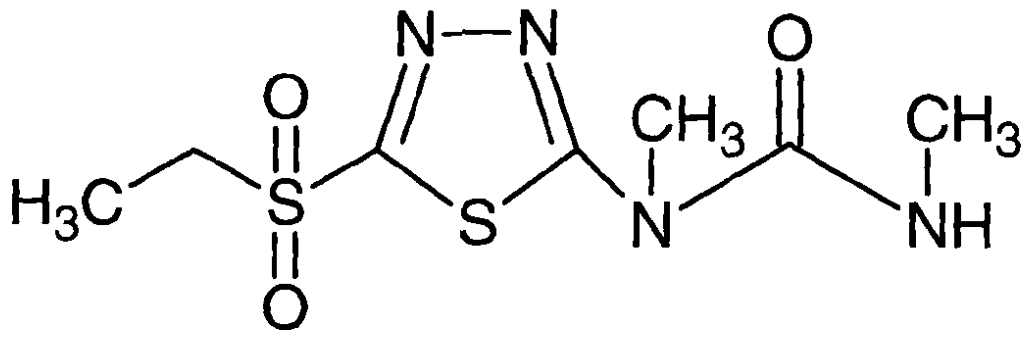
Figure 2: The chemical structure of Tebuthiuron.

2.2.1.1.2 Ethidimuron

There are three registered formulations of Ethidimuron (Figure 3), Ustilan 10 GR and Ustilan 70 WP for the control of annual and perennial grasses as well as broad-leaved weeds. Ustilan 20 GG can be applied to control bush encroachment (Vermeulen et al., 1996). Table 2 indicates the physical and chemical properties of Ethidimuron.

Table 2: The physical and chemical properties of Ethidimuron as indicated by Herbicide Handbook (1994), Tomlin (1997), Vermeulen et al., (1996), Worthing (1993), and Zweig (1980).

Properties	**Ethidimuron**
Empirical formula	$C_7H_{12}N_4O_3S_2$
Structural formula	See Figure 3
Common name	Ethidimuron
Chemical name (IUPAC)	1-(5-ethylsulphonyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea
Trade names	Ustilan 10 GR, Ustilan 70 WP, Ustilan 20 GG
Physical state	Colourless crystals
Molecular mass	264.2
Melting point	156 °C
Degradation	Decomposes at 217 °C
Vapour pressure	<0.001mPa at 20 °C
Solubility	3g kg^{-1} water at 20 °C, also soluble in dichloromethane (106g L^{-1}), propan-2-ol (6g L^{-1}) and is unstable in alkali

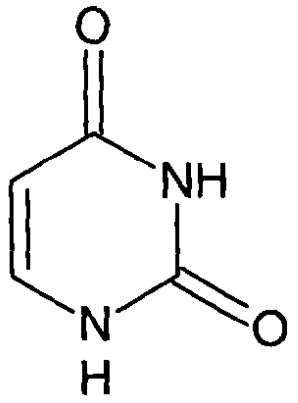


Ethidimuron

Figure 3: The chemical structure of Ethidimuron.

2.2.1.2 Uracil Herbicides

Uracil herbicides share the same core but differ in substitutions. Uracil is a symmetrical ring with two nitrogens (Figure 4).



Uracil

Figure 4: The chemical structure of uracil.

In 1961 Du Pont announced the discovery of substituted uracils, which is a family of highly effective herbicides (Myburgh, 1999). Isocil was the first uracil herbicide that was commercially introduced but other members of the class followed shortly (Gardiner, 1969).

These herbicides are soil-borne, easily absorbed by plant roots and translocated apoplastically to the leaves. At low concentrations they are toxic in leaves, are potent inhibitors of photosynthesis and have low mammalian toxicity. They differ from urea herbicides due to the fact that they have no effect on bacteria, fungi and other non-photosynthetic organisms, except at very high concentrations (Ashton and Crafts, 1981).

The uracil herbicides, like ureas block the Hill reaction, thus interfering with a step in the photosynthetic pathway (Hilton et al., 1964). Studies conducted by Hoffman (1971) showed that uracils closely resemble ureas in their mode of action. A uracil, Hyvar X was described by Bingeman et al, (1962). This compound is more active than isocil, an effective grass killer and can control a wide spectrum of broadleaf plants. The common name for the active ingredient was bromacil.

Uracil compounds are absorbed from the soil by roots, transported to the foliage via the transpiration stream and distributed throughout the tissue (Herbicide Handbook, 1994). Bromacil has the most activity in the shoots of plants and is evenly distributed throughout the plant (McFarlane et al., 1987b). Uracil compounds are xylem-mobile, when it is applied to the soil it penetrates the root zone where it is translocated to the foliar region of

the plants and kill the plants through their inhibition of photosynthesis (McFarlane, 1987b). Citrus plants exposed to uracil herbicides show tolerance which has been ascribed to the limited absorption and translocation of herbicides in citrus (Gardiner et al., 1969). The uracil herbicides are similar to urea herbicides in their mode of action (Ashton and Crafts, 1981; Gardiner et al., 1975). Like urea herbicides they are absorbed by roots and translocated apoplastically to the leaves where they inhibit photosynthesis. Bromacil, isocil and terbacil effectively inhibit the Hill reaction (Hoffman, 1971). Hoffman (1971) proposed that uracil herbicides block the pathway between the chloroplast and O_2 evolution. This could be responsible for the accumulation of a phytotoxic product, possibly a reactive free radical.

Foliar chlorosis is the primary symptom caused by uracil herbicides. Bromacil causes chlorosis and necrosis in leaves (Herbicide Handbook, 1994) and it has been shown to inhibit root and shoot growth (Ashton et al., 1969; Ashton and Monaco, 1991; Zimdahl, 1993).

Studies conducted by Gerstl and Yaron (1983) to determine the behaviour of two herbicdes (bromacil and napropamide) found that both herbicides follow first-order kinetics and are affected by soil moisture and temperature, whereas the effect of soil type and initial concentration was less distinct. However, temperature had a larger effect on the degradation process than soil type (Zimdahl et al., 1970). The leaching properties of a herbicide can be related to the water solubility of that specific herbicide. Leaching increases by rainfall or irrigation, especially for compounds that are highly water soluble (Rhodes, 1970; Reddy, 1992). Bromacil is relatively more soluble than other herbicides, thus it would leach more easily (Reddy, 1992).

Persistence of uracils in soil depends on soil type, rainfall, temperature and other soil factors. Depending on the soil properties and climate (Rhodes, 1970; Reddy, 1992), bromacil can either be persistent (Machado-Neto and Victoria-Filho, 1995) or mobile (Russo et al., 1998) in the environment. It is strongly absorbed by organic matter and to a lesser extent by clay particles, thus it is more persistent and less mobile in soils with a high organic matter content (Rhodes et al., 1970). Shipman (1983) found that organic matter content, cation exchange capacity, total nitrogen and soluble salt concentration

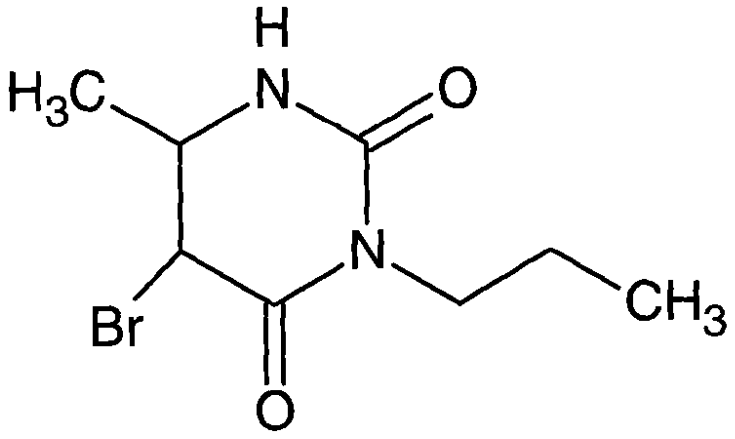
were significantly correlated with the persistence and increasing depth of Bromacil residues in four soil types.

2.2.1.2.1 Bromacil

Uracils consist of a broad-spectrum class of herbicides. Four Bromacil formulations (Figure 5) are registered in South Africa (Vermeulen et al., 1996). Hyvar X (10% granular formulation), Atlacide 60GR (60% granular formulation), Bromacil WP and Hyvar X (both 80% wettable powders). There are other combinations of Bromacil also in use for total weed control which include, Hydi G (bromacil/diuron), Hykarpon (bromacil/diuron-proprop), Viper (bromacil/hexazinone), Slam GR (bromacil/tebuthiuron) and Orbit 100GR (bromacil/terbuthylazine). Hyvar X and Bromacil WP are registered not only for the control of annual and perennial grasses but also for the control of bush encroachment (Vermeulen et al., 1996). Table 3 indicates the chemical and physical properties of bromacil.

Table 3: The physical and chemical properties of Bromacil as indicated by Herbicide Handbook (1994), Tomlin (1997), Vermeulen et al., (1996), Worthing (1993), and Zweig (1980).

Properties	**Bromacil**
Empirical formula	$C_9H_{13}BrN_2O_2$
Structural formula	See Figure 5
Common name	Bromacil
Chemical name (IUPAC)	5-bromo-3-*sec*-butyl-6-methyluracil
Trade names	Hyvar XG10, Atlacide 60GR, Bromacil WP, Hyvar X
Physical state	White, crystalline, odourless solid
Molecular mass	261.1
Density	1.55g mL^{-1} at 25 °C
Melting point	158 °C-159° C
Degradation	Thermally stable up to melting point, stable in aqueous bases and is slowly decomposed by concentrated acids
Vapour pressure	0.033 mPa at 25 °C
Solubility	815 mg L^{-1} in water at pH 7 at 25 °C, it is also soluble in ethanol, acetone, acetonitrile and xylene



Bromacil

Figure 5: The chemical structure of Bromacil.

2.3 Soil Contamination by Herbicides

The production and use of herbicides are still largely accepted but it is extremely important to control or minimise the health and environmental problems associated with their application. It has been cited by Lagaly (2001) that it is a greater ecological problem to reduce the applied amounts of herbicides than to decompose or degrade the non-bioactive amounts.

Herbicides are distributed into the environment but only a small amount of the applied pesticide is bioactive. For example, herbicides are sprayed on the veldt to obstruct the growth of weeds but only part of the applied amount exerts an effect. The disappearance of pesticides (bromacil, etc.) from soils results from interactions between various processes such as chemical decomposition, microbiological degradation, volatilisation, runoff, leaching, photodecomposition and uptake by plants (Gomez et al., 1996). Some of the herbicides remain attached to the soil particles or are adsorbed by dust particles, are leached out, migrate into the ground water or are distributed by surface runoff (Craven and Hoy, 2004). There is more and more interest in soil-applied herbicides for the control of low-value plants due to the fact that their timing of application is generally less critical than with foliar sprays (Petersen and Ueckert, 1991).

When considering the persistence of a pesticide it is important to examine soil composition, including factors such as soil acidity or alkalinity. Therefore, the assessment of pesticide persistence in a suitable range of soils specifically tailored to the properties and proposed use of the individual pesticide is very important (Alexander, 1999).

2.4 The Soil Ecosystem

The soil ecosystem represents an intricate balance between living and non-living components and forms the building blocks for all life in the biosphere but due to the different activities of several organisms, permanent changes take place in the soil environment. These changes involve shifts between growth and decay and thus in a biological and biochemical way, soil represents the most important link for the global interchange of matter and energy (Filip, 2002).

Soils are made up of various sized inorganic mineral particles (sand, silt and clay), reactive and stable forms of organic matter, several living organisms (earthworms, insects, bacteria, fungi, algae, nematodes, etc.), water and gases including O_2, CO_2, N_2 and CH_4. Soil biological activities are influenced by physical and chemical properties of soil which regulates the interchange of molecules/ions between the solid, liquid and gaseous phases. This, in turn, controls the cycling of nutrients, plant growth and decomposition of organic materials. Soil plays a very important role in completing the cycling of major elements (C, N, P and S) required for biological systems, degradation of organic wastes and detoxifying of certain hazardous compounds. The role of soil in the recycling of organic materials into carbon dioxide (CO_2) and water and the degrading of chemical pollutants is achieved by microbial decomposition, chemical hydrolysis, complexation and sorption reactions (Doran and Parkin, 1994; Doran et al., 1996).

2.4.1 Carbon Cycle

Carbon (C) is essential to all forms of life on earth. Compounds that comprise living tissues are made-up of carbon atoms arranged in chains or rings and associated with many other elements. Carbon can be present in reduced forms, which include methane (CH_4) and organic matter; it can also be oxidised in forms of carbon monoxide (CO) and carbon dioxide (CO_2) (Atlas and Bartha, 1998).

Carbon fixation and decomposition are responsible for the cycling of carbon within an ecosystem and occurs through the activity of autotrophs and heterotrophs, respectively. It cycles in the form of inorganic carbon dioxide and various organic compounds (Atlas, 1997). The synthesis of organic compounds from carbon dioxide and water through photosynthesis results in the fixation of carbon which occurs through the activities of cyanobacteria, algae, photosynthetic bacteria and aerobic chemolithoautotrophs (Prescott et al., 1999; Raven and Johnson, 1999). Carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) are forms of inorganic carbon found in soil which are products of chemical erosion of rocks, biogenetic deposits such as coal, petroleum and humus, whereby carbon is deposited into soil (Atlas, 1997).

The trophic structure within an ecosystem allows the activity of various groups of organisms each having their own metabolic needs. Chemoorganotrophic and chemoautotrophic organisms use organic and inorganic compounds, respectively, as sources for generating cellular energy, whereas photoautotrophic organisms uses light as the energy source and carbon dioxide as a source of carbon. Reduced carbon, which is the product of fixation and/or decomposition can be transferred from population to population within the soil community (Atlas, 1997; Atlas and Bartha, 1998).

Soil organic matter contains 3-4 times as much carbon as is found in all the world's living vegetation. Hence, soil organic matter plays an important role in the global carbon balance that is thought to be the major factor affecting global warming (or the greenhouse effect) (Ashman and Puri 2002).

2.4.2 Phosphorous Cycle

Phosphorous (P) is one of the main limiting elements for crop production. In many ecosystems phosphorous availability limits overall ecosystems productivity through its effect on plant production and nitrogen fixation (Rowell, 1994). The most important plant function associated with phosphorous is the storage and transfer of energy. The energy obtained from photosynthesis and metabolism of carbohydrates is stored in phosphate compounds vital for plant growth, such as ADP or ATP (adenosine di- and triphosphates). Synthesised through both respiration and photosynthesis, ATP contains high-energy phosphate groups that drives most energy-requiring biochemical processes.

Phosphorous is also an essential component of deoxyribonucleic acid (DNA), the seat of genetic inheritance, and of ribonucleic acid (RNA), which directs protein synthesis in both plants and animals. Phosphorous is also part of phospholipids, which play critical roles in cellular membranes (Ashman and Puri, 2002).

The native phosphorous in soils originated largely from the weathering of rocks containing mineral apatite $Ca_{10}(PO_4)_6(F, Cl, OH)_2$. Phosphorous present in this form is unavailable to plants. Secondary minerals of phosphorous include Fe and Al phosphates and Ca phosphates that are very insoluble and as such not available to plants.

Phosphorous is absorbed by plants as the primary and secondary orthophosphate ions ($H_2PO_4^-$ and HPO_4^{2-}), which are present in the soil solution. The amount of each form present depends on soil solution pH. At pH 7 both phosphate ions are present in about equal amounts. At pH<7, $H_2PO_4^-$ is the main form, while at pH>7, HPO_4^{2-} dominates. The ionic forms of phosphorous (i.e., phosphate ions) are susceptible to adsorption and subsequent precipitation either by Fe, Al or Mn present in the soil solution (case in acidic soils) or by Ca-compounds (in alkaline soils). At neutral pH phosphates tend to bound with clay minerals. Phosphate compounds with either Fe, Al, Mn, or Ca tend to have low solubility and consequently low availability to plants. This is referred to as phosphate fixation to indicate that phosphorous is tied up in a form unavailable for uptake by plants (Entry et al., 2002; Rowell, 1994).

Mineralisation is the overall process of conversion of phosphorous from an organic to an inorganic form as a result of microbial decomposition. The inorganic phosphorous released by mineralisation is readily available to higher plants and to microorganisms. Phosphatase enzymes are responsible for the degradation of organic-bound phosphorous which releases inorganic phosphorous to soil as plant residues and other organic debris (Garcia et al., 2002).

2.4.3 Nitrogen Cycle

Nitrogen is an integral component of all amino acids, which are the building blocks of proteins (including the enzymes), nucleic acids and chlorophyll. All living organisms depend in some or other way on the results of nitrogen fixation to synthesise proteins, nucleic acids and other necessary nitrogen-containing compounds. These occur in various

states within an ecosystem as ammonium (NH_4), nitrate (NO_3), nitrite (NO_2) and molecular or atmospheric nitrogen (N_2). Atmospheric nitrogen gas constitutes 78% of the earth's atmosphere, however the biologically available fixed nitrogen in the soil, oceans and the bodies of organisms only constitutes about 0.03% of that amount (Atlas and Bartha, 1998).

Only a few organisms which are all bacteria can convert or fix atmospheric nitrogen into forms that can be utilised in biological processes. The triple bond that links the two atoms of the diatomic atmospheric nitrogen together is very stable and the reaction that catalyses the cleavage of atmospheric nitrogen is dependent on a complex of three proteins, ferredoxin, nitrogen reductase and nitrogenase. This reaction uses ATP as a source of energy, electrons derived from photosynthesis or respiration as well as a powerful reducing agent (Prescott et al., 1999; Raven and Johnson, 1999).

Amines and amino acids released from the decomposition (by mostly bacteria in neutral and alkaline environments and mostly fungi in acidic environments) of proteins are further decomposed by heterotrophic microorganisms during ammonification process. The microorganisms carrying out ammonification can be either aerobic or anaerobic. Some of the ammonium (NH_4) released into the soil solution will be either converted to nitrites (NO_2) or nitrates (NO_3) by the process of nitrification carried out by photoautotrophs, chemoheterotrophs and a few bacterial species of chemoautotrophs. The different forms of nitrogen will then absorbed by plants, used by heterotrophic microorganims to build new tissues, adsorbed to clay minerals, or released to the atmosphere as elemental nitrogen (Ashman and Puri, 2002).

Claassens (2003) cited that plants and microbes use nitrate (NO_3) as a source of nitrogen much the same as ammonium (NH_4) whereby it undergoes a series of microbially mediated processes until it is returned to the atmosphere in the form of N_2 by denitrification.

2.4.4 Sulphur Cycle

Sulphur (S) is a constituent of the amino acids (methionine and cysteine), vitamins (biotin, thiamine, B1), and many protein enzymes that regulate photosynthesis and nitrogen fixation. Sulphur is closely associated with nitrogen in the processes of protein and enzyme synthesis (Atlas, 1997).

Microorganisms contribute greatly to the sulphur cycle. During the microbial decomposition of organic C-bonded sulphur compounds, sulphides are formed along with elemental sulphur and thiosulphates ($S_2O_3^{2-}$). These (reduced) substances are subject to oxidation and autotrophic bacteria *Thiobacillus sp.* carry out this oxidation. The oxidation of hydrogen sulphide (H_2S) is an acidifying process, since for every sulphur atom oxidised, two hydrogen ions are formed (Atlas and Bartha, 1998).

The acidifying effect of sulphide oxidation can bring about extremely acid soil conditions (to a pH as low as 1.5). Photosynthetic microorganisms transform sulphur by using sulphide as an electron source but when sulphate diffuses into reduced habitats, it provides an opportunity for different groups of microorganisms to carry out sulphate reduction. The use of sulphate as external electron acceptor to form sulphide in the environment is known as dissimilatory assimilation and anaerobic respiration. The reduction of sulphate for use if amino acid and protein biosynthesis is described as assimilatory reduction. Sulphite is another critical intermediate that can be reduced to sulphide by a wide variety of microorganisms (Prescott et al., 1999; Raven and Johnson, 1999).

2.5 Soil Quality

All ecosystems share air, surface water and groundwater environmental components, which has a profound effect on the health and productivity of a given ecosystem and the environment related to it, but the quality of soil should not be ignored because it is the foundation of the entire biosphere and thus has multi-functional attributes (Harris et al., 1996; Filip, 2002). Soil forms the skin of unconsolidated mineral and organic matter on the earth's surface and functions to maintain the ecosystems on which all life depends (Doran et al., 1996). It provides the physical layer for almost all human activities, e.g. agriculture, buildings and transport and it produces resources for industrial use and waste management (Brussaard, 1997).

For a long time modern agriculture has judged new farming techniques and products primarily on their ability to increase food production and the potential for environmental damage associated with modern agriculture was overlooked (Doran et al., 1996). Three general ways through which soil interact with, and affect the health of higher animals exists. First, the possibility of direct poisoning of animals and people from contaminated

soils which is mostly related to industrial accidents, improper use or disposal of agrochemicals, industrial chemicals or radioactive waste products. Second, the interaction between soil health and animal/human health occurs indirectly, through soil's influence on the quality of water and air. Lastly, the impact of soil on animal and human health is also indirect and occurs through the quality of food plants grown in soil which can be contaminated by presence of agrochemicals (such as pesticides), or through decreased or imbalanced content of necessary plant nutritional compounds (Doran et al., 1996). The disturbance of soils can lead to critical changes in the biosphere, which in the end, may threaten the very existence of human beings (Snakin et al., 1996).

Defining and quantifying soil quality is not an easy task, some have believed that soil quality is an abstract characteristic and can not be defined. However, to evaluate, maintain and manage our soils for the benefit of future generations it is extremely important to have guidelines by which management protocols can be designed. Doran and Parkin (1994) defined soil quality as: "The capacity of a soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality and promote plant and animal health." Biological productivity translates to the ability of soil to enhance plant and biological productivity, environmental quality relates to the ability of soil to attenuate environmental contaminants, pathogens and offsite damage; and animal health represents the interrelationship between soil quality and plant, animal and human health (Doran and Parkin, 1994).

To assess soil quality, indicators representing the physical, chemical and biological controlling components of soil are necessary (Kennedy and Papendick, 1995). This is complicated because of the interactions of these components with time, space and intensity. Defining soil quality indicators should involve a holistic approach rather than a reductionistic approach (Doran and Parkin, 1994). Soil quality indicators usually include soil organic matter, biological activity and soil biodiversity, soil structure and porosity, available water capacity, plant available nutrients, cation exchange capacity, soil acidity, soil salinity and depth of rooting and crop vigor (Shaxson, 1998).

Soil health changes over time due to natural events or human impacts. It can also be enhanced by management and land-use decisions that evaluate the multiple functions of soil and do not only focus on single functions such as crop productivity. Therefore a balance in soil functions, for productivity, environmental quality and plant and animal health is required for optimal soil health (Doran, 2002).

2.5.1 Physical Characteristics

2.5.1.1 Soil Composition and Structure

Disturbance of the soil layer is often characterised by the loss of the humus-rich organogenic layer caused by wind and water erosion, by mechanical rupture or the accumulation of sediments (Snakin et al., 1996). Soil composition is a physical factor and gives information about the relative amount of sand, silt and clay as well as the organic matter present in soil. Soil can be divided into thee particle-size classes namely, sand, silt and clay. Adsorption of herbicides to soil particles may reduce their bio-availability to soil microorganisms (Alva and Singh, 1991).

Soil structure describes the manner in which sand, silt and clay particles are bonded together in aggregates. Texture is a critical soil property which is determined largely by particle size since there is a 1000-fold difference in the size of the sand and clay particles. Thus, gaps are formed between particles of different sizes which are called soil pores. These pores affect water drainage, gas exchange with the atmosphere, soil strength and amount of water held for plants (Ashman and Puri, 2002). The disruption of soil structure caused by compaction from heavy agricultural machines or other compactive processes can be monitored by evaluating the soil density (Snakin et al., 1996).

2.5.2 Chemical Characteristics

2.5.2.1 Soil Acidity and Salinity

The availability of nutrients are influenced by many chemical reactions, such as adsorption and precipitation by the soil chemical environment of which soil pH is particularly important. As pH influences so many biological and chemical relationships at once, it provides inconclusive information as to which soil process is affected by it and this in turn influences the productive capacity of soil. pH is an important chemical property of soil that can influence the persistence of some herbicides (Alexander 1999).

2.5.2.2 Organic Matter

Soil organic matter contributes to soil quality through its effects on specific soil functions which include serving as a medium for the growth of plant roots; regulating the flow of water, air and nutrients; portioning precipitation into plant-available-, ground-, and surface-water; serving as a repository for atmospheric carbon and mitigating the impacts of pollutants on human and ecosystem health (Doran and Parkin, 1994).

Soil organic matter or soil organic carbon is generally seen as intricate chemical parameters of soil quality. It influences soil porosity by means of its role as aggregate stabiliser which contributes to changes in gas exchange reactions and water relations. Soil organic matter constitute a significant amount of the carbon cycle and is a repository for nutrients. It also has an influence on many essential biological and chemical processes and plays a crucial role in nutrient release and availability (Henderson, 1995; Nambiar, 1997).

Soil organic matter consists of three fractions which decompose at different rates (Voroney et al., 1981). Sikora and McCoy (1990) listed them as a readily available or soluble fraction that microorganisms decompose quickly; a fraction which is also degradable but at a much slower rate because of substrate complexity or a protection mechanism; and a fraction which is highly resistant to degradation because of structural complexity and/or protection mechanisms which lead to stability in the soil. Microorganisms decompose soil organic matter in proportion to their growth rate and maintenance (Parnas, 1975), whereby they form a reservoir of nutrients which are released into the soil when the biomass dies (Sikora and McCoy, 1990).

2.5.3 Biological Characteristics

2.5.3.1 Biological Activity and Soil Biodiversity

Microorganisms inhabit soil and together with exocellular enzymes and soil mesofauna and macrofauna conduct all known metabolic reactions (Alef and Nannipieri, 1995). Soil organisms contribute to the maintenance of soil quality since they control the decomposition of plant and animal materials, biogeochemical cycling, the formation of soil structure and the fate of organics applied to soil. Soil microbial processes are essential to soil quality and a better understanding of these processes and microbial community structure is necessary (Turco et al., 1994).

Soil microorganisms control the transformation and mineralisation of natural compounds and xenobiotics. The soil microbiota which is both high in density and diversity, are able to modify their energetic performance and activity rates in an ever changing environment. The microbial consortium therefore possesses the ability to accommodate environmental obstacles by adjusting the activity rates, biomass and community structure of microorganisms (Scholter et al., 2003).

2.5.3.2 Microbial Activity

There are possibly millions of species of microorganisms present in soil (Brussaard, 1997). It has even been estimated by Jong (1989) that microorganisms constitute about one quarter of the total biomass on earth. Microorganisms are responsible for the decomposition and transformation of organic matter including all nitrogen and carbon transformations (Alexander, 1977; Apsimon et al., 1990). The decomposition of compounds such as cellulose, hemicellulose, polysaccharides, hydrocarbons and lignin provide energy to heterotrophic microorganisms which in turn are responsible for nutrient transformations, including nitrogen fixation and protein and amino acid transformations (Roper, 1983; Sikora and McCoy, 1990). Therefore, microorganisms are responsible for a major amount of mineral transformations which include P, S, Fe, K, Ca, Mg, Mn, Al, As, Zn and Se (Alexander, 1977; Doran, 1982; Doran and Parkin, 1994).

Microbial activity can be defined as the large variety of activities carried out by microorganisms in soil, but should not be confused with biological activity which include activities of other soil organisms as well as plant roots (Nannipieri, 1990; Nannipieri et al., 2002). Soil microbial activity contributes to the liberation of nutrients available for plants but also to the mineralisation and mobilisation of pollutants and xenobiotics. Therefore, microbial activity is of exceptional importance in biochemical cycling (Zahir et al., 2001). Nutritional conditions, temperature, water availability, proton concentrations and oxygen supply regulates microbial activities. Scholter et al. (2003) cited that microbial activity measurements include enzymatic assays that catalyse substrate-specific transformations and can be used to help ascertain effects of soil management, land use and specific environmental conditions (Kandeler et al., 1996).

There are two general locations of enzymes in soil, those associated with intracellular- and extracellular enzymes. The function of intracellular enzymes is obvious with their central role in the numerous life processes of cells. Extracellular or abiontic enzymes as defined by Skujins (1967) are those outside living cells. Soil enzymes may originate from plants, animals, fungi and bacteria and even though it is agreed that the microbial component is the main source of enzymes in soils, the specific properties of isoenzymes (enzymes that catalyses the same reactions but may differ in origin and have slight differences in kinetic properties or amino acid sequencing) from each source cannot be determined.

Taylor et al. (2002) mentioned two main reasons for measuring soil enzymes. As indicators of process diversity they give insight into the biochemical potential, possible resilience and potential for manipulation of the soil system. Soil quality indicators change in key functions and activities and they provide information about progress of remediation operations or the sustainability of particular types of land management. However, enzyme assays have their restrictions and only provide a measure of the potential activity (Pettit, 1977). Table 4 lists the major groups of common enzymes as well as their function in the ecosystem.

Table 4: Grouping of commonly used soil enzymes and their ecological function (Dick et al., 1996).

Enzyme group	**Enzyme**	**Ecological or soil health function**
Oxidoreductases	Dehydrogenase	Exists as integral part of intact cell and reflect total oxidative activities of soil microflora/important in oxidising soil organic matter
	Glucose oxidase	Oxidises glucose
	Catalase, peroxidase	Release oxygen from hydrogen peroxide
	Polyphenol oxidases	Oxidise phenolic compounds and are involved in humification of soils

Enzyme group	Enzyme	Ecological or soil health function
Hydrolases	Phosphatase (mono- and diester)	Releases plant available PO_4 from organic matter
	Sulphatase	Releases plant available SO_4 from organic matter
	Amylase	Hydrolyses starch into maltose
	Cellulase	Endohydrolysis of 1,4-β-D glucosidic linkage in cellulose, a major component of wood and plant fibres
	Xylanase	Cleaves 1,3-β-D-xylosidic linkages of xylan, a polysaccharide found with cellulose
	B- and α-glucosidase	Release glucose, an important energy source for microbial activity
	β - and α-galactosidase	Hydrolysis of melibiose and lactose, respectively
	Invertase, saccharase, sucrase	Hydrolyses sucrose to glucose and fructose, providing energy for microbial activity
	Proteinase	Hydrolyses proteins, releasing amino compounds/important in N cycling and N mineralization
	Petidase	Hydrolyses dipeptides, releasing 2 amino acids/important in N cycle and N mineralisation
	Asparginase	Act on C-N bonds (other than peptide bonds) on respective amino acids releasing NH_3 important in N mineralisation to provide plant available N
	Amidase	Hydrolyses C-N bonds of amides releasing NH_3 important for N mineralisation to provide plant available N form
	Urease	Belongs to group of enzymes acting on C-N bonds of urea, a fertiliser source and a major constituent in urine of grazing animals

Enzyme group	Enzyme	Ecological or soil health function
Transferases	Dextranssucrase	Hydrolyses sucrose, releasing glucose and fructose
	Thiosulphate S-transferase (rhodanese)	Performs intermediate step in oxidation of elemental S which is found in small amounts in soils or is added as a S fertiliser
Lyases	Glutamate decarboxylase	Hydrolyses aspartic acid
	Tyrosine decarboxylase	Hydrolyses tyrosine, a product of proteinase activities and involved in N mineralization
	L-Histidine ammonia lyase	Deaminates histidine and involved in N mineralisation
Broad spectrum enzymes	Fluorescein diacetate hydrolysis	Provides general indicator of soil hydrolytic activity by assaying 3', 3'-diacetylfluorescein hydrolysis which is carried out by proteases, lipases and esterases

A group of methods on soil microbial activities uses biochemical procedures which reveals information about the metabolic processes of microbial communities (Schloter et al., 2003). Nutrient cycling in soils involves biochemical, chemical and physical reactions, with biochemical processes being mediated by microorganisms, plant roots and soil animals (Zahir et al., 2001). All biochemical reactions are catalysed by enzymes which are catalysts meaning they are substances that without undergoing permanent alteration cause chemical reactions to proceed at faster rates (Tabatabai, 1994). Dehydrogenase, acid- and alkaline phosphatase, arylsulphatase and urease were investigated for their involvement in the major biochemical cycles (C, P, N and S).

2.5.3.2.1 Dehydrogenase

One of the most studied enzymes is dehydrogenase because it normally only exists in viable cells and has an important role in the oxidation of organic matter where it transfers hydrogen from substrates to acceptors. Dehydrogense uses oxygen directly as a hydrogen acceptor. This is called aerobic dehydrogense but the enzyme can also operate through other hydrogen acceptors to carry out anaerobic dehydrogenase. Dehydrogenase has also

been reported to be associated with microbial biomass and other biological activity (Stevenson, 1959; Ladd & Tabatabai, 1978).

2.5.3.2.2 β-Glucosidase

The enzyme β-glucosidase (EC 3.2.1.21) is a rate limiting enzyme during the degradation of cellulose to glucose. Its mode of action is the catalysis of glucosides through the processes of hydrolysis. This enzyme is important in the C cycle and hydrolysis products ensure energy sources for microorganisms in soils. It belongs to the glucosidases which hydrolyse α-D-glucopyranoside and is known as α-glucosidase. It is more abundant than α-glucosidase in soil and has been found in animal, microorganisms and plants (Turner et al., 2002).

2.5.3.2.3 Phosphatase

Phosphorous (P) is provided for plant uptake though the phosphorous cycle. Phosphate is released by the activity of phosphatase and activity of phosphatase is strongly influenced by soil pH (Eivazi and Tabatabai, 1977), thus phosphatases are assayed at acidic and alkaline conditions. Under acidic conditions phosphatase provides an index for soil to mineralise organic phosphorous. Phosphatases are enzymes with a relatively broad specificity which are able to act on various different structurally related substrates even though they act at different rates. Phosphatases catalyse the hydrolysis of phosphate esters and are named according to their specific substrates. There are five groups, phosphomonoesterases, nucleases, phosphoric trimester hydrolases, phosphoryl-containing anhydrides and phosphoamidases (Alef and Nannipieri, 1995).

Phosphomonoesterases catalyse the hydrolysis of organic phosphomonoester to inorganic phosphorous, thereby enabling plants to absorb or translocate phosphorous compounds. Phosomonoesterases are divided into groups by a range of pH. Alkaline enzymes have only been found in animals and microorganisms whereas acid and neutral enzymes have been detected in plant and microbial cells (Aon and Colaneri, 2001; Sannino and Gianfreda, 2001). Acid and alkaline phospomonoesterases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2, pH 6.5 and orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, pH 11.0) contribute to plant nutrition due to their activity in the ectorhizosphere which is higher in bulk soil while the organic phosphorous content is lower.

2.5.3.2.4 Urease

Urea (urea amidohydrolase, EC 3.5.1.5) is hydrolysed to carbon dioxide and ammonium through the catalytic reaction driven by the enzyme, urease which forms an intermediate called carbamate. This enzyme also catalyses the hydrolysis of hydroxyurea, dihydroxyurea and semicarbazide. Urease is present in microbial, animal and plant cells and thus widely distributed within the environment where it is tightly bound to soil and organic matter (Turner et al., 2002).

2.5.3.2.5 Arylsulphatase

Sulphatases catalyse the hydrolysis of organic sulphate esters and is grouped by the type of the ester in arylsulphatases (EC 3.1.6.1) namely, alkylsulphatases, steroid sulphatases, glucosulphatases, chondrosulphatases and myrosulphatases (Tabatabai, 1982). Arylsulphatase is one of the most studied soil sulphatases and is thought to play an important role in the hydrolysis of ester sulphate, which comprises 40 to 70% of the total sulphur (S) in many soils (Tabatabai, 1994). Ester sulphates are found only in fungi and thus high sulphatase activity indicates an increased level of ester sulphate produced by fungi (Dick et al., 1996). Arylsulphatase drives an irreversible reaction, and is present in microorganisms, animals and plants (Li and Sarah, 2003). This enzyme is also responsible for catalysing the hydrolysis of p-nitrophenyl sulphate, potassium phenyl sulphate, potassium nitrocatechol sulphate and potassium phenolphthalein sulphate. Arylsulphatase activity decreases as depth increases and correlates with the content of soil organic carbon, total nitrogen and cation exchange capacity (Tabatabai and Bremner, 1970). The role of arylsulphatase in the cycling of sulphur, particularly in soil, is not clear. Most of the available sulphur found in surface soil is in the form of organic sulphates, thus making this enzyme activity important (Tabatabai, 1982).

2.5.3.3 Microbial Diversity

There exists competition between communities of plants and animal (Loreau et al., 2001). Biodiversity is an expression of the variety of living things, at genetic, species and ecosystem levels (Doran and Parkin, 1994). Diversity is described as a function of two components, the total number of species present and the distribution of individuals among those species. The relative abundance of the diverse components within a community and the interactions that occur between the components are important attributes of

biodiversity. These interactions are critical in regulating the nutrient cycling and energy flow in soil (Pankhurst, 1995).

2.5.3.3.1 Microbial Biomass

The microbial biomass is defined as the living component of soil organic matter but excludes macrofauna and plant roots (Jenkinson and Ladd, 1981). According to Jenkinson and Ladd (1981), soil microbial biomass is the eye of the needle through which all organic matter needs to pass. Although it comprises less than 5% of organic matter in soil, it performs at least three crucial functions for plant production in the soil ecosystem. It is a labile source of carbon, nitrogen, phosphorous and sulphur; it is an intermediate sink of carbon, nitrogen, phosphorous and sulphur; and is an agent of nutrient transformation and pesticide degradation. (Dalal, 1998). Microorganisms form symbiotic associations with roots, act as biological agents against plant pathogens, contribute towards soil aggregation (Angers et al., 1992) and participate in soil formation (Nannipieri et al., 2002).

Microbial biomass can be used to assess the biological status of soil because it represents the fraction of the soil responsible for the energy and nutrient cycling, and the regulation of organic matter transformations which is sensitive to management or pollution. Sparling (1997) have proposed that microbial biomass is a sensitive indicator of soil quality and health which can be determined by several methods such as chloroform fumigation incubation or extraction, substrate-induced respiration (SIR), adenosine triphosphate (ATP) analysis and phospholipid fatty acid analysis (PLFA) (Dalal, 1998).

2.5.3.3.2 Community Structure

The composition and activity of soil microbial communities are responsible for determining biogeochemical cycles, the turnover processes of organic matter and the fertility and quality of soils. The quantitative description of microbial diversity is therefore necessary but it is not an easy task due to the fact that most microorganisms cannot be characterised by conventional cultivation techniques. At present only two methods can overcome the problem of selective culturing while providing unbiased information towards the structure of the complex microbial communities (Zelles, 1999). These methods are the examination of microbial populations using ribosomal RNA and phospholipid fatty acid (PLFA) analysis (Findlay, 1996; Zelles, 1999).

2.5.3.2.2.1 Phospholipid fatty acid Analysis (PLFA)

Phospholipids are important and essential membrane components of all living cells. They are not found in storage products. Therefore, under the conditions expected in naturally occurring communities, phospholipid fatty acids make up a relatively constant proportion of the biomass of organisms (Lechevalier, 1989). Living microorganisms have an intact membrane which contains fatty acids as components of its phospholipids and according to Albers et al. (1994) and White et al. (1979) rapid changes in microbial community structure can be identified by changes in PLFA patterns. Following cell death, PLFAs are rapidly degraded by endogenous and exogenous phospholipases (Peacock et al., 2001) which makes them important as signature molecules and indicators of viable microbial biomass (Calderon, 2000; Claassens 2003; Rutters et al., 2002).

Phospholipid fatty acids are integrally part of microbial membranes and are specific to groups of organisms (Frostgård and Baath, 1996; Olsson et al., 1999; Zelles, 1999). Therefore, PLFAs can signify changes in the microbial composition of a soil (Ibekwe and Kennedy, 1998; Hill et al., 2000).

Phospholipid fatty acids can be divided into major groups which include normal saturated fatty acids (Nsats), mid-branched saturated fatty acids (MBsats), terminally branched saturated fatty acids (TBsats), branched monosaturated fatty acids (Bmonos), monosaturated fatty acids (Monos) and Polyunsaturated fatty acids (Polys) (Claassens, 2003). Normal saturated fatty acids are considered ubiquitous because they are found in most microorganisms (prokaryotic and eukaryotic). Mid-branched saturated fatty acids are primarily indicative of actinomycetes. Terminally branched saturated fatty acids are indicative of gram-positive bacteria whereas monosaturated fatty acids are indicative of gram-negative bacteria (Ratledge and Wilkinson, 1988; Zelles, 1999). Monosaturated fatty acids are indicative of actinomycetes bacteria found mostly in soil and polyunsaturated fatty acids are indicative of fungi and other micro-eukaryotic organisms (Olsson, 1999; Pondor and Tadros, 2002; Rütters et al., 2002; Steger et al., 2003; White et al., 1996).

Phospholipid turnover is rapid due to materials in dead cells which will be readily consumed by the living biomass. Therefore, the total phospholipid content gives a reasonable estimation of the living biomass size (Balkwill et al., 1988). Fractionation and

analysis of individual fatty acids (signature fatty acids) permits the identification of specific functional groups of microorganisms (bacteria, fungi, actinomycetes) from fatty acid profiles and provides information about the nutritional status and the degree of stress of the microbial community (Frostgård and Bååth, 1996; Pennanen et al., 1996).

Changes in phospholipid profiles are usually related to changes and variation in the abundance of microbial groups and it is possible to identify groups by refering to a database of pure cultures and known biosynthetic pathways (Zelles, 1999). Phospholipid fatty acids, with a chain length of < 20-C atoms are considered to be of mainly bacterial origin. While 18-C chain PLFAs constitute on average 43% of the total PLFAs in soil fungi (Frostgård and Bååth, 1996). Bacterial biomass can be calculated by summing up several fatty acids which include 15:0, a15:0, i15:0, i16:0, i17:0, cy17:0, cy19:0 and 16:1ω7c, whereas fungal biomass can be obtained from the 18:2ω6c fatty acid (Frostegård and Bååth, 1996).

A fungal-to-bacterial (F:B) biomass ratio of 1.0 indicates that the fungi and bacteria are contributing equally to the microbial biomass in the soil. These ratios have been used to measure microbial community structure shifts (Wardle and Parkinson, 1990), such as those due to moisture gradients, management changes, or along a transect from a pollution point source (Bewley and Parkinson, 1985). Fungal-to-bacterial ratios have also been applied to determine the dominant group of microorganisms degrading plant residues. Previous studies have indicated ratios of saturated to unsaturated fatty acids, increased ratios of *trans*- to *cis*- monoenoic fatty acids and increased ratios of cyclopropyl fatty acids to their monoenoic precursors to be indicative of stress in microbial environments (Kieft et al., 1994).

2.6 Plant Physiology

Plants play a very important role in ecosystems and the environment has a significant role in plant growth and development. Environmental stresses encompass a wide range of physical conditions, which can significantly alter plant metabolism, growth and development, leading at their extremes to plant death. These stresses include drought, high salinity, extremes of temperatures, metal toxicity, ultraviolet radiation, nutrient deprivation, high light stress and hypoxia. 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 (Strasser et al., 1995).

Nie et al. (1992) found that a decrease in photosynthetic competence is combined with changes in the photosynthetic apparatus which include a reduction in pigment content, lower activities of several enzymes of photosynthetic carbon metabolism (Stamp, 1984; Stamp, 1987), modifications in thylakoid membranes, depression in the composition of photochemical activities of photosystem I (PS I) and photosystem II (PS II) (Robertson et al., 1993) as well as a decrease in the rate of carbon dioxide assimilation when young maize leaves are exposed to stress conditions like sub-optimal temperatures (Nie et al., 1992).

Solar radiation is essential for life on earth as a major source of energy driving the photosynthetic process but any light in excess can be detrimental for plants, causing a disturbance in their development and decreasing their production abilities. Excess light generates a flux of excess electrons, leading to over-reduction of the electron transport chains and overproduction of NADPH. This may result in increased formation of harmful reactive oxygen species in the electron transport chains (Mehler reaction, generating $O_2^{\bullet-}$, which is then transformed to H_2O_2), and thus damage the photoprotection process. The damage from electron transfer systems result in the formation of free radicals (singlet radicals, superoxide radicals and hydroxyl radicals). It is necessary to activate the biochemical protection mechanism of the plant in order to eliminate these extremely hazardous radicals. The defence mechanism protects the unsaturated membrane lipids, nucleic acids, enzymes and other cellular structures from harmful effects of free radicals (Foyer et al., 1994; Caasi-Lit et al., 1997).

Part of the light absorbed by plant pigments, mostly chlorophyll is lost through re-emission as fluorescence or as heat. The decay processes of excited chlorophyll are competitive, therefore, changes in photosynthetic rate and/or dissipative heat will lead to complementary changes in emitted fluorescence intensity. One photon of red light contains enough energy for a chlorophyll molecule to reach the first excited state, but it remains stable for less than 8-10 seconds, during which charge separation takes place within the reaction centre, forming the primary photochemical step of photosynthesis. If no charge separation occurs, the absorbed energy is released as fluorescence and/or heat

when the excited molecule returns to the original energy level (Bolhar-Nordenkampf and Öquist, 1993).

2.6.1 Carbon Metabolism

Plants face constant change in the environment and part of their success in dealing with the ability within an ever changing environment is their ability to metabolise carbon. Carbon metabolism is responsive to changes in the relationship between the supply of carbon dioxide (CO_2) or the products of electron transport and the demand for assimilated carbon (Leegood, 1993).

Photosynthetic carbon metabolism is only able to respond rapidly to external factors, since it is a highly integrated and complex process. The Calvin cycle (or Reductive Pentose Phosphate pathway) lies at the interface between electron transport and product synthesis. On the other hand there are changing inputs from electron transport (ATP and NADPH) and the regulation of metabolism by electron transport (light-activation of enzymes) while on the other hand, the processes of photorespiration, respiration and the synthesis of products such as starch and sucrose are carefully adjusted to meet the rate at which the Calvin cycle can supply carbon. In addition, C_4 plants ensure a saturating supply of carbon dioxide to the enzyme rubulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) and thereby suppressing photorespiration by the possession of an ATP-dependant CO_2 pump, which is closely co-ordinated with the Calvin cycle (Calvin, 1992; Leegood, 1993). The Calvin cycle has three phases: carboxylation, reduction and regeneration. The carboxilation involves addition of carbon dioxide and water (H_2O) to rubulose-1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglyceric acid (3-PGA). In the reduction phase 3-PGA is reduced to an aldehyde by ATP and NADPH which arises from phosphorylation and light-activated reactions, respectively. The regeneration phase involves RuBP, which is needed to react with additional carbon dioxide diffusing into leaves through stomates. This step is complex and involves the phosphorylated sugars (sucrose and starch) (Salisbury and Ross, 1992)

2.6.2 Chlorophyll *a* Fluorescence

The efficiency of the PS II system during photosynthesis is influenced by fluctuations in the plant growth environment, and can be quantified by the means of chlorophyll *a* fluorescence. Fluorescence induction patterns and derived indices have been used as

empirical diagnostic tools in stress physiology (Strasser et al., 2000). Therefore, photosystem II fluorescence can be regarded as a biosensing device for stress detection in plants. Illuminating a dark-adapted leaf results in characteristic changes in the intensity of chlorophyll *a* fluorescence, known as the Krautsky effect (Krautsky and Hirsch, 1931). The Krautsky effect transient shows a fast rise completed in less than 1s, with a subsequent slower decline toward steady state. It has been proposed that the rising phase of the transient reflects the primary reactions of photosynthesis (Krause and Weis, 1991; Strauss et al., 2005). The fluorescence rise kinetics of the Krautsky transient is polyphasic when plotted on a logarithmic timescale, showing steps J and I (Strasser and Govindjee, 1992) or I_1 and I_2 (Schreiber and Neubauer, 1987) between the initial O (F_0) and maximum P level (F_P). Strasser and Strasser (1995) have developed the JIP-test, which is used to translate the original fluorescence measurements of the these O-J-I-P transients into several phenomenological and biophysical expressions that quantify photosystem II (PSII) function. The transient changes its shape in accordance to environmental conditions and provides information about the structure, conformation and function of the photosynthetic apparatus (Strasser et al., 1995).

2.6.3 Carbon Dioxide Assimilation

Photorespiration provides an effective electron sink when carbon dioxide assimilation is low (Kozaki and Takeba, 1996) and it has been found that photorespiration protect leaves against high irradiance through not only acting as a sink for reducing equivalents but also preventing over-reduction of the electron carriers between photosystem II (PS II) and photosystem I (PS I) (Kozaki and Takeba, 1996, Osmond and Grace, 1995).

Studying carbon dioxide gas assimilation rates can give a means to screen *in vivo* limitations to the photosynthetic carbon assimilation. These studies allow the quantitative assessment of environmental factors on the different levels of the diffusion pathway. Strauss (2001) described that measurements of the growth of plants are usually described in terms of dry weight and carbon gain, which consists of drying, weighing and chemical analysis of the dried material. The direct measuring of carbon dioxide uptake provides an easy solution because it has the advantage that it is instantaneous and non-destructive.

2.6.4 Plant Proteins

A wide variety of proteins essential for photosynthesis are embedded in the thylakoid membrane. The reaction centres (RC), the antenna pigment-protein complexes and most of the electron transport enzymes are all integral proteins. The photosynthetic carbon reduction cycle is dependant on Rubisco, which is the most abundant protein in the world, accounting for approximately 50 percent of the soluble protein in most leaves (Hopkins and Hüner, 2004; Taiz and Zeiger, 2002). Rubisco catalyses two known reactions, the carboxylation of RuBP to form two molecules of PGA and alternatively, the oxygenation of the same substrate to form one molecule each of PGA and 2-phosphoglycate. Phosphoenolpyruvate carboxylase (PEP) is another important enzyme, it catalyses the irreversible carboxylation of PEP to oxaloacetate (OAA) in the cytosol of mesophyll cells of C_4 plants (Ashton et al., 1990). This forms the primary carboxylation reaction of C_4 photosynthesis (Slack and Hatch, 1967)

2.6.5 Extractable Leaf Pigments

Pigments are integrally related to the physiological function of leaves and because pigments are so important for leaf function, variations in pigment content may provide information concerning the physiological state of leaves (Liu et al., 2004). Even though there was not a high correlation between the chlorophyll content and photosynthesis rate, Marini (1986) found that the assessment of photosynthetic pigments and their relationships, is an important indicator of senescence. Chlorophyll tends to decline more rapidly than carotenoids when plants are under stress or during leaf senescence (Gitelson and Merzlyak, 1994; Merzlyak et al., 1999).

Carotenoids are widespread yellow and orange pigments of bacteria, algae, plants and animals. Carotenoids are known to be involved in several physiological processes such as antennae function and photoprotection in the photosynthetic apparatus (Liu et al., 2004). In all environments carotenoid photoprotection is realised via the quenching of singlet oxygen, scavenging free radicals and the quenching of excited triplet state of molecules of photosensitiser (Krinsky, 1979).

Chlorophylls act as a sponge, absorbing all light energy regardless of whether they need the energy or have the capability to process it and transfer it into the photosynthetic apparatus. Carotenoids play a crucial part in the processes of light harvesting complex and in the photoprotection of the photosystems. When incident light energy exceeds what is needed for photosynthesis, the carotenoids form zeaxanthin from violaxanthin via antheraxanthin through the xantophyll cycle for photoprotection (Demmig-Adams and Adams, 1996). Zeaxanthin participates intensely in the regulation of heat dissipation of the photosystem II energy when this has an energetic overload (Ort, 2001; Ramalho et al., 2000).

Chlorophyll loss is associated with environmental stress and the variation in total chlorophyll/carotenoids ratio may be a good indicator of stress in plants (Blankenship, 2002). The ratio between chlorophyll a and b remains stable under stress conditions experienced by plants even if synthesis of chlorophyll does not desist (Wilcox and Koller, 1992). Ratios between zeaxanthin, violaxanthin and antheraxanthin indicate the potential of the plant to form zeaxanthin from violaxanthin via antheraxanthin. Therefore, zeaxanthin is necessary to dissipitate excess energy (Adams and Demmig-Adams, 1994).

2.7 Bioremediation

Attempts have been made since the 1960s to address the magnitude of environmental contamination (Sayler et al., 1999). In today's era of heightened environmental awareness and government regulation, efforts to cleanup contaminated sites represent both commitment to responsible stewardship of our limited natural resources and good business. Today, environmental managers can choose from a variety of approaches from intensive engineering techniques (excavation, incineration, phytoremediation, bioremediation, etc.) to natural attenuation, a "hands-off" approach relying entirely on natural processes to remediate sites with no human intervention (Environmental Protection Agency, 2001).

Recent years have witnessed an enormous growth in the controlled, practical use of microorganisms for the destruction of chemical pollutants (Sawyer et al., 1994). These technologies rely on the biodegradative activities of microorganisms and focus on enhancing existent but slow biodegradation processes in nature or technologies that bring chemicals into contact with microorganisms in some type of reactor that allows for rapid

transformation (Bellon-Maurel et al., 2003). In many instances, the focus of attention is on existing sites of pollution and such technologies are encompassed by the term "bioremediation" (Alexander, 1999). The goal of bioremediation is to degrade organic pollutants to concentrations that are either undetectable or if detectable, to concentrations below the limits established as safe or acceptable by regulatory agencies (Alexander, 1999).

Bioremediation allows natural processes to clean up harmful contaminants in the environment. It uses microorganisms, such as bacteria, protozoa and fungi to degrade contaminants by optimising conditions for biodegradation through aeration, addition of nutrients and control of pH and temperature (Atlas and Bartha, 1992; Morgan and Watkinson, 1989; Norris, 1994). The most important aspect of bioremediation is the fact that it involves the management of a contaminated site using biologically based technologies to prevent, minimise or mitigate damage to human health or the environment. There are three basic components of any bioremediation processes: microorganisms, a potentially biodegradable contaminant and a bioreactor in which the process can take place. Bioremediation provides a technique for cleaning up pollution by enhancing some biodegradation processes that occur in nature (Pankhurst et al., 1995).

Table 5 indicates a variety of different technologies and procedures being used in bioremediation and a number of new and promising approaches that have been suggested or have reached advanced stages of development. Some of these technologies are *in situ* treatments in which soil is not removed from the original site. Other bioremediation technologies require removal of the contaminated material in some manner from its original location. These technologies represent *ex situ* treatments and can be split into two techniques, slurry-phase- and solid-phase treatment (Environmental Protection Agency, 2001).

Table 5: Examples of various bioremediation techniques.

***In situ* bioremediation**	***Ex situ* bioremediation**
Landfarming	Slurry-phase treatment: Use of biorectors
Phytoremediation	Solid-phase treatment: Landfarming Biopiles Composting
Natural attenuation	

The processes of bio-stimulation implies that additional nutrients, oxygen or other electron donors and acceptors are added to increase the number or activity of naturally occurring microorganisms available for bioremediation. Successful application of bio-stimulation was demonstrated at the DuPont Niagara Falls Plant manufacturing various organic and inorganic chemicals (Buchanan et al., 1995). Chlorinated solvents were produced from 1930 to 1975. In-field evaluations determined that biological reductive anaerobic dechlorination was occurring naturally. A field program was implemented in a pre-selected area of the plant through use of an *in situ* borehole bioreactor to attempt to stimulate indigenous biological reductive dechlorination by the addition of yeast extract (substrate) and sulphate (electron acceptor). An active microbiological population developed, which reduced the *in situ* concentrations of chlorinated compounds by more than 94%. Concentration of the typical biological degradation products did not increase, probably due to alternative biodegradation pathways (Meinhardt, 2003).

Bio-augmentation is the processes by which microorganisms are added that can bio-transform or biodegrade a particular contaminant. Fungi and bacteria present in contaminated soils or water cause bioremediation. Bacteria are usually the organisms of choice because they have more rapid metabolic rates, numerous metabolic pathways of various organic pollutants and can be genetically manipulated to improve their bioremediation capabilities (Bouwer and Zehner, 1993).

The bioremediation agent used in this study (as prescribed by ESKOM) to evaluate the bio-stimulation and bio-augmentation on soil microbial properties and plant physiology, was B350. It contains a high concentration of naturally occurring Group I non pathogenic microorganisms since their natural ability to of degradation of a wide range of chemicals. It should be said that B350 is a blend of air-dried living microorganisms that are dormant, until rehydrated. They feed on the contaminant (hydrocarbons etc) and breed up to a large biomass. The agent, B350, is designed to improve the performance of biological systems in that it establishes and maintains a resistant biomass that is able to degrade varying wastes, it increases biochemical oxygen demand (BOD) and chemical oxygen demand (COD) removal efficiency, it produces sludge flocs with superior settling characteristics that assist hydraulically stressed systems and it prevents the blocking, ponding and possible collapse of filterbed media (Anon, 2005). Therefore it was chosen to degrade residual herbicides within soil as a means to remediate contaminated soil.

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Chapter 3: The Assessment of Potential Soil Enzymatic Activities During the Treatment of Soil Containing Bromacil, Tebuthiuron and Ethidimuron.

Abstract

Microorganisms inhabit soil and contribute to the maintenance of soil quality due to their influence on the decomposition of plant and animal material, biogeochemical cycling, the formation of soil structure and the fate of organic compounds applied to soil. Herbicides are commonly used in agriculture, but their persistence and leaching in soil keeps affecting the quality of soil long after application. In this study, the effect of a specific bioremediation agent, B350 on Bromacil, Tebuthiuron and Ethidimuron was evaluated. The bioremediation agent, B350, which is used for the remediation of hydrocarbon and other organic materials in soil was applied. Each growth medium was characterised by chemical and physical analyses of soil collected on site and the microbial activity in soil was evaluated by means of enzymatic analyses (dehydrogenase, β-glucosidase, acid- and alkaline phosphatase, arylsulphatase and urease). The relationship between soil chemical and physical characteristics and the enzymatic activities were investigated by means of multivariate ordination techniques. Results of the assays of potential enzymatic activities in soil showed that uncontaminated soil was characterised by activity of most enzymes throughout the trial, whereas contaminated soil was characterised by having no enzymatic activities. No significant differences ($p > 0.05$) in enzymatic activities were observed between treatments (with or without the application of the specific bioremediation agent) for the same growth medium (uncontaminated-, contaminated-, irradiated uncontaminated-, irradiated contaminated soil and silica sand).

Keywords: Bioremediation, Bromacil, Ethidimuron, herbicides, enzymatic activity, soil quality and Tebuthiuron.

3.1 Introduction

The application of herbicides for industrial weed control at power stations, under power lines, servitude's and substations has been used for many years by ESKOM (South Africa). Herbicides including Bromacil, Tebuthiuron and Ethidimuron were applied to the area of soil between security fences at Zeus, a substation in Mpumalanga, South Africa. Herbicide build-up is inevitable in treated areas and is known to cause damage to vegetation (crops, vegetables, surrounding re-growth, etc.) and pollution of groundwater. These herbicides may also lead to contamination of adjacent agricultural land through their mobility in soil (Goszszynska, 2001).

Reclamation of these treated areas is necessary to limit contamination of adjacent agricultural land, but is problematic. Physical removal of contaminated soil is a costly procedure (Alexander, 1999) and alternatives should be considered. Management of soil communities (microorganisms and plants) to remove contaminants in the soil system can be implemented for the remediation of contaminated soil.

The herbicides applied (Bromacil, Tebuthiuron and Ethidimuron) belong to the uracil and phenylurea family which are known as photosystem II (PS II) herbicides because they affect PS II during the photosynthesis process. These herbicides bind to *b*-quinone, a protein that allows the transfer of electrons, subsequently blocking electron flow and inhibiting photosynthesis, better known as the inhibition of the Hill reaction (Camillieri, 1987; Pfister and Schreiber, 1983).

While it is desirable for herbicides to control weeds during the season of application, it is not desirable for them to persist and subsequently affect plant growth. Herbicides vary from each other in terms of their potential to persist in soil and several factors affect herbicide persistence in the soil environment (Tomlin, 1997). According to Karlen et al. (2003), these factors can be divided into categories that interact with each other, including soil factors, climatic conditions and herbicide properties.

Soil composition, soil chemistry and microbial activity (biological activity) are elements of soil quality (Doran and Parkin, 1994) that can influence herbicide persistence. Soil composition is a physical factor and gives information about the relative amount of sand,

silt and clay as well as the organic material present in soil. Adsorption of herbicides to soil particles may reduce their bio-availability to soil microorganisms (Alexander 1999). There is increasing concern that herbicides not only affect the target organisms (weeds) but also the microbial community present in soil and that these non-target effects may influence the performance of important soil functions. These critical soil functions include organic matter degradation, the nutrient cycles and methane oxidation (Brussaard, 1997).

Microbial community function in soil is a key component of several soil functions (Riffaldi et al., 2002). Soil enzymes have been suggested as potential indicators of soil quality and the activity of any enzyme assayed in a soil sample is the sum of active and potentially active enzymes from all the different sources (Nannipieri et al., 2002). Soil enzymatic activities are often closely related to important soil quality parameters which include organic material, soil physical properties and microbial activity and may therefore be used as indicators of soil quality (Dick et al., 1996). Dick et al., (1996) found that enzyme assay results present a means of determining the potential of soil to degrade or transform substrates. Soil enzymes representative of the main biogeochemical cycles (C, P, S, N) and of microbial biomass are often used as indicators of soil health. These enzymes include β-glucosidase, phosphatase, sulphatase and urease, representing the carbon, phosphorous, sulphur and nitrogen cycles, respectively (Aon et al., 2001; Pascual et al., 2000; Trasar-Cepeda et al., 2000). Enzymes assayed in this study include dehydrogenase, β-glucosidase, alkaline phosphatase, acid phosphatase, arylsulphatase and urease. Enzymatic assays were performed on soil samples at the start of the trial, after 3 weeks and again after 6 weeks.

The measurement of dehydrogenase activity is feasible because the activity is an integral part of microorganisms and their role in the oxidation of organic matter (Aon and Colaneri, 2001; Casida et al., 1964). Dehydrogenase has also been reported to be associated with microbial biomass and other biological activity (Nannipieri et al., 1996; Stevenson, 1959; Skujins, 1973). β-glucosidase is important in the C cycle and hydrolysis products ensure energy sources for microorganisms in soils (Trasar-Cepeda et al., 2000). Phosphorus is provided for plant uptake though the P cycle. Phosphate is released by the activity of phosphatase. The activity of phosphatase is strongly influenced by soil pH (Aon and Colaneri, 2001; Eivazi and Tabatabai, 1977), thus phosphatases are assayed at

acidic and alkaline conditions. At acidic conditions phosphatase provides an index for soil to mineralise organic P. Arylsulphatase is one of the most studied soil sulphatases and is thought to play an important role in the hydrolysis of ester sulphate, which comprises 40 to 70% of the total S in many soils (Tabatabai, 1994). Ester sulphates are found only in fungi and thus high sulphatase activity indicates an increased level of ester sulphate produced by fungi (Dick et al., 1996). Urease catalyses the hydrolysis of urea to CO_2 and NH_3 (Aon and Colaneri, 2001).

The objectives of the present investigation were to quantify the effect of the specific bioremediation agent (B350) as prescribed by ESKOM, to monitor the biostimulation and bioaugmentation on the functional diversity of the microbial communities in the relevant soil.

3.2 Materials and Methods

3.2.1 Experimental Layout

Grass and soil were collected from the Zeus substation, Mpumulanga (South Africa) and transferred to the glass-house facilities of the PPRI, Roodeplaat Dam, Pretoria where it was kept until transported to the North-West University, Potchefstroom campus, Potchefstroom. Soil was sieved (1.5cm^2 mesh) and the homogenised soil was stored in plastic containers until the trial started. Each treatment (with the addition of agent, irradiated agent and without the addition of agent) consisted of three replicates and was kept in the glass-house at 25°C (day time) and 15°C (night time). Table 1 shows the different growth mediums which consisted of contaminated- (CS), uncontaminated- (US), irradiated contaminated- (ICS), and irradiated uncontaminated soil (IUS) as well as irradiated silica sand. For the irradiation of the soil, 2kg contaminated- and uncontaminated soil was placed in plastic bags, heat sealed and taken to Isotron Inc. for gamma irradiation (KGy 70). A portion of the bioremediation agent (B350) was also irradiated at KGy 70. Treatments were conducted in pots, 25cm in diameter. Watering of pots occurred twice a day to ensure a constant moisture content. Soil moisture content was measured and kept below field capacity to prevent leaching of the herbicides.

The trial started with the sampling for soil enzymatic activity assays. After sampling, the addition of the bioremediation agent, B350, took place. After a week, three *Zea mays* seedlings and a *Cynodon dactylon* grass-plug were planted in each pot. *Zea mays* seedlings were germinated in a growth chamber (Conviron PGW 36, Controlled environment Ltd, Winnipeg, MB. Canada R3H 0R9) under controlled conditions with sufficient incandescent light bulbs (General Electric, Neodynium R80, 100W) and fluorescent lamps (General Electric, Cool White, 1500W) to maintain light intensities as high as 1300μmole photns.$m^{-2}.s^{-1}$ at 1 m distance. The plants were subjected to a daily light period of 15 hours (23°C) and dark period of 9 hours (20°C). Grass-plugs were cut from tufts growing in uncontaminated soil collected from Zeus. Plugs were treated with a rooting powder (Seradix B no. 1) and planted in a mixture of sand and vermiculite at 24°C and 70% humidity for a week before transplanting to the experimental pots. *Zea mays* seedlings were used in the trial to serve as an internal control and due to its comparative plant physiology to that of the *C. dactylon*. Both plants are parallel veined, equipped with an additional CO_2 fixation pathway (C_4-plant), easy to grow and germinate and the phototoxic effects are visually detectable.

The duration of the trial was six weeks, with sampling for soil enzymatic activities from day one with a three week interval for a period of six weeks.

Table 1: Pilot trial layout, conducted with uncontaminated (uncon), contaminated (con), irradiated uncontaminated, irradiated contaminated soil and irradiated silica sand as growth mediums with agent (A), irradiated agent (B) and without the addition of the specific bioremediation agent (C). These treatments were conducted in triplicate.

Treatments	**Uncon. Soil**	**Con. soil**	**Irradiated silica sand**	**Irradiated con. soil**	**Irradiated uncon. Soil**
Agent (A)	1 2 3	4 5 6	7 8 9	10 11 12	13 14 15
Irradiated agent (B)	1 2 3	4 5 6	7 8 9	10 11 12	13 14 15
Without agent (C)	1 2 3	4 5 6	7 8 9	10 11 12	13 14 15

3.2.2 Chemical and Physical Analyses

The chemical and physical analyses of the soil were performed as described by Van Rensburg et al. (1998). Twenty-four soil chemical and physical variables were analysed (Tables 2, 3, 4, 5 and 6).

3.2.3 Characterisation of the Functional Diversity of Soil Microbial Communities

3.2.3.1 Sampling Procedure

Sampling was conducted at a depth of 0-15cm deep in the soil (Aon and Colaneri, 2001; Taylor et al., 2002). Aseptic techniques as described by Dick et al. (1996) were used during sampling. Samples for soil enzymatic activities were analysed immediately after sampling.

3.2.3.2 Determination of Soil Dry Mass

The weight of each soil sample was determined in its field moist state and again after oven drying (105°C overnight). The difference attributed to the loss of the water was calculated and related to the soil moist weight (Claassens, 2003). The dry mass and soil moisture content (% soil moisture) was determined as reported by Alef and Nannipieri (1995). Soil dry mass was used to determined the activity of enzymes (dehydrogenase, β-glucosidase, phosphatase activities, arylsulphatase and urease) at dry weight.

3.2.3.3 Soil Enzymatic Activities

For the determination of dehydrogenase activity, soil was kept field moist. While samples used for determination of β-glucosidase (EC 3.2.1.21), urease (urea amidohydrolase, EC 3.5.1.5), acid (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2, pH 6.5) and alkaline (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, pH 11.0) phosphatase activities and arylsulphatase (EC 3.1.6.1) were air-dried (Alef and Nannipieri, 1995). All analyses were carried out in triplicate.

3.2.3.3.1 Dehydrogenase Activity

Dehydrogenase activity can be estimated by the procedure described by Alef and Nannipieri (1995). Soil (1.0g, field moist) was weighed and incubated (in the dark for 2h at 40°C) with 1.5ml Tris (hydroxy-methyl)-aminomethane buffer and 2ml iodonitrotetrazolium chloride (INT) (5mg ml^{-1} in 2% v/v *N,N*-dimethylformamide). Sterilised soil (1.0g samples, autoclaved at 121°C for 20min) were used as controls.

Shaking soil suspensions (at 20min intervals for 1h) followed the addition of 10ml *N,N*-dimethylformamide/ethanol (1:1 v/v), whereupon suspensions were filtered (Whatman no. 2 filter paper) and the absorbance of each sample was determined spectrophotometrically at 464nm. The dehydrogenase activity was expressed as μg INF g^{-1} dry weight $2h^{-1}$.

3.2.3.3.2 β-Glucosidase, Phosphomonoesterase and Arylsulphatase Activity

β-glucosidase (EC 3.2.1.21), acid (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2, pH 6.5) and alkaline (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, pH 11.0) phosphatase as well as arylsulphatase (EC 3.1.6.1) activities were all based on *p*-nitrophenol release after cleavage of a synthetic substrate (*p*-nitrophenyl glucoside, *p*-nitrophenyl phosphate and *p*-nitrophenyl sulphate, respectively) (Dick et al., 1996). The β-glucosidase assay was performed according to the procedure described by Dick et al. (1996). Soil (1.0g air-dried) was weighed and incubated (for 1h at 37°C) with 0.25ml toluene, 4ml modified universal buffer (pH 6.0) and 1ml *p*-nitrophenyl-β-D-glucosidase (PNG). After incubation the reaction was terminated by the addition of 1ml 0.5 M calcium chloride (Ca_2Cl) and 4ml 0.1M Tris (hydroxyl methyl)-aminomethane buffer (pH 12.0). Adding substrate to samples after incubation were used as controls. Soil suspensions were filtered (Whatman no. 2 filter paper) and the absorbance of each sample was determined spectrophotometrically at 410nm and expressed as mg *p*-nitrophenol g^{-1} dry weight h^{-1}.

Acid (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2, pH 6.5) and alkaline (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, pH 11.0) phosphatase activities were assayed using the method described by Alef and Nannipieri (1995). Modified universal buffer pH 6.5 and 11.0 were used for acid and alkaline phosphomonoesterase, respectively, and was expressed as mg *p*-nitrophenol g^{-1} dry weight h^{-1}.

Arylsulphatase assays only differed in the choice of buffer used. The arylsulphatase assay was performed according to the procedure described by Nannipieri et al., (2002) and was expressed as mg *p*-nitrophenol g^{-2} dry weight.

3.2.3.3.3 Urease Activity

Urease (urea amidohydrolase, EC 3.5.1.5) activity was assayed using the procedure as described by Alef and Nannipieri (1995). Soil (5.0g, air-dried) was weighed and incubated (at 37°C for 2h) with 2.5ml urea solution. The addition of 50ml 1.0 M potassium chloride (KCl) following incubation was accompanied with shaking (for 30min). Soil suspensions were filtered (Whatman no. 2 filter paper) and the absorbance of each sample was determined spectrophotometrically at 600nm. Controls were prepared with 2.5ml distilled water with the addition of urea only after incubation but before the addition of the KCl solution. Urease activity was expressed as μg NH_4-N g^{-1} dry weight 2 h^{-1}.

3.3 Statistical Analysis

Parametric and non-parametric statistical analyses were performed on all data obtained using STATISTICA 6 (StaSoft, Inc ©). The data was tested for normality using the Shapiro-Wilk's test. In the case of the data being normally distributed (parametric) a breakdown and one-way ANOVA was performed and the Turkey's honest significant difference (HSD) test was used to determine statistical significance between the various treatments. In the case of non-parametric data analysis was performed and the Kruskal-Wallis ANOVA and Median test was used to determine statistically significant differences between treatments.

The relationship between soil chemical characteristics and the microbiological variables (enzymatic activities and PFLA profiles) was investigated using Redundancy Analysis (RDA) multivariate ordination techniques using CANOCO (Canoco for Windows Version 4.5, GLW-CPRO ©). The most significant soil chemical variables were selected through the forward selection procedure provided in CANOCO, thereby ensuring that only the most significant environmental gradients were investigated. Redundancy Analysis (RDA) was performed with the activities of the six enzymes assayed as species dependent variables and most significant soil variables as independent environmental factors (Claassens et al., 2005).

3.4 Results and Discussion

3.4.1 Chemical and Physical Analyses

From the results of the particle size distribution analysis (Table 2) it is clear that the soil under investigation was typified by a large clay fraction (on average ± 16.95%) of the overall growth medium. Any residual herbicide would therefore be tightly adsorbed to the clay fraction. Also, in combination with the relatively high silt fraction this soil will be prone to compaction and subsequent infiltration of water would be limited; both these factors will minimise possible leaching.

Table 2. Particle size distribution of all growth mediums.

		Sand	**Silt**	**Clay**
Growth mediums	(%) > 2mm	(%)	(%)	(%)
Contaminated soil (CS)	11.1	47.4	27.0	25.6
Uncontaminated soil (US)	0.9	53.4	28.1	18.5
Irradiated contaminated soil (ICS)	14.0	50.6	26.9	22.5
Irradiated uncontaminated soil (IUS)	1.6	52.9	22.8	24.3
Silica sand (SS)	7.3	98.4	0.2	1.4

Table 3. Soil nutrient status for all growth mediums as determined on an ammonium acetate extraction.

Growth mediums	**Ca***	**Mg***	**K***	**Na***	**P***	**EC****
Contaminated soil (CS)	1148.5	390.7	51.4	39.3	7.7	54
Uncontaminated soil (US)	1132.0	453.1	100.0	36.0	2.8	44
Irradiated contaminated soil (ICS)	1228.5	409.4	55.4	45.8	8.1	62
Irradiated uncontaminated soil (IUS)	1057.5	439.9	86.6	44.8	5.9	54
Silica sand (SS)	150.5	16.6	4.5	22.0	4.4	47

* Ca, Mg, K, Na, P values mg/kg

** EC value mS/m

From the results presented in Table 3, 4 and 5 it is clear that all samples analysed were typified by lower P concentrations. When the exchangeable cation ratios were determined for the respective growth mediums it became evident that, with the exception of the silica sand as is to be expected, it did not differ significantly from one another or from the norm percentages of Ca 65: Mg 25: K 8: Na 2 (De Beer et al., 2004), by on average being: 61:

36: 2: 2. However this fluctuation indicates a serious potassium (K) deficiency in the soil as sampled at Zeus, sub-station. The $pH_{(KCl)}$ also indicated that the pH of the medium could decrease below 5.5 with normal cultivation practices, warranting some concern for potential metal toxicity in the long term. The average cation exchange capacity (CEC) for the samples fall within the 15 to 20 cmole(+)kg^{-1} range, which is high and to a large degree a function of the high clay fraction in the samples analysed (Table 2).

Table 4: Exchangeable cation concentrations and other variables as determined by means of an ammonium acetate extraction for all growth mediums.

Growth mediums	Ca*	Mg*	K*	Na*	CEC **	S-value	Base-saturation (%)	pH (H_2O)	pH (KCl)
Contaminated soil (CS)	5.73	3.22	0.13	0.17	19.30	9.25	47.91	5.31	4.07
Uncontaminated soil (US)	5.65	3.73	0.26	0.16	20.00	9.79	48.95	5.93	4.50
Irradiated contaminated soil (ICS)	6.13	3.37	0.14	0.20	20.52	9.84	47.95	5.32	4.22
Irradiated uncontaminated soil (IUS)	5.28	3.62	0.22	0.19	17.57	9.31	53.03	6.04	4.84
Silica sand (SS)	0.75	0.14	0.01	0.10	1.63	0.99	60.85	6.38	6.07

* Ca, Mg, K, Na values mg/kg

**CEC cmol(+)/kg

It became evident that the irradiation of contaminated- and uncontaminated soil caused an increase in the water soluble manganese, copper and iron in uncontaminated soil (Table 5). Also the percentage of organic matter in contaminated soil increased, while it decreased in uncontaminated soil. Silica sand, as expected had very low nutrients due to the absence of binding sites for elements. Therefore, all nutrients or materials in the bioremediation agent will be leached.

Table 5: 1:2 Extraction (micro-elements and other data) for all growth mediums.

Growth mediums	Fe*	Mn*	Cu*	Zn*	B**	pH	EC#	% C
Contaminated soil (CS)	23.34	4.45	0.21	0.05	1	4.86	0.25	0.81
Uncontaminated soil (US)	36.88	2.89	0.46	0.16	<1	4.74	0.24	3.51
Irradiated contaminated soil (ICS)	30.63	19.52	0.46	0.09	<1	5.17	0.23	1.18
Irradiated uncontaminated soil (IUS)	28.15	25.13	7.67	14.63	33	6.01	0.20	2.92
Silica sand (SS)	31.92	14.28	0.39	0.18	7	6.22	0.23	0.30

* Fe, Mn, Cu, Zn mmol/l ** B values µmol/l

#EC value mS/cm

Table 6: 1:2 Extraction (macro elements) for all growth mediums.

Growth mediums	Ca*	Mg*	K*	Na*	P*	SO_4*	NO_3*	NH_4*	Cl*	HCO_3*
Contaminated soil (CS)	0.45	0.34	0.12	0.70	<0.01	0.54	1.21	0.04	0.17	0.05
Uncontaminated soil (US)	0.37	0.34	0.26	0.58	<0.01	0.26	1.60	0.07	0.23	0.05
Irradiated contaminated soil (ICS)	0.42	0.27	0.09	0.67	<0.01	0.64	0.59	0.06	0.19	0.25
Irradiated uncontaminated soil (IUS)	0.21	0.13	0.23	0.25	0.01	0.30	0.47	0.75	0.21	0.65
Silica sand (SS)	0.64	0.17	0.12	0.28	<0.01	0.84	0.29	0.19	0.14	0.20

* Ca, Mg, K, Na, P SO_4, NO_3, NH_4, Cl and HCO_3 mmol/l

3.4.2 Characterisation of the Functional Diversity of Soil

3.4.2.1 Soil Enzymatic Activities

Redundancy Analysis (RDA) ordination diagrams (Figure 1, 2 and 3) illustrate the relationship between specific environment variables, potential enzymatic activities and different treatments for three time intervals (at the start of the trial, three weeks after and six weeks after the trial commenced). The soil variables pH(H_2O), phosphorus (P), percentage organic carbon (%C), nitrate (NO_3) and sulphate (SO_4) were used in the ordination because these environmental variables form the basis for enzymatic activities in the soil environment. Enzymatic activity for treatments over three time intervals are summarised in Table 9, 10 and 11.

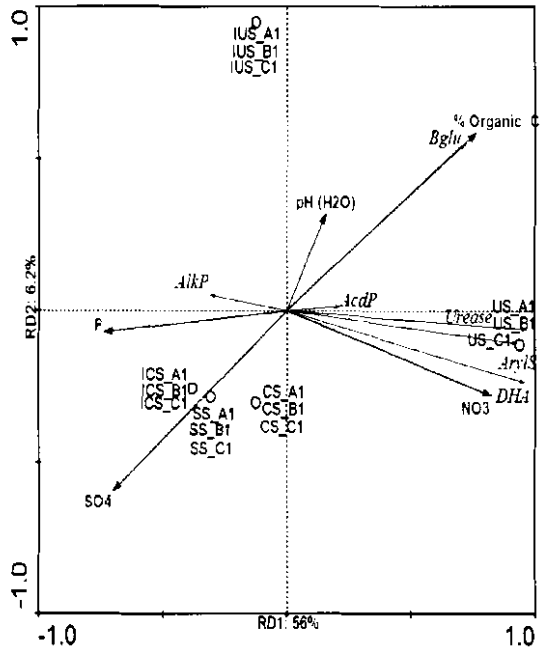

Figure 1. Redundancy analysis ordination diagram illustrating the relationship between the dominant environmental variables and soil enzymatic activities for all treatments.

Environmental variables were determined before the addition of the bioremediation agent and would not be influenced since the study was conducted in a closed system over a short period of time. Therefore, environmental variables do not change quantitatively but their influence on enzymatic activities should be considered.

Figure 1 shows the redundancy ordination analysis for all treatments before the trial started with eigenvalues for the three axes before the addition of bioremediation agent to the specific growth mediums were 0.56, 0.062 and 0.019, respectively and the total observed variance of the first two axes was 96.3%. The first axis associated strongly with NO_3 (r^2 = 0.8168), the second axis with SO_4 (r^2= -0.4954) and the third axis (not shown) with pH(H_2O) (r^2 = -0.187). The effect of the specific environmental variables on the enzymatic activities was statistically significant (p = 0.02). Based on these results obtained it is evident that before the addition of the bioremediation agent to soil), uncontaminated soil was characterised by a presence of dehydrogenase, urease and arylsulphatase activity and less with organic carbon and β-glucosidase. This can possibly be attributed to the fact that the soil was stored before use for a period of four months. What is clear from the ordination is that organic carbon had a positive association with β-glucosidase. According to Turner et al. (2002), β-glucosidase is closely related to the carbon cycle and plays an important role in hydrolytic processes which occur during organic matter degradation, thus explaining the close association between organic carbon and β-glucosidase. Uncontaminated soil was characterised with urease activity and shows a close association with NO_3. Urease is associated with nitrogen transformations (Aon and Colaneri, 2001).

No association between SO_4 and arylsulphatase could be seen, even though sulphatase is associated with SO_4 production in soil by fungi (Li and Sarah 2003). Previous studies have shown a weak association between microbial biomass S, microbial biomass C and arylsulphatase and to some extent show that arylsulphatase activity is substrate mediated (Skujins, 1967). This could explain the lack of association with SO_4 and arylsulphatase activity found in this study.

Contaminated soil, irradiated contaminated soil and silica sand was characterised with lower enzymatic activity. No significant difference (p > 0.05) existed between treatments, with agent (A), with irradiated agent (B) and without the addition of agent (C). The

redundancy ordination analysis in figure 2 shows trail after three weeks with eigenvalues for the three axes after three weeks after adding the bioremediation agent to the specific growth mediums were 0.512, 0.826 and 0.053 and the total observed variance of the first two axes was 40.3%. The first axis associated strongly with NO_3 (r^2= 0.8529), the second axis with pH(H_2O) (r^2 = -0.8313) and the third axis (not shown) with SO_4 (r^2 = 0.3323).

The effect of the specific environmental variables on microbial enzymatic activities was statistically significant (p = 0.02) and indicates that uncontaminated soil was characterised with the presence of organic carbon and β-glucosidase. The increase of organic carbon may be as a result of microbial activity which is higher due to availability of plant material and the addition of the bioremediation agent to soil. Uncontaminated soil still showed the presence of dehydrogenase activity.

After three weeks, uncontaminated soil (Figure 2) no longer showed a presence of urease activity, which could be as a result of plants that translocate nitrogen quickly for physiological functions and biosynthesis. Plants deplete nitrogen (N) available in the closed system and with no additional available nitrogen, urease activity decreases. Once again, it is clear that no significant difference existed between treatments.

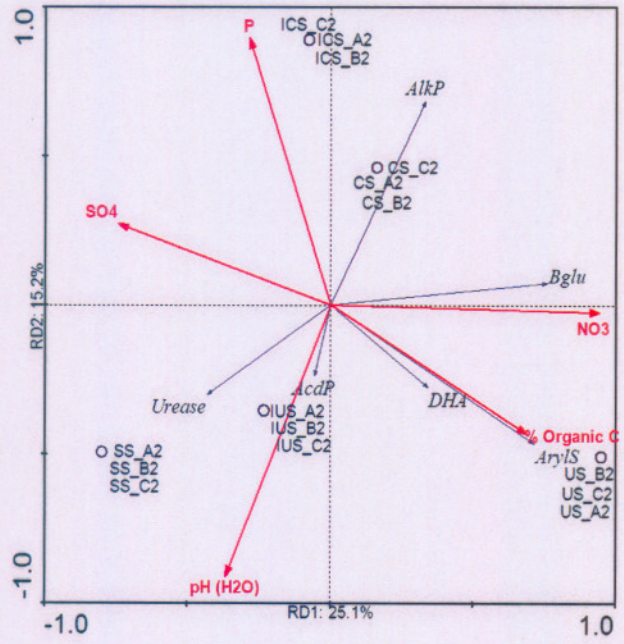

Figure 2. Redundancy analysis ordination diagram illustrating the relationship between the dominant environmental variables and soil enzymatic activities for all treatments.

Table 8. Potential Enzymatic activity for all treatments after 3 weeks of adding the bioremediation agent to the specific growth mediums.

Growth medium_ treatment	Dehydrogenase (μg INF g^{-1} $2h^{-1}$)	β-Glucosidase (mg INF g^{-1} h^{-1})	Alkaline phosphatase (mg INF g^{-1} h^{-1})	Acid phosphatase (mg INF g^{-1} h^{-1})	Urease (μg NH_4-N g^{-1} $2h^{-1}$)	Arylsulphatase (g INF g-1 2h-1)
US_A	81.03 ± 4.10	1506.10 ± 538.48	127.97	451.34	1.06 ± 0.32	3.67 ± 0.45
CS_A	76.54 ± 44.27	518.71 ± 74.16	281.00	80.58 ± 42.50	0.62 ± 0.44	0.26 ± 0.02
SS_A	0.87 ± 0.24	136.15 ± 36.18		325.06 ± 148.95	10.22 ± 4.92	0.15 ± 0.10
ICS_A	37.73 ± 0.73	1230.31 ± 542.84	644.63	297.53	8.30 ± 6.94	0.40± 0.10
IUS_A	38.30 ± 2.91	614.64 ± 223.47		467.30 ± 62.66	1.85 ± 0.74	1.03± 0.43
US_B	78.96 ± 6.27	1188.09 ± 107.62	191.24 ± 63.93	350.40 ± 154.06	3.83 ± 0.87	3.00 ± 0.24
CS_B	28.08 ± 3.59	642.25 ± 312.39	243.91 ± 120.24		2.07 ± 1.23	2.42 ± 2.06
SS_B	4.17 ± 1.84	601.98 ± 565.17		340.96	4.88 ± 1.36	1.96 ± 1.58
ICS_B	32.17 ± 2.99	558.38 ± 111.29	666.49	338.89 ± 78.63	4.03 ± 1.70	0.85 ± 0.25
IUS_B	42.91 ± 9.50	421.54 ± 155.89	276.66 ± 145.44		8.35 ± 7.74	1.57 ± 0.23
US_C	55.18 ± 11.18	943.88 ± 88.32	426.64	312.61 ± 115.16	7.29 ± 6.73	3.34 ± 0.13
CS_C	18.50 ± 1.63	1243.62 ± 936.29	328.59 ± 100.16		2.96 ± 0.47	0.65 ± 0.21
SS_C	118.87 ± 58.80	75.98 ± 54.81	41.66 ± 12.05		9.09 ± 1.96	
ICS_C	19.52 ± 7.85	425.16 ± 83.49	205.23 ± 151.18	225.62	1.86 ± 0.62	0.47 ± 0.06
IUS_C	32.64 ± 6.22	604.00 ± 73.14	129.83 ± 40.31	2457.14 ± 2199.97	3.99 ± 2.46	1.69 ± 0.45

[1] US (uncontaminated soil), CS (contaminated soil), SS (silica sand), ICS (Irradiated contaminated soil) and IUS (Irradiated uncontaminated soil)

[2] Treatment- A (with agent), B (with irradiated agent) and C (without agent)

[3] All values ± SEM represents the results obtained from three independent samples (n = 3)

[4] INF: iodonitrotetrazolium chloride-formazan

Table 9. Potential enzymatic activity for all treatments after six weeks of adding the bioremediation agent to the specific growth mediums.

Growth medium_ treatment	Dehydrogenase (µg INF g^{-1} $2h^{-1}$)	β-Glucosidase (mg INF g^{-1} h^{-1})	Alkaline phosphatase (mg INF g^{-1} h^{-1})	Acid phosphatase (mg INF g^{-1} h^{-1})	Urease (µg NH_4-N g^{-1} $2h^{-1}$)	Arylsulphatase (g INF g-1 2h-1)
US_A	142.69 ± 10.77	1210.77 ± 581.42	97.29	243.24 ± 29.07	6.77 ± 1.33	3.89 ± 0.38
CS_A	56.57 ± 4.74	400.74	104.63 ± 15.73	178.11	10.50 ± 0.70	0.20 ± 0.08
SS_A	6.31 ± 2.39	577.78 ± 291.26		112.94 ± 36.03	5.07 ± 1.32	0.22
ICS_A	62.54 ± 11.97	573.61 ± 436.16	504.56	169.97 ± 40.69	2.67 ± 1.65	0.35 ± 0.15
IUS_A	146.67 ± 11.35	1807.00 ± 701.00	114.81	225.96 ± 116.09	1.57 ± 0.90	0.63 ± 0.20
US_B	133.95 ± 15.64	1411.45 ± 646.34	528.85	552.75	10.22 ± 4.36	4.39 ± 0.22
CS_B	65.56 ± 5.46	1331.06 ± 641.67	116.86 ± 41.82	301.06 ± 137.82	6.04 ± 3.13	0.22 ± 0.01
SS_B	7.10 ± 2.54	58.99 ± 20.26	328.74 ± 218.55	201.68 ± 174.93	12.28 ± 5.22	0.30 ± 0.03
ICS_B	71.72 ± 16.23	145.84		175.16 ± 61.77	13.84 ± 10.00	0.27 ± 0.09
IUS_B	114.58 ± 15.95	6052.26 ± 428.92	346.59 ± 170.41	347.34 ± 86.90	1.67 ± 1.40	1.37 ± 0.42
US_C	153.05 ± 33.50	2161.28 ± 284.66		874.96 ± 270.25	3.01 ± 1.31	8.35 ± 0.96
CS_C	55.93 ± 8.93	256.50 ± 83.84	63.62	293.21 ± 104.20	0.62 ± 0.42	0.45 ± 0.19
SS_C	5.68 ± 0.63	97.76 ± 50.95	324.07 ± 113.55	133.87 ± 113.99	0.61	0.03 ± 0.00
ICS_C	47.65 ± 2.23	504.96 ± 228.01	530.37 ± 58.08	53.88 ± 49.95	1.11	0.73 ± 0.33
IUS_C	126.03 ± 17.38	1083.26 ± 89.28	287.66 ± 161.00	693.13 ± 173.32	1.84	1.28 ± 0.42

[1] US (uncontaminated soil), CS (contaminated soil), SS (silica sand), ICS (Irradiated contaminated soil) and IUS (Irradiated uncontaminated soil)

[2] Treatment- A (with agent), B (with irradiated agent) and C (without agent)

[3] All values ± SEM represents the results obtained from three independent samples (n = 3)

[4] INF: iodonitrotetrazolium chloride-formazan

Table 7. Potential enzymatic activity for all treatments before the addition of the bioremediation agent.

Growth medium_ treatment	Dehydrogenase (μg INF g^{-1} $2h^{-1}$)	β-Glucosidase (mg INF g^{-1} h^{-1})	Alkaline phosphatase (mg INF g^{-1} h^{-1})	Acid phosphatase (mg INF g^{-1} h^{-1})	Urease (μg NH_4-N g^{-1} $2h^{-1}$)	Arylsulphatase (g INF g-1 2h-1)
US_A	173.76 ± 5.09	396.90 ± 68.57	273.90 ± 32.42	243.74	31.09 ± 0.61	10.62 ± 2.37
CS_A	86.09 ± 28.14	218.12 ± 15.23	508.77	152.03	4.62 ± 0.10	2.34 ± 1.68
SS_A	16.32 ± 4.09	34.82 ± 20.89	139.72 ± 79.28	337.61 ± 233.97	1.32 ± 0.76	1.47 ± 1.27
ICS_A	9.03 ± 1.08	120.67 ± 61.73	399.79 ± 51.41	329.55	4.93 ± 1.24	1.98 ± 1.28
IUS_A	13.42 ± 2.06	314.24 ± 101.55	23.41	267.43 ± 106.78	3.34 ± 1.01	2.61± 1.79
US_B	184.10 ± 35.37	506.44 ± 97.63		235.75	22.62 ± 0.55	7.80 ± 0.98
CS_B	53.49 ± 1.80	229.43 ± 29.22		464.30 ± 170.36	2.88 ± 1.68	3.92 ± 0.13
SS_B	14.60 ± 1.15	232.82	104.39 ± 31.65	289.81 ± 75.40	3.26 ± 1.85	0.71 ± 0.25
ICS_B	7.46 ± 2.66	184.64 ± 22.31	286.58 ± 110.67		5.37 ± 0.29	1.04 ± 0.97
IUS_B	14.24 ± 7.63	459.25 ± 177.05	81.84 ± 30.27	61.35 ± 50.81	5.84 ± 2.88	
US_C	180.48 ± 5.69	617.64 ± 56.22		470.05 ± 17.68	27.84 ± 1.41	9.81 ± 1.03
CS_C	50.42 ± 6.37	146.19 ± 71.18		153.49 ± 139.00	7.47 ± 1.59	0.81 ± 0.57
SS_C	30.96 ± 11.70	165.28 ± 114.53	224.22 ± 9.15	86.32 ± 58.55	1.23 ± 0.55	3.76 ± 0.67
ICS_C	12.26 ± 9.30	59.06 ± 25.07	317.14 ± 283.43	361.64	1.06 ± 0.48	1.51± 0.59
IUS_C	6.28 ± 1.45	553.33 ± 165.71	568.60 ± 184.14	437.35 ± 265.34	5.39 ± 2.67	3.02 ± 1.68

[1] US (uncontaminated soil), CS (contaminated soil), SS (silica sand), ICS (Irradiated contaminated soil) and IUS (Irradiated uncontaminated soil)

[2] Treatment-A (with agent), B (with irradiated agent) and C (without agent)

[3] All values ± SEM represents the results obtained from three independent samples ($n = 3$)

[4] INF: iodonitrotetrazolium chloride-formazan

Figure 3 shows the redundancy ordination analysis on conclusion of the trial, after six weeks with eigenvalues for the first three axes after six weeks 0.735, 0.919 and 0.034, respectively and the total observed variance of the first two axes was 40.3%. The first axis associated strongly with percentage organic carbon (r^2= 0.9217), the second axis with NO_3 (r^2= -0.4560) and the third axis (not shown) also with NO_3 (r^2 = - 0.1548). The effect of the specific environmental variables on microbial enzymatic activities was statistically significant (p = 0.04), uncontaminated soil showed a presence with arylsulphatase but not with dehydrogenase and β-glucosidase, which could be attributed to the depletion of available nutrients as the trial progressed, resulting in a decline in utilisation of nutrients and inhibition of microbial activity. Both alkaline- and acid phosphatase activity was inconsistent throughout the trial with no visible trends. The low phosphorus availability in soil could be the contributing factor in this case.

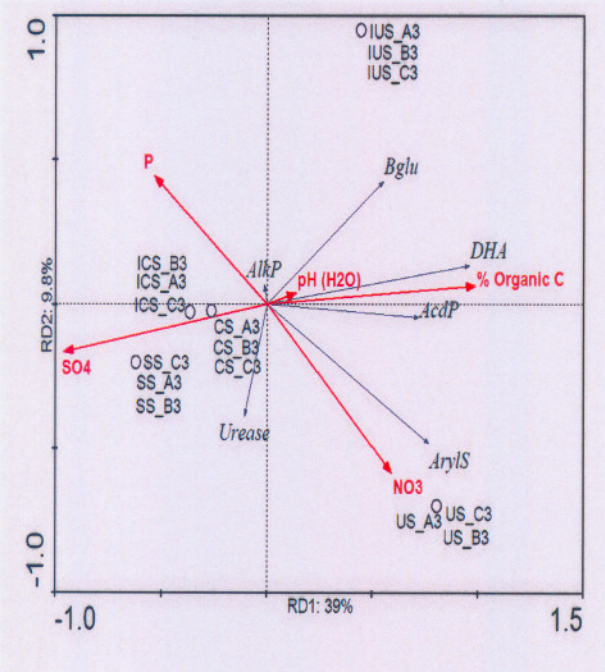

Figure 3. Redundancy analysis ordination diagram illustrating the relationship between the dominant environmental variables and soil enzymatic activities for all treatments.

It is clear that the bioremediation agent used in this study had no significant effect on microbial activity. Over the six week study no significant differences based on potential enzymatic activity analyses could be observed between treatments on different growth mediums.

3.5 Conclusions

Soil ecosystems have complex dynamics and no single characteristic is well enough defined for studying microbial activity (Garcia et al., 2002). Therefore it is necessary to study interrelationships between chemical, physical, biochemical and biological properties to obtain an accurate representation of the function and structure of soils (Trasar-Cepeda et al., 2000). Measurement of only one or some of these properties will give only a partial evaluation of the state of the soil ecosystem. Thus, no single soil property is sufficient to evaluate the state of anthropogenic or natural impacts on an ecosystem, because all methods are subject to limitations. It would therefore be most sensible to use the combination of several types of techniques to assess soil quality (Claassens, 2003).

Results showed that the contaminated growth medium showed no improvement with the addition of agent, or irradiated agent or without the addition of agent. Furthermore, the uncontaminated growth medium improved from before adding the bioremediation agent to three weeks after, but declined as a result of using a closed system where the depletion of nutrients occurred within the last three weeks. It is therefore, clear that the specific bioremediation agent used in this study had no significant effect on microbial activity and over the six week study no significant differences based on enzymatic activity analyses was observed between treatments on different growth mediums.

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Chapter 4: The Assessment of the Microbial Community Structure During the Treatment of Soil Containing Bromacil, Tebuthiuron and Ethidimuron.

Abstract

Microorganisms inhabit soil and contribute to the maintenance of soil quality due to their influence on the decomposition of plant and animal material, biogeochemical cycling, the formation of soil structure and the fate of organic compounds applied to soil. Since microbial communities play an important role in mediating essential biochemical cycles of carbon, nitrogen, sulphur, hydrogen and oxygen, it is important to detect changes in microbial community structures. Herbicides are commonly used in agriculture, but their persistence and leaching in soil keeps affecting the quality of soil long after application. In this study, the effect of a specific bioremediation agent, B350 on Bromacil, Tebuthiuron and Ethidimuron was evaluated. B350 is used for the remediation of hydro carbon and other organic materials in soil. Each growth medium was characterised by analyses of the chemical and physical properties and the community structure was evaluated by means of phosholipid fatty acid (PLFA) analyses. The relationship between soil chemical and physical characteristics and the community structure was investigated by means of multivariate ordination techniques. Results from the PLFA analyses indicated that uncontaminated soil was characterised with most PLFA groups, whereas contaminated soil was characterised with the absence of major PLFA groups at the end of the trial, possibly indicating a low diversity in microbial community. Results from the PLFA analyses indicated no significant ($p > 0.05$) difference in community structure for treatments (with or without the application of the bioremediation agent) of the same growth medium.

Keywords: Bioremediation, Bromacil, Ethidimuron, herbicides, PLFA, soil quality and Tebuthiuron.

4.1 Introduction

The application of herbicides for industrial weed control at power stations, under power lines, servitude's and substations has been used for many years by Eskom (South Africa). Herbicides including Bromacil, Tebuthiuron and Ethidimuron were applied to the area of soil between security fences at Zeus, a substation in Mpumalanga, South Africa. Herbicide build-up is inevitable in treated areas and is known to cause damage to vegetation (crops, vegetables, surrounding re-growth, etc.) and pollution of groundwater. These herbicides may also lead to contamination of adjacent agricultural land through their mobility in soil (Goszszynska, 2001).

Reclamation of these treated areas is necessary to limit contamination of adjacent agricultural land, but is problematic. Physical removal of contaminated soil is a costly procedure (Alexander, 1999) and alternatives should be considered. Management of soil communities (microorganisms and plants) to remove contaminants in the soil system can be implemented for the remediation of contaminated soil.

The herbicides applied (Bromacil, Tebuthiuron and Ethidimuron) belong to the uracil and phenylurea family which are known as photosystem II (PS II) herbicides because they affect PS II during the photosynthesis process. These herbicides bind plastoquinone, a protein that allows the transfer of electrons, subsequently blocking electron flow and inhibiting photosynthesis, better known as the inhibition of the Hill reaction (Camillieri, 1987; Pfister and Schreiber, 1983).

While it is desirable for herbicides to control weeds during the season of application, it is not desirable for them to persist and subsequently affect plant growth. Herbicides vary from each other in terms of their potential to persist in soil and several factors affect herbicide persistence in the soil environment (Tomlin, 1997). According to Karlen et al. (2003) these factors can be divided into categories that interact with each other, including soil factors, climatic conditions and herbicide properties.

Soil composition, soil chemistry and microbial activity (biological activity) are elements of soil quality (Doran and Parkin, 1994) that can influence herbicide persistence. Soil composition is a physical factor and gives information about the relative amount of sand,

silt and clay as well as the organic material present in soil. Adsorption of herbicides to soil particles may reduce their bio-availability to soil microorganisms (Alexander 1999). There is increasing concern that herbicides not only affect the target organisms (weeds) but also the microbial community present in soil and that these non-target effects may influence the performance of important soil functions. These critical soil functions include organic material degradation, the nitrogen cycle and methane oxidation (Brussaard, 1997).

Microorganisms play an important role in soil formation, ecosystem biogeochemistry, contaminant degradation and maintenance of groundwater quality (Konopka and Turco, 1991). Microbial community composition may be one important control in soil processes (Balser et al., 2002; Cavigelli and Robertson, 2000). There exists a large obstacle when evaluating and comparing microbial communities in soil since there is a lack of effective methods for addressing community-level characteristics (Cavigelli et al., 1995; Olsson and Alstrom, 2000). This is due to the fact that only a small percentage of all soil microorganisms that are culturable using traditional microbiological analysis (Ward et al., 1990). Biochemical methods do not have the problems associated with the traditional microbiological methods since they do not rely on the culturability of microorganisms (Tunlid and White, 1992). Therefore, biochemical extractions are considered to be quantitatively reliable (White, 1995). The analysis of phospholipids fatty acids (PLFA) of the cell membranes is used as a fast and reliable method for characterising the microbial community structure (Frostegård, 1995). Phospholipid fatty acids are structural components of all biological membranes, they have no storage function and represent a relatively consistent fraction of cell mass. They also degrade quickly upon an organisms death and extraction and derivatisation methods permit the recovery of PLFAs from living organisms (White, 1988). Each analysis yields a specific profile composed of numerous PLFAs defined on the basis of compound structure and the quantity of each compound present in the sample. According to Stoeck et al., (2002) certain PLFAs isolated from prokaryotic and eukaryotic cell membranes can serve as unique signatures for certain functional groups of microorganisms.

The objectives of the present investigation were to quantify the effect of the specific bioremediation agent (B350) as prescribed by ESKOM, to monitor the biostimulation and bioaugmentation on the structural diversity of the microbial communities in the relevant soil.

4.2 Materials and Methods

4.2.1 Experimental Layout

Grass and soil were collected from the Zeus substation, Mpumulanga (South Africa) and transferred to the glass-house facilities of the Plant Protection Research Institute (PPRI), Roodeplaat Dam, Pretoria where it was kept until transported to the North-West university, Potchefstroom campus, Potchefstroom. Soil was sieved (1.5cm^2 mesh) and the homogenised soil was stored in plastic containers until the trial started. Each treatment (with the addition of agent, irradiated agent and without the addition of agent) consisted of three replicates and was kept in the glass-house at 25°C (day time) and 15°C (night time). Table 1 shows the different growth mediums which consisted of contaminated- (CS), uncontaminated- (US), irradiated contaminated- (ICS), and irradiated uncontaminated soil (IUS) as well as irradiated silica sand. For the irradiation of the soil, 2kg contaminated- and uncontaminated soil was placed in plastic bags, heat sealed and taken to Isotron Inc. for gamma irradiation (KGy 70). A portion of the bioremediation agent (B350) was also irradiated at KGy 70. Treatments were conducted in pots, 25cm in diameter. Watering of pots occurred twice a day to ensure a constant moisture content. Soil moisture content was measured and kept below field capacity to prevent leaching of the herbicides.

The trial started with the sampling for PLFA's. After sampling, the addition of the bioremediation agent, B350, took place. After a week, *Zea mays* seedlings and a *Cynodon dactylon* grass-plug were planted in each pot. *Zea mays* seedlings were germinated in a growth chamber (Conviron PGW 36, Controlled environment Ltd, Winnipeg, MB. Canada R3H 0R9) under controlled conditions with sufficient incandescent light bulbs (General Electric, Neodynium R80, 100W) and fluorescent lamps (General Electric, Cool White, 1500W) to maintain light intensities as high as 1300µmol photns.m^{-2}.s^{-1} at 1 m distance. The plants were subjected to a daily light period of 15 hours (23°C) and dark period of 9 hours (20°C). Grass-plugs were cut from tufts growing in uncontaminated soil

collected from Zeus. Plugs were treated with a rooting powder (Seradix B no. 1) and planted in a mixture of sand and vermiculite at 24°C and 70% humidity for a week before transplanting to the experimental pots. *Zea mays* seedlings were used in the trial to serve as an internal control and due to its comparative plant physiology to that of the *C. dactylon*. Both plants are parallel veined, equipped with an additional CO_2 fixation pathway (C_4-plant), easy to grow and germinate and the phototoxic effects are visually detectable.

The duration of the trial was six weeks, with sampling for community structure from day one with a three week interval for a period of six weeks.

Table 1: Pilot trial layout, conducted with uncontaminated (uncon), contaminated (con), irradiated uncontaminated, irradiated contaminated soil and irradiated silica sand as growth mediums with agent (A), irradiated agent (B) and without the addition of the specific bioremediation agent (C). These treatments were conducted in triplicate.

Treatments	Uncon. soil	Con. soil	Irradiated silica sand	Irradiated con. Soil	Irradiated uncon. Soil
Agent (A)	1 2 3	4 5 6	7 8 9	10 11 12	13 14 15
Irradiated agent (B)	1 2 3	4 5 6	7 8 9	10 11 12	13 14 15
Without agent (C)	1 2 3	4 5 6	7 8 9	10 11 12	13 14 15

4.2.2 Chemical and Physical Analyses

The chemical and physical analyses of the soil from the growth mediums were performed as described by Van Rensburg et al. (1998). Twenty-four soil chemical and physical variables were analysed (Tables 2, 3, 4, 5 and 6).

4.2.3 Characterisation of the Structural Diversity of Soil Microbial Communities

4.2.3.1 Phospholipid Fatty Acid Extraction, Fractionation and Analysis

Glassware used for the extraction, fractionation and analyses of PLFAs were washed with phosphate-free soap, rinsed five times with tap water and five times with distilled water, then air dried and heated in a muffle furnace at 450°C for a minimum of 4 hours to remove any lipid contaminants. The solvents used for the analyses were of the highest

purity and the silicic acid and internal standard methyl nonadecanonate (C19:0) were obtained from Sigma Aldrich.

A modified Bligh and Dyer procedure was used to extract total lipids from 5g lyophilised soil (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 transesterified to the corresponding fatty acid methyl esters (FAMEs) by mild alkaline methanolysis (Guckert et al., 1985). The FAMEs were analysed by capillary gas chromatography with flame ionisation detection on an Agilent 6890 series II chromatograph fitted with a 60m SPB-1 column (0.250 mm I.D., 0.250 μm film thickness). Definitive identification of peaks was undertaken using gas chromatography-mass spectrometry of samples using an Agilent 6890 series II chromatograph interphased with an Agilent 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).

4.3 Statistical Analysis

Parametric and non-parametric statistical analyses were performed on all data obtained using STATISTICA 6 (StaSoft, Inc ©). The data was tested for normality using the Shapiro-Wilk's test. In the case of the data being normally distributed (parametric) a breakdown and one-way ANOVA was performed and the Turkey's honest significant difference (HSD) test was used to determine statistical significance between the various treatments. In the case of non-parametric data analysis was performed and the Kruskal-Wallis ANOVA and Median test was used to determine statistically significant differences between treatments.

The relationship between soil chemical characteristics and the microbiological variables (enzymatic activities and PFLA profiles) was investigated using Redundancy Analysis (RDA) multivariate ordination techniques using CANOCO (Canoco for Windows Version 4.5, GLW-CPRO ©). The most significant soil chemical variables were selected through the forward selection procedure provided in CANOCO, thereby ensuring that only the most significant environmental gradients were investigated. Redundancy

Analysis (RDA) was performed with major PFLA groups as species dependent variables and the most significant soil variables as independent environmental factors. The most significant soil physical and chemical variables were selected through the forward selection procedure provided in CANOCO, thereby ensuring that only the most pertinent environmental gradients were investigated (Claassens et al., 2005).

4.4 Results and Discussion

4.4.1 Chemical and Physical Analyses

From the results of the particle size distribution analysis (Table 2) it is clear that the soil under investigation was typified by a high clay fraction (on average ± 16.95%) of the overall growth medium. Any residual herbicide would therefore be tightly adsorbed to the clay fraction. Also, in combination with the relatively high silt fraction, this soil will be prone to compaction and subsequent infiltration of water would be limited; both these factors will minimise possible leaching.

Table 2. Particle size distribution of all growth mediums.

		Sand	**Silt**	**Clay**
Growth mediums	(%) > 2mm	(%)	(%)	(%)
Contaminated soil (CS)	11.1	47.4	27.0	25.6
Uncontaminated soil (US)	0.9	53.4	28.1	18.5
Irradiated contaminated soil (ICS)	14.0	50.6	26.9	22.5
Irradiated uncontaminated soil (IUS)	1.6	52.9	22.8	24.3
Silica sand (SS)	7.3	98.4	0.2	1.4

Table 3. Soil nutrient status for all growth mediums as determined on an ammonium acetate extraction.

Growth mediums	**Ca***	**Mg***	**K***	**Na***	**P***	**EC****
Contaminated soil (CS)	1148.5	390.7	51.4	39.3	7.7	54
Uncontaminated soil (US)	1132.0	453.1	100.0	36.0	2.8	44
Irradiated contaminated soil (ICS)	1228.5	409.4	55.4	45.8	8.1	62
Irradiated uncontaminated soil (IUS)	1057.5	439.9	86.6	44.8	5.9	54
Silica sand (SS)	150.5	16.6	4.5	22.0	4.4	47

* Ca, Mg, K, Na, P values mg/kg ** EC value mS/m

From the results presented in Table 3, 4 and 5 it is clear that all samples analysed were typified by low P concentrations. When the exchangeable cation ratios were determined for the respective growth mediums it became evident that, with the exception of the silica sand as is to be expected, it did not differ significantly from one another or from the norm percentages of Ca 65: Mg 25: K 8: Na 2 (De Beer et al., 2004), by on average being: 61: 36: 2: 2. However the fluctuation indicates a serious potassium (K) deficiency in the soil as sampled at Zeus, sub-station. The $pH_{(KCl)}$ also indicated that the pH of the medium could decrease below 5.5 with normal cultivation practices, warranting some concern for potential metal toxicity in the long term. The average cation exchange capacity (CEC) for the samples fall within the 15 to 20 cmole(+)kg^{-1} range, which is high and to a large degree a function of the high clay fraction that dominate in the samples analysed (Table 2).

Table 4: Exchangeable cation concentrations and other variables as determined by means of an ammonium acetate extraction for all growth mediums.

Growth mediums	Ca*	Mg*	K*	Na*	CEC **	S-value	Base- saturation (%)	pH (H_2O)	pH (KCl)
Contaminated soil (CS)	5.73	3.22	0.13	0.17	19.30	9.25	47.91	5.31	4.07
Uncontaminated soil (US)	5.65	3.73	0.26	0.16	20.00	9.79	48.95	5.93	4.50
Irradiated contaminated soil (ICS)	6.13	3.37	0.14	0.20	20.52	9.84	47.95	5.32	4.22
Irradiated uncontaminated soil (IUS)	5.28	3.62	0.22	0.19	17.57	9.31	53.03	6.04	4.84
Silica sand (SS)	0.75	0.14	0.01	0.10	1.63	0.99	60.85	6.38	6.07

* Ca, Mg, K, Na values mg/kg

** CEC cmol(+)/kg

It became evident that the irradiation of contaminated- and uncontaminated soil resulted in an increase in the water soluble manganese, copper and iron in uncontaminated soil (Table 5). Also the percentage of organic material in contaminated soil increased, while it decreased in uncontaminated soil. Silica sand, as expected had very low nutrients due to the absence of binding sites for elements. Therefore, all nutrients or materials in the bioremediation agent may be leached.

Table 5: 1:2 Extraction (micro-elements and other data) for all growth mediums.

Growth mediums	Fe*	Mn*	Cu*	Zn*	B**	pH	EC#	% C
Contaminated soil (CS)	23.34	4.45	0.21	0.05	1	4.86	0.25	0.81
Uncontaminated soil (US)	36.88	2.89	0.46	0.16	<1	4.74	0.24	3.51
Irradiated contaminated soil (ICS)	30.63	19.52	0.46	0.09	<1	5.17	0.23	1.18
Irradiated uncontaminated soil (IUS)	28.15	25.13	7.67	14.63	33	6.01	0.20	2.92
Silica sand (SS)	31.92	14.28	0.39	0.18	7	6.22	0.23	0.30

* Fe, Mn, Cu, Zn mmol/l ** B values µmol/l
#EC value mS/cm

Table 6: 1:2 Extraction (macro elements) for all growth mediums

Growth mediums	Ca*	Mg*	K*	Na*	P*	SO_4*	NO_3*	NH_4*	Cl*	HCO_3*
Contaminated soil (CS)	0.45	0.34	0.12	0.70	<0.01	0.54	1.21	0.04	0.17	0.05
Uncontaminated soil (US)	0.37	0.34	0.26	0.58	<0.01	0.26	1.60	0.07	0.23	0.05
Irradiated contaminated soil (ICS)	0.42	0.27	0.09	0.67	<0.01	0.64	0.59	0.06	0.19	0.25
Irradiated uncontaminated soil (IUS)	0.21	0.13	0.23	0.25	0.01	0.30	0.47	0.75	0.21	0.65
Silica sand (SS)	0.64	0.17	0.12	0.28	<0.01	0.84	0.29	0.19	0.14	0.20

* Ca, Mg, K, Na, P SO_4, NO_3, NH_4, Cl and HCO_3 mmol/l

4.4.2 Characterisation of the Structural Diversity of Soil Microbial Communities.

4.4.2.1 Phospholipid Fatty Acid Extraction, Fractionation and Analysis

Phospholipid fatty acids (PLFAs) can be divided into major groups which include normal saturated fatty acids (Nsats), mid-branched saturated fatty acids (MBsats), terminally branched saturated fatty acids (TBsats), branched monounsaturated fatty acids (Bmonos), monounsaturated fatty acids (Monos) and Polyunsaturated fatty acids (Polys). Normal saturated fatty acids are considered ubiquitous because they are found in most microorganisms (prokaryotic and eukaryotic) (Claassens 2003). According to Zelles, (1999) terminally branched saturated fatty acids are indicative of gram-positive bacteria whereas monounsaturated fatty acids are indicative of gram-negative bacteria (Ratledge and Wilkinson, 1988). Monounsaturated fatty acids are indicative of actinomycetes bacteria found mostly in soil and polyunsaturated fatty acids are indicative of fungi and other micro-eukaryotic organisms (White et al., 1996). The microbial community structure (based on the mole percentage (mole %) of the different PLFA groups) for treatments are presented in Figures 1, 2 and 3. The mole % and ratios including statistical differences for major PLFA groups for the respective treatments are summarised in Tables 7, 8, 9, 10, 11 and 12.

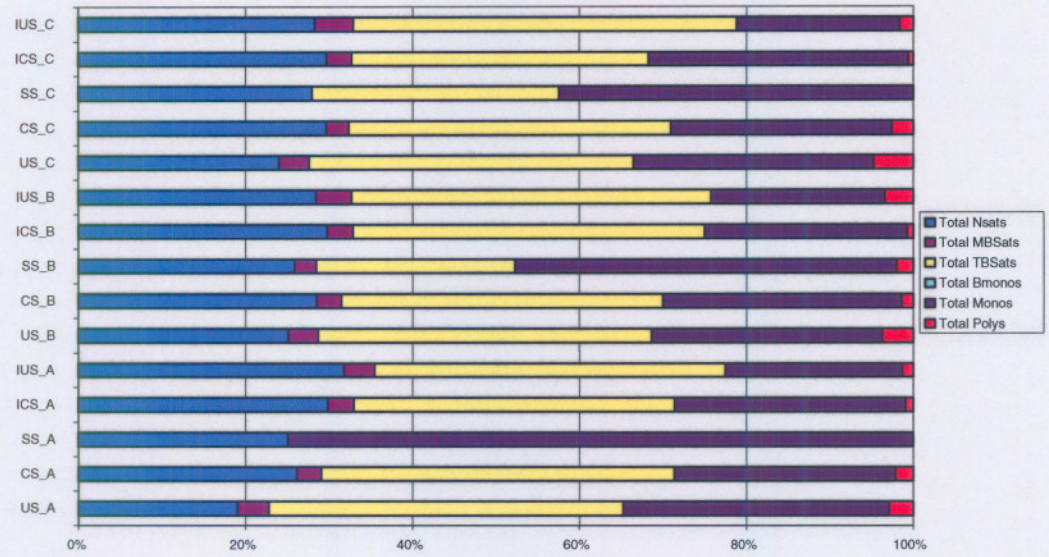

Figure 1. Microbial community structure based on the mole % of the major PLFA groups for all treatments (with agent A, with irradiated agent B and without the addition of agent) before the addition of the bioremediation agent.

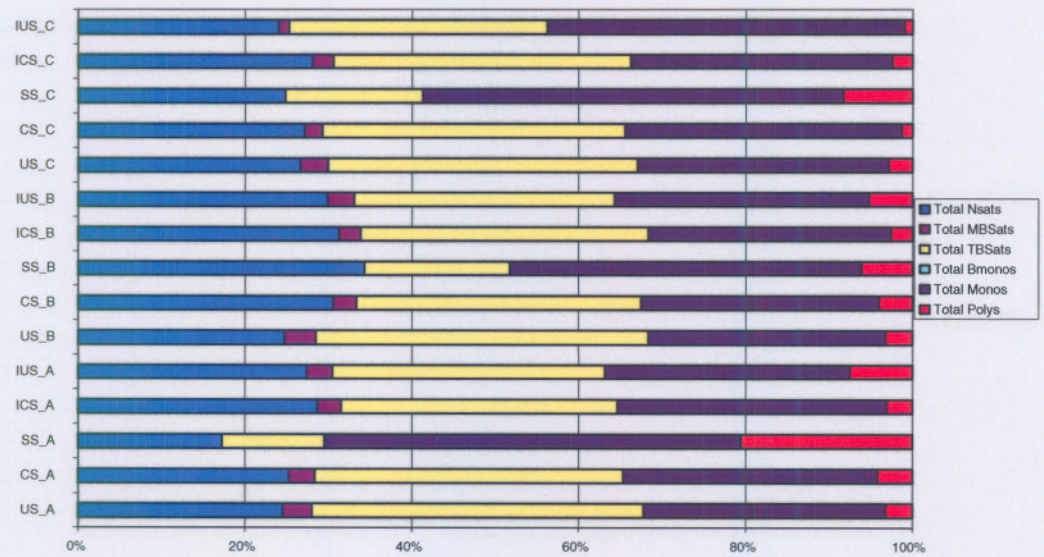

Figure 2. Microbial community structure based on the mole % of the major PLFA groups for all treatments (with agent A, with irradiated agent B and without the addition of agent C) three weeks after the addition of bioremediation.

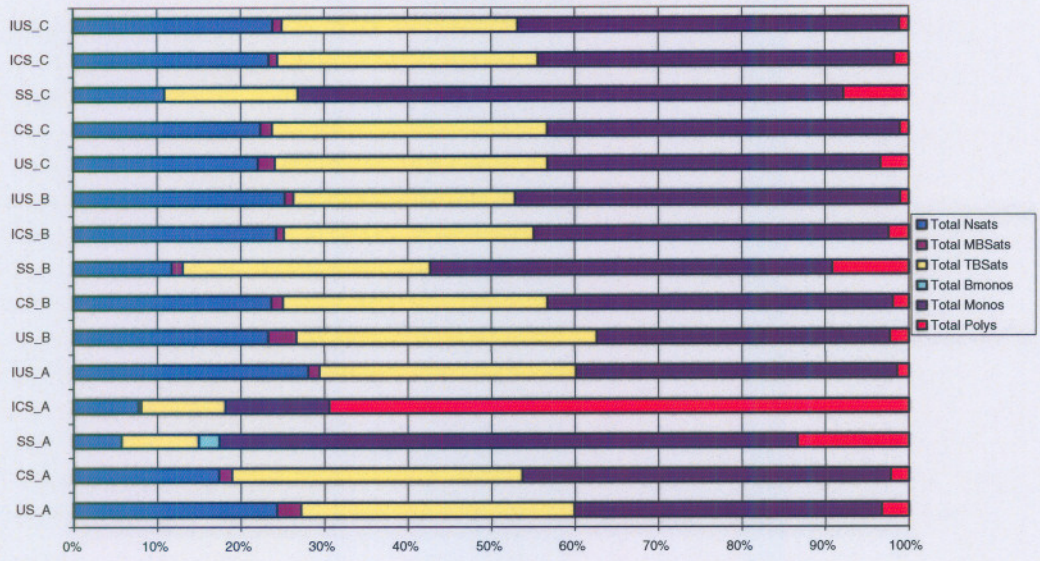

Figure 3. Microbial community structure based on the mole % of the major PLFA groups for all treatments (with agent A, with irradiated agent B and without the addition of agent C) six weeks after the addition of the bioremediation agent.

The relationship between specific environmental variables (soil chemical and physical analysis) and microbial PLFAs for treatments at three different time intervals are illustrated by Redundancy Analysis (RDA) ordination diagrams in Figures 4, 5 and 6.

Uncontaminated soil was characterised by higher estimated viable microbial biomass, Polys, percentage organic carbon (%C) and nitrate (NO_3) before the addition of the bioremediation agent.

Figure 4 shows ordination for environmental variables and PFLA groups with different treatments before the addition of the specific bioremediation, eigenvalues for the first three axes were 0.578, 0.247 and 0.011, respectively. The total observed variance for the first two axes were 82.5%. The first axis correlated strongly with SO_4 (r^2 = 0.9155), the second axis with P (r^2 = -0.8486) and the third axis (not shown) with NO_3 (r^2 = o.095). The overall effect of the environmental variables, were statistically significant (p= 0.02).

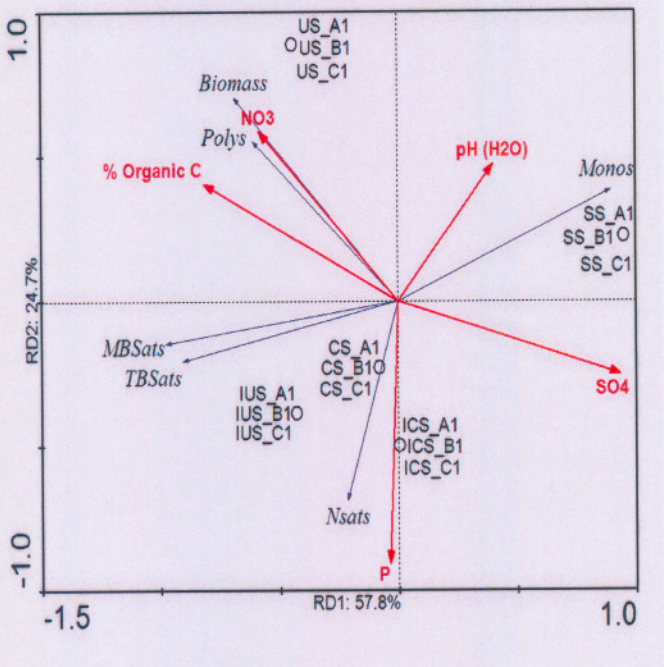

Figure 4. Redundancy analysis ordination diagram illustrating the association between specific environmental variables and PLFA groups with different treatments before the addition of the bioremediation agent to the respective growth mediums.

Contaminated soil and irradiated uncontaminated was characterised by higher MBSats, TBSats and NSats whereas irradiated contaminated soil was characterise with higher NSats. Actinomycetes, containing MBSats, have been found to survive better in harsh environments due to their ability to span interstitial spaces to collect water and nutrient sources (Olsson, 1999). The lack of organic carbon, radiation of uncontaminated soil and high clay content contribute to harsh conditions and may explain the presence of MBSats. Normally saturated fatty acids tend to show an increase as microbial diversity decreases (Rütters et al., 2002). This may indicate lower microbial diversity in contaminated soil than uncontaminated soil.

Silica sand was characterised by the presence of Monos which are indicative of Gram-negative bacteria. These bacteria are fast growing bacteria, utilise many carbon sources and can adapt to a variety of environments (Ponder and Tadros, 2002; Rütters et al., 2002). Therefore, the presence of gram negative bacteria within the silica sand growth medium, may be attributed to the ability of these microorganisms to grow on the limited nutrients available in this medium.

Table 7. Estimated viable biomass and mole % of major PLFA groups for all treatments before the addition of the bioremediation agent.

Growth medium _Treatment	Estimated viable biomass (pmol. g^{-1} dry weight)	Total Nsats	Total MBSats	Total TBSats	Total Monos	Total Polys
US_A	52316.2 ± 30240.61	18.85 ± 5.09	3.72 ± 0.34	42.05 ± 2.79	31.52 ± 3.16	2.86 ± 1.27
CS_A	13372.28 ± 7729.64	25.90 ± 0.17	2.88 ± 0.11	41.95 ± 0.84	26.28 ± 0.51	2.07 ± 0.35
SS_A	942.72 ± 544.92	16.32 ± 13.18	0.00 ± 0.00	18.95 ± 0.00	48.75 ± 0.00	0.00 ± 0.00
ICS_A	12434.86 ± 7187.78	29.58 ± 4.56	3.05 ± 0.48	38.06 ± 4.25	27.40 ± 5.16	0.87 ± 0.24
IUS_A	19163.36 ± 11077.08	31.43 ± 4.14	3.60 ± 0.31	41.57 ± 4.24	20.96 ± 3.39	1.28 ± 0.51
US_B	51980.06± 30046.28	24.84 ± 0.41	3.52 ± 0.11	39.56 ± 1.11	27.34 ± 0.67	3.67 ± 0.46
CS_B	7668.25 ± 4432.51	28.22 ± 2.19	2.93 ± 0.22	38.14 ± 1.41	28.33 ± 2.76	1.36 ± 0.12
SS_B	1489.71 ± 861.10	26.59 ± 0.00	0.00 ± 0.00	24.44 ± 0.00	46.93 ± 0.00	2.04 ± 0.00
ICS_B	11204.40 ± 6476.53	29.38 ± 1.52	3.03 ± 0.20	41.55 ± 1.66	23.92 ± 0.95	0.73 ± 0.16
IUS_B	17686.58 ± 10223.45	28.13 ± 2.35	4.21 ± 0.49	42.59 ± 1.72	20.58 ± 0.77	3.36 ± 1.96
US_C	48859.02 ± 28242.21	23.80 ± 0.17	3.55 ± 0.14	38.50 ± 0.62	28.45 ± 0.90	4.71 ± 0.26
CS_C	13507.72 ± 7807.93	29.39 ± 2.99	2.67 ± 0.08	38.25 ± 3.18	26.23 ± 1.12	2.53 ± 0.94
SS_C	1379.55 ± 797.43	27.94 ± 0.00	0.00 ± 0.00	29.62 ± 0.00	42.44 ± 0.00	0.00 ± 0.00
ICS_C	9395.45 ± 5430.90	29.43 ± 2.52	2.97 ± 0.44	35.22 ± 4.40	30.82 ± 5.77	0.57 ± 0.07
IUS_C	16464.87 ± 9517.26	27.84 ± 0.34	4.52 ± 0.20	45.19 ± 1.27	19.26 ± 1.19	1.56 ± 0.10

[1] Growth mediums US (uncontaminated soil), CS (contaminated soil), SS (silica sand), ICS (Irradiated contaminated soil) and IUS (Irradiated uncontaminated soil)

[2] Treatments A (with agent), B (with irradiated agent) and C (without agent)

[3] All values ± SEM represents the results obtained from three independent samples ($n = 3$)

[4] Nsats (normal saturated), MB Sats (Mid-chain branched saturated), TB Sats (Terminally branched saturated), Monos (monounsaturated) and Polys (polyunsaturated)

Table 8. Specific PLFA ratios for all treatments before addition of the bioremediation agent.

Growth medium _Treatment	Saturated/ Unsatarated	Fungal/ Bacterial	cy19:0 / 18:1ω7c	cy17:0 / 16:1ω7c	18:1ω7t / 18:1ω7c	16: 1ω7t / 16:1ω7c
US_A	2.10 ± 0.25	0.06 ± 0.03	0.00 ± 0.00	0.66 ± 0.01	0.06 ± 0.00	0.27 ± 0.04
CS_A	2.69 ± 0.08	0.04 ± 0.01	0.08 ± 0.08	0.83 ± 0.05	0.00 ± 0.00	0.28 ± 0.02
SS_A	1.05 ± 0.00	0.00 ± 0.00	0.43 ± 0.00	0.16 ± 0.00	0.12 ± 0.00	0.00± 0.00
ICS_A	2.80 ± 0.60	0.02 ± 0.00	0.84 ± 0.61	0.74 ± 0.19	0.06 ± 0.03	0.30 ± 0.06
IUS_A	3.87 ± 0.69	0.02 ± 0.01	0.37 ± 0.10	1.14 ± 0.08	0.18 ± 0.09	0.35 ± 0.03
US_B	2.49 ± 0.08	0.08 ± 0.01	0.00 ± 0.00	0.72 ± 0.04	0.06 ± 0.01	0.26 ± 0.00
CS_B	2.52 ± 0.38	0.03 ± 0.00	1.13 ± 1.13	0.90 ± 0.04	0.00 ± 0.00	0.30 ± 0.03
SS_B	1.09 ± 0.00	0.02 ± 0.02	0.00 ± 0.00	0.10 ± 0.00	0.13 ± 0.00	0.08 ± 0.00
ICS_B	3.10 ± 0.14	0.02 ± 0.00	0.25 ± 0.16	0.95 ± 0.08	0.00 ± 0.00	0.34 ± 0.03
IUS_B	3.65 ± 0.10	0.07 ± 0.04	0.30 ± 0.01	1.15 ± 0.12	0.16 ± 0.03	0.39± 0.02
US_C	2.32 ± 0.11	0.11 ± 0.01	0.00 ± 0.00	48.72 ± 48.13	5.24 ± 5.19	0.26 ± 0.01
CS_C	2.69 ± 0.13	0.06 ± 0.02	0.16 ± 0.08	0.73 ± 0.11	0.02 ± 0.02	0.32 ± 0.03
SS_C	1.36 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.00	0.12 ± 0.00	0.00 ± 0.00
ICS_C	2.41 ± 0.58	0.01 ± 0.00	0.82 ± 0.55	1.37 ± 0.45	0.02 ± 0.02	0.28 ± 0.04
IUS_C	4.06 ± 0.30	0.03 ± 0.00	0.38 ± 0.08	1.17 ± 0.20	0.19 ± 0.04	0.40 ± 0.03

[1] Growth mediums US (uncontaminated soil), CS (contaminated soil), SS (silica sand), ICS (Irradiated contaminated soil) and IUS (Irradiated uncontaminated soil).

[2] Treatments A (with agent), B (with irradiated agent) and C (without agent)

[3] All values ± SEM represents the results obtained from three independent samples (n = 3)

Table 9. Estimated viable biomass and mole % of major PLFA groups for all treatment three weeks after the addition of the bioremediation agent.

Growth medium _Treatment	Estimated viable biomass (pmol. g^{-1} dry weight)	Total Nsats	Total MBSats	Total TBSats	Total Monos	Total Polys
US_A	37220.07 ± 3806.88	24.23 ± 0.93	3.45 ± 0.11	39.21 ± 0.59	28.67 ± 0.18	3.15 ± 0.40
CS_A	14093.85 ± 1387.76	24.99 ± 0.62	3.05 ± 0.16	36.54 ± 0.55	30.14 ± 0.71	4.12 ± 0.64
SS_A	2047.43 ± 770.69	13.49 ± 10.95	0.00	18.11	38.99	16.08
ICS_A	13598.91 ± 2596.96	28.35 ± 1.31	2.72 ± 0.10	32.71 ± 2.81	31.86 ± 0.98	3.02 ± 0.56
IUS_A	22442.83 ± 3673.72	27.17 ± 1.08	3.02 ± 0.12	32.35 ± 0.37	29.06 ± 1.84	7.43 ± 1.26
US_B	38429.94 ± 5965.25	24.42 ± 0.52	3.67 ± 0.07	39.33 ± 0.66	28.04 ± 0.48	3.17 ± 0.32
CS_B	16097.93 ± 1572.08	29.46 ± 1.79	2.67 ± 0.20	32.85 ± 0.95	27.44 ± 0.36	3.86 ± 0.69
SS_B	1479.03	34.29 ± 0.00	0.00	17.47	42.11	6.13
ICS_B	14393.16 ± 644.24	30.92 ± 1.25	2.53 ± 0.08	34.11 ± 1.48	28.72 ± 0.39	2.52 ± 0.20
IUS_B	16700.33 ± 535.22	29.64 ± 0.47	3.15 ± 0.18	30.97 ± 0.62	30.21 ± 0.09	5.17 ± 0.18
US_C	29612.25 ± 3556.03	26.38 ± 0.27	3.26 ± 0.13	36.69 ± 0.84	29.76 ± 0.66	2.83 ± 0.07
CS_C	6511.97 ± 2761.07	26.94 ± 1.01	2.08 ± 1.04	36.00 ± 4.53	32.87 ± 7.50	1.28 ± 0.74
SS_C	2620.95	24.82 ± 0.00	0.00	16.50	50.36	8.32
ICS_C	12910.10 ± 1580.66	27.83 ± 2.05	2.47 ± 0.76	35.29 ± 3.00	31.04 ± 5.65	2.37 ± 0.29
IUS_C	21695.39 ± 3340.36	23.98 ± 0.54	1.21 ± 0.01	30.84 ± 1.19	42.69 ± 1.61	0.96 ± 0.12

[1] Growth mediums US (uncontaminated soil), CS (contaminated soil), SS (silica sand), ICS (Irradiated contaminated soil) and IUS (Irradiated uncontaminated soil).

[2] Treatments A (with agent), B (with irradiated agent) and C (without agent)

[3] All values ± SEM represents the results obtained from three independent samples ($n = 3$)

[4] Nsats (normal saturated), MB Sats (Mid-chain branched saturated), TB Sats (Terminally branched saturated), Monos (monounsaturated) and Polys (polyunsaturated)

Table 10. Specific PLFA ratios for all treatments three weeks after the addition of bioremediation agent.

Growth medium _Treatment	Saturated / Unsatarated	Fungal / Bacterial	cy19:0 / 18:1ω7c	cy17:0 / 16:1ω7c	18:1ω7t / 18:1ω7c	16:1ω7t / 16:1ω7c
US_A	0.00 ± 0.01	0.07 ± 0.01	0.68 ± 0.01	2.33 ± 0.00	0.04 ± 0.00	0.21 ± 0.00
CS_A	0.13 ± 0.06	0.09 ± 0.02	0.94 ± 0.01	2.15 ± 0.01	0.00 ± 0.00	0.20 ± 0.00
SS_A	0.00 ± 0.00	0.29 ± 0.10	0.20 ± 0.06	1.15 ± 0.03	0.08 ± 0.02	0.00 ± 0.01
ICS_A	0.14 ± 0.10	0.07 ± 0.02	1.27 ± 0.01	2.01 ± 0.01	0.00 ± 0.00	0.16 ± 0.00
IUS_A	0.14 ± 0.15	0.17 ± 0.03	1.26 ± 0.02	2.17 ± 0.01	0.00 ± 0.01	0.16 ± 0.00
US_B	0.00 ± 0.07	0.07 ± 0.01	0.68 ± 0.01	2.41 ± 0.00	0.03 ± 0.00	0.22 ± 0.00
CS_B	0.13 ± 0.10	0.08 ± 0.02	0.78 ± 0.01	2.37 ± 0.01	0.00 ± 0.00	0.19 ± 0.00
SS_B	0.14 ± 0.00	0.23 ± 0.09	0.19 ± 0.05	1.23 ± 0.03	0.00 ± 0.02	0.00 ± 0.01
ICS_B	0.12 ± 0.05	0.05 ± 0.01	1.13 ± 0.00	2.35 ± 0.00	0.00 ± 0.00	0.15 ± 0.00
IUS_B	0.04 ± 0.01	0.11 ± 0.00	1.48 ± 0.00	2.11 ± 0.00	0.01 ± 0.00	0.15 ± 0.00
US_C	0.00 ± 0.07	0.06 ± 0.00	0.67 ± 0.00	2.23 ± 0.00	0.03 ± 0.00	0.20 ± 0.00
CS_C	1.20 ± 0.58	0.03 ± 0.02	0.65 ± 0.01	2.24 ± 0.01	0.00 ± 0.00	0.17 ± 0.00
SS_C	0.13 ± 0.00	0.00 ± 0.05	0.14 ± 0.03	0.82 ± 0.02	0.08 ± 0.01	0.00 ± 0.01
ICS_C	0.40 ± 0.70	0.04 ± 0.00	1.52 ± 0.00	2.36 ± 0.00	0.10 ± 0.00	0.28 ± 0.00
IUS_C	1.28 ± 0.09	0.02 ± 0.00	1.09 ± 0.00	1.32 ± 0.00	0.40 ± 0.00	0.13 ± 0.00

[1] Growth mediums US (uncontaminated soil), CS (contaminated soil), SS (silica sand), ICS (Irradiated contaminated soil) and IUS (Irradiated uncontaminated soil).

[2] Treatments A (with agent), B (with irradiated agent) and C (without agent)

[3] All values ± SEM represents the results obtained from three independent samples ($n = 3$)

Table 11. Estimated viable biomass and mole % of major PLFA groups for all treatments six weeks after the addition of the bioremediation agent.

Growth medium Treatment	Estimated viable biomass (pmol. g^{-1} dry weight)	Total Nsats	Total MBSats	Total TBSats	Total Monos	Total Polys
US_A	524.95 ± 2.48	641.52 ± 55.69	75.78 ± 16.27	864.35 ± 96.31	970.58 ± 97.13	85.63 ± 11.64
CS_A	508.83 ± 5.00	136.24 ± 21.87	11.83 ± 1.05	271.94 ± 36.44	345.33 ± 33.00	16.91 ± 1.55
SS_A	500.68 ± 2.83	3.81 ± 3.06	0.00 ± 0.00	11.28 ± 0.00	46.10 ± 0.00	8.85 ± 0.00
ICS_A	511.02 ± 7.10	242.06 ± 30.22	10.33 ± 0.98	314.66 ± 26.49	386.89 ± 19.92	2165.99 ± 2149.54
IUS_A	512.69 ± 10.65	388.76 ± 174.82	18.09 ± 6.33	425.68 ± 196.91	532.13 ± 246.70	19.71 ± 7.18
US_B	542.40 ± 2.14	679.78 ± 8.18	98.65 ± 2.27	1048.85 ± 40.02	1024.99 ± 137.02	65.67 ± 8.44
CS_B	527.29 ± 9.55	186.06 ± 10.96	10.95 ± 1.37	249.22 ± 44.70	325.25 ± 25.08	14.98 ± 2.46
SS_B	507.83	10.34 ± 0.00	1.20 ± 0.00	26.18 ± 0.00	42.57 ± 0.00	8.09 ± 0.00
ICS_B	536.84 ± 7.04	200.92 ± 55.82	7.56 ± 2.12	247.71 ± 67.85	352.26 ± 89.04	19.82 ± 2.92
IUS_B	539.20 ± 4.73	302.40 ± 55.16	12.38 ± 1.64	317.95 ± 35.52	552.07 ± 85.11	12.13 ± 1.48
US_C	566.74 ± 8.73	612.45 ± 26.09	55.67 ± 17.24	905.92 ± 47.53	1107.42 ± 61.60	93.45 ± 35.15
CS_C	541.31 ± 3.33	107.53 ± 44.20	6.58 ± 3.30	158.33 ± 75.87	203.02 ± 78.62	5.06 ± 1.78
SS_C	556.24	8.75 ± 0.00	0.00 ± 0.00	12.84 ± 0.00	52.60 ± 0.00	6.27 ± 0.00
ICS_C	573.71 ± 3.28	153.99 ± 33.86	6.69 ± 1.40	205.30 ± 46.77	281.08 ± 65.97	11.36 ± 4.86
IUS_C	564.04 ± 22.15	259.69 ± 49.59	12.22 ± 2.49	307.64 ± 36.71	498.26 ± 80.45	11.98± 2.67

[1] Growth mediums US (uncontaminated soil), CS (contaminated soil), SS (silica sand), ICS (Irradiated contaminated soil) and IUS (Irradiated uncontaminated soil).

[2] Treatments A (with agent), B (with irradiated agent) and C (without agent)

[3] All values ± SEM represents the results obtained from three independent samples (n = 3)

[4] Nsats (normal saturated), MB Sats (Mid-chain branched saturated), TB Sats (Terminally branched saturated), Monos (monounsaturated) and Polys (polyunsaturated)

Table 12. Specific PLFA ratios for all treatments six weeks after the addition of the bioremediation agent.

Growth medium _Treatment	Saturated/ Unsatarated	Fungal/ Bacterial	Cy19:0 /18:1ω7c	cy17:0 / 16:1ω7c	18:1ω7t/ 18:1ω7c	16: 1ω7t/ 16:1ω7c
US_A	1.68 ± 0.30	0.06 ± 0.01	1.57 ± 0.79	0.67 ± 0.01	0.39 ± 0.35	0.20 ± 0.01
CS_A	1.23 ± 0.07	0.03 ± 0.00	2.32 ± 0.06	0.93 ± 0.04	0.78 ± 0.01	0.23 ± 0.00
SS_A	0.41 ± 0.00	0.15 ± 0.05	0.21 ± 0.00	0.44 ± 0.00	0.27 ± 0.00	0.00 ± 0.00
ICS_A	1.46 ± 0.08	0.06 ± 0.03	1.51 ± 0.13	1.01 ± 0.07	0.42 ± 0.02	0.16 ± 0.01
IUS_A	1.59 ± 0.11	0.05 ± 0.02	1.45 ± 0.12	1.49 ± 0.13	0.45 ± 0.21	0.10 ± 0.05
US_B	1.85 ± 0.27	0.04 ± 0.00	1.71 ± 0.88	0.67 ± 0.02	0.41 ± 0.36	0.22 ± 0.02
CS_B	1.36 ± 0.07	0.03 ± 0.01	1.97 ± 0.20	0.87 ± 0.03	0.70 ± 0.03	0.19 ± 0.01
SS_B	0.89 ± 0.00	0.22 ± 0.10	0.23 ± 0.00	3.62 ± 0.00	3.53 ± 0.00	0.00 ± 0.00
ICS_B	1.27 ± 0.04	0.05 ± 0.01	1.36 ± 0.08	0.97 ± 0.07	0.42 ± 0.03	0.13 ± 0.00
IUS_B	1.15 ± 0.01	0.02 ± 0.00	1.56 ± 0.03	1.38 ± 0.09	0.58 ± 0.06	0.13 ± 0.00
US_C	1.42 ± 0.02	0.06 ± 0.02	2.25 ± 0.09	0.64 ± 0.01	0.78 ± 0.39	0.19 ± 0.00
CS_C	1.14 ± 0.26	0.02 ± 0.01	3.22 ± 0.38	0.84 ± 0.07	1.16 ± 0.31	0.29 ± 0.03
SS_C	0.41 ± 0.00	0.00 ± 0.05	0.19 ± 0.00	0.37 ± 0.00	0.19 ± 0.00	0.00 ± 0.00
ICS_C	1.31 ± 0.04	0.03 ± 0.00	1.58 ± 0.13	0.79 ± 0.05	0.35 ± 0.02	0.16 ± 0.01
IUS_C	1.17 ± 0.01	0.02 ± 0.00	1.21 ± 0.04	1.09 ± 0.02	0.51 ± 0.03	0.11 ± 0.00

[1] Growth mediums US (uncontaminated soil), CS (contaminated soil), SS (silica sand), ICS (Irradiated contaminated soil) and IUS (Irradiated uncontaminated soil).

[2] Treatments A (with agent), B (with irradiated agent) and C (without agent)

[3] All values ± SEM represents the results obtained from three independent samples ($n = 3$)

Figure 5 shows the redundancy analysis with eigenvalues for the first three axes 0.601, 0.105 and 0.004, respectively. The total observed variance for the first two axes was 70.7%. The first axis correlated strongly with SO_4 ($r^2 = 0.7792$), the second axis with P ($r^2 = -0.8325$) and the third axis (not shown) with NO_3 ($r^2 = 0.089$). The overall effect of the environmental variables were statistically significant (p= 0.02) with estimated viable biomass, percentage organic carbon and nitrate.

Uncontaminated soil after three weeks was characterised with higher MBSats and TBSats content and this was stronger than before adding the bioremediation agent. Contaminated and irradiated contaminated soil still had a presence of NSats, highlighting the possibility that contaminated and irradiated contaminated soil had low microbial diversity.

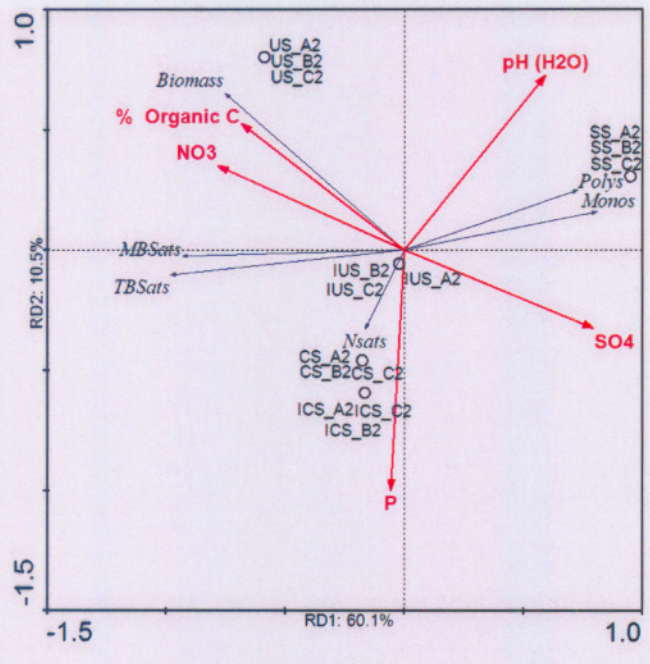

Figure 5. Redundancy analysis (RDA) ordination diagram illustrating the association between specific environmental variables and PLFA groups with different treatments three weeks after the addition of the bioremediation agent to the respective growth mediums.

The strong presence of Monos within in silica sand were still visible after three weeks. An additional association with Polys was also visible. Polyunsaturated fatty acids are indicative of eukaryotic microorganisms, primarily fungi (White et al., 1996). Similar to Actinomycetes, fungi are opportunistic and more tolerant to harsh environments than

other bacteria (Olsson, 1999). This could explain the presence of these microorganisms in nutrient depleted silica sand.

The redundancy analysis ordination shows the situation after six weeks with eigenvalues for the first three axes 0.795, 0.047 and 0.011, respectively. The total observed variance for the first two axes was 84.5%. The first axis correlated strongly with %C (r^2 = 0.8814), the second axis with pH (H_20) (r^2 = -0.307) and the third axis (not shown) with SO_4 (r^2 = -0.3894). The overall effect of the environmental variables was statistically significant (p= 0.02), weeks of adding the bioremediation agent, indicating microbial diversity. A decrease in estimated viable biomass occurred from week three to week six (Table 7, 9 and 11), possibly due to the depletion of available nutrients during the trial, which was conducted in closed systems.

Contaminated soil and silica sand was characterised with no major PLFA groups, therefore a decrease in microbial diversity and change in community structure may be expected. Irradiated contaminated soil was the only treatment which was characterised by an increase in Polys (fungi) indicating a shift in the microbial community structure of this treatment.

Throughout the trial no significant difference ($p > 0.05$) could be observed between different treatments (with the addition of agent, with the addition of irradiated agent and without the addition of agent) in the same growth medium (uncontaminated-, contaminated, irradiated contaminated-, irradiated uncontaminated soil and silica sand). This can clearly be seen from the grouping of treatments on the RDA ordination diagrams (Figures 4, 5 and 6).

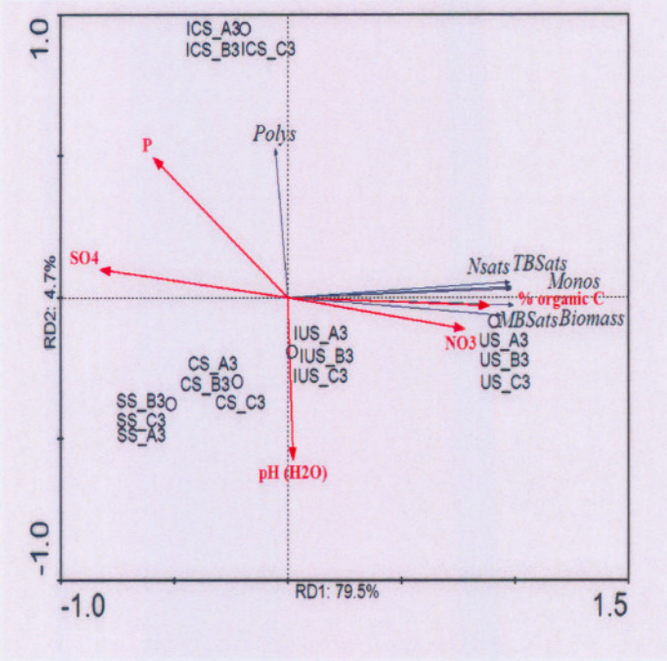

Figure 6. Redundancy analysis (RDA) ordination diagram illustrating the association between specific environmental variables and PLFA groups with different treatments after six weeks of adding the bioremediation to the respective growth mediums.

A fungal-to-bacteria (F:B) biomass ratio of 1.0 indicates that the fungi and bacteria are contributing equally to the microbial biomass in the soil. These ratios have been used to measure microbial community structure shifts (Wardle and Parkinson, 1990), such as those due to moisture gradients, management changes (Frey et al., 1999), or along a transect from a pollution point source (Bewley and Parkinson, 1985). Fungal-to-bacterial ratios have also been applied to determine the dominant group of microorganisms degrading plant residues. Previous studies have indicated ratios of saturated to unsaturated fatty acids, increased ratios of *trans*- to *cis*- monoenoic fatty acids and increased ratios of cyclopropyl fatty acids to their monoenoic precursors to be indicative of stress in microbial environments (Kieft et al., 1994; Claassens, 2003). Based on these ratios (Table 8, 10 and 12), it is evident that the microbial community experienced nutritional stress for the duration of the trial.

4.5 Conclusions

During this study, it was evident that PLFA analyses were sufficiently sensitive to detect differences in the community structure of different treatments in various growth mediums. Uncontaminated soil was stable before the addition of the bioremediation agent as it was the only growth medium which was characterised by viable biomass, percentage organic carbon, nitrate and Polys. As the trial proceeded, uncontaminated soil was the only growth medium that was characterised by a diversified profile including TBSats, NSats, Monos and MBSats as well as viable biomass, percentage organic carbon and nitrate thus showing a diversity in microbial community structure. Whereas contaminated soil remained constant throughout six weeks which was characterised by no detection of any major PLFA groups. Indicating that this growth medium has probably a small microbial diversity. The remaining growth mediums, all showed at some time a presence of one or other PLFA group but on conclusion of the trial it was only irradiated contaminated soil that showed the presence of Polys which is indicative of fungi. The ratios of saturated to unsaturated fatty acids, increased ratios of trans- to cis- monoenoic fatty acids and increased ratios of cyclopropyl fatty acids to their monoenoic precursors are indicative of stress in the environments (Claassens, 2003; Kieft et al, 1994). Based on these ratios it is evident that the microbial community experienced nutritional stress for the duration of the trial. Furthermore, throughout the trial it was clear that there was no significant difference between the different treatments. Thus the specific bioremediation agent used in this study had no effect on the community structure.

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Chapter 5: The Assessment of Plant Physiological Changes During the Treatment of Soil Containing Bromacil, Tebuthiuron and Ethidimuron.

Abstract

Herbicides are commonly used in agriculture but their persistence in soil keeps affecting the quality of soil. Herbicides differ from each other in persistence and leaching depending on several factors e.g. soil and climatic factors and herbicide characteristics. Plants contribute to the removal of herbicides through uptake and since some herbicides cause inhibition of photosynthesis, their phytotoxic effects may be assessed by quantification of this inhibition. In this study, the effect of a specific bioremediation agent (B 350) on Bromacil, Tebuthiuron and Ethidimuron toxicity was evaluated in seedlings of two C_4-grass species, *Cynodon dactylon* and *Zea mays*. The phytotoxic effect of the herbicides on the physiology of the two species was assessed through measurement of photochemical activity and CO_2 gas exchange rates as well as evaluating changes in pigment (chlorophylls and xantophylls) and protein content (specifically Rubisco). Inhibition of photosynthesis occurred within a week and chlorophyll *a* fluorescence measurements indicated that photosystem II (PS II) electron transport was inhibited by the herbicides. Furthermore, it was found that inhibition was similar for all treatments with photosynthetic rates inhibited by up to 95%. On a physiological level the results indicated no significant difference between treatments with bioremediation agent, with irradiated agent and without the addition of agent. A more detailed study was done in which a dilution of growth medium (1:3 with silica sand) was prepared to determine the precise mechanism of inhibition seen in the previous study. Results showed a rapid inhibition of photosynthesis by 48% within a few days of treatment. Immunodetection with specific antibodies showed that no degradation of Rubisco protein took place and that the decrease in photosynthesis can be attributed primarily to the inhibition of PS II function. Chlorophyll a fluorescence measurements indicated severe inhibition of electron transport beyond the primary electron acceptor Q_A^-. There was also an increase in the absorption of light and trapping of excitation energy by PS II reaction centres in an attempt to compensate for the loss of electron transport capacity. Heat dissipation by PS II reaction centres was increased but analysis of xantophyll cycle pigments revealed that increased inter-conversion of violaxanthin to zeaxanthin did not occur. Again no

significant differences were seen between the different treatments with bioremediation agent, irradiated agent and in the absence of agent.

Keywords: Bioremediation, Bromacil, *Cynodon dactylon*, Ethidimuron, herbicides, photosynthesis, photosystem II, Tebuthiuron, *Zea mays*.

5.1 Introduction

Eskom (South Africa) has applied herbicides for industrial weed control at power stations, under power lines, in servitude's and substations for many years. Herbicides including Bromacil, Tebuthiuron and Ethidimuron were applied to the area of soil between security fences at Zeus, a substation in the Mpumalanga province. Herbicide build-up was inevitable in treated areas and is known to cause damage to vegetation (crops, vegetables, surrounding re-growth, etc.) and pollution of groundwater (Goszszynska, 2001). Reclamation of these treated areas is necessary to limit contamination of adjacent agricultural land, but this is a problematic task. Physical removal of contaminated soil is a costly procedure (Alexander, 1999) and alternatives should be considered. Management of soil communities (micro-organisms and plants) to remove contaminants in the soil system can be implemented for the remediation of contaminated soil (Sawyer et al., 1994).

The herbicides applied (Bromacil, Tebuthiuron and Ethidimuron) belong to the uracil and phenylurea families that are known as photosystem II (PS II) herbicides since they affect PS II function during photosynthesis (Hilton et al., 1994). These herbicides bind to plastoquinone, a thylakoid membrane protein, blocking electron transport and inhibiting photosynthesis (Camillieri, 1987; Pfister and Schreiber, 1983).

While it is desirable for herbicides to control weeds during the season of application, it is not desirable for them to persist and subsequently affect plant growth in the long term. Persistence or residual life is terms used to describe the length of time a herbicide remains active in soil. Herbicides vary from each other in terms of their potential to persist in soil and several factors affect herbicide persistence in the soil environment (Tomlin, 1997).

Plants contribute to the removal of herbicides through uptake and translocation (Cunningham et al., 1997). As previously mentioned, herbicides could cause inhibition of photosynthesis. The degree of inhibition can be quantified by means of chlorophyll *a* fluorescence measurements, since herbicides alter the kinetics of polyphasic chlorophyll fluorescence transients O-J-I-P (Kautsky transients) (Strasser et al, 2000). Chlorophyll *a* fluorescence can therefore be used to identify physiological changes or stress induced by herbicides. Furthermore, CO_2 gas exchange provides an approach to measure the overall effect of herbicides on the rate of photosynthesis (Farquhar and Sharkey, 1982; Long and Hällgren, 1993).

A wide variety of proteins essential for photosynthesis are embedded in the thylakoid membranes within the chloroplasts. The PS I and II reaction centres (RC), the antenna pigment-protein complexes and most of the electron transport proteins are all integral membrane associated proteins. The operation of the photosynthetic carbon reduction cycle is dependant on ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which is the primary enzyme involved in CO_2 assimilation and the most abundant protein in the world, accounting for approximately 30 - 50 % of the total protein in most leaves (Hopkins and Hüner, 2004; Taiz and Zeiger, 2002).

Pigments are integrally related to the physiological function of leaves, since chlorophyll absorbs light energy and transfers it into the photosynthetic apparatus. When incident light energy exceeds the requirement for photosynthesis, the carotenoid pigment violaxanthin is converted to zeaxanthin via antheraxanthin in the so-called xanthophyll cycle. This cycle conveys photoprotection against excess light (Demmig-Adams and Adams, 1996) and the ratios of violaxanthin, antheraxanthin and zeaxanthin indicate the degree of stimulation of xantophyll cycle activity by plants (Adams et al., 1994).

The objectives of the present investigation were to determine the physiological effects of persistence of herbicides on two grass species, to evaluate the biostimulation and bioaugmentation of the specific bioremediation agent (B350) prescribed by ESKOM and to identify the site of action in the photosynthetic carbon metabolism of the two species.

5.2 Materials and Methods

5.2.1 Experimental Layout for Pilot study

Grass and soil was collected from the Zeus substation, Mpumulanga (South Africa) and transferred to the glasshouse facilities of the PPRI, Roodeplaat Dam, Pretoria where it was kept until transported to North-West University, Potchefstroom. Soil was sieved (1.5cm^2 mesh) and the homogenised soil stored in plastic containers until the trial started. Each treatment consisted of three replicates and was kept in the glasshouse at 25°C (day time) and 15°C (night time). Table 1 shows the growth mediums that consisted of contaminated- (CS), uncontaminated- (US), irradiated contaminated- (ICS), and irradiated uncontaminated soil (IUS), as well as irradiated silica sand which acted as control for this study. For the irradiation of the soil, 2kg contaminated- and uncontaminated soil was packed in plastic bags, heat sealed and taken to Isotron Inc. (Kemptonpark, South Africa) for gamma irradiation (KGy 70). A portion of the bioremediation agent (B350) was also irradiated at KGy 70. Treatments were conducted in pots of 25cm in diameter. Watering of plants occurred twice a day to ensure a constant moisture percentage. Leaching was prevented by keeping the soil moisture below field capacity.

The addition of the specific bioremediation agent (B350) took place and after a week, three *Z. mays* seedlings and a *C. dactylon* grass-plug were planted in each pot. *Zea mays* seedlings were germinated in a growth chamber (Conviron PGW 36, Controlled environment Ltd, Winnipeg, MB. Canada R3H 0R9) under controlled conditions with sufficient incandescent light bulbs (General Electric, Neodynium R80, 100W) and fluorescent lamps (General Electric, Cool White, 1500W) to maintain light intensities as high as 1300 μmol m^{-2} s^{-1} at 1 m distance from the plants. The plants were subjected to a daily light period of 15 hours (26°C) and dark period of 9 hours (20°C). Grass-plugs were cut from tuft growing from grass in uncontaminated soil. Plugs were treated with a rooting powder (Seradix B no.1) and planted in a mixture of sand and vermiculite at 24°C and 70% humidity for a week before transplanting to the experimental pots. *Z. mays* seedlings were used in the trial to serve as an internal control and due to its comparative plant physiology to that of *C. dactylon*. Both species are monocots with C_4 photosynthetic pathways, easy to grow and suitable for visual detection of phototoxic effects.

The duration of the trial was approximately six weeks, during which time the necessary plant material was sampled, frozen in liquid N_2 and kept frozen until pigment and protein analysis.

Table 1: Pilot trial layout, conducted with uncontaminated (uncon), contaminated (con), irradiated uncontaminated, irradiated contaminated soil and irradiated silica sand as growth mediums with agent (A), irradiated agent (B) and without the addition of the specific bioremediation agent (C). These treatments were conducted in triplicate.

Treatments	Uncon. Soil	Con. Soil	Irradiated silica sand	Irradiated con. Soil	Irradiated uncon. Soil
Agent (A)	1 2 3	4 5 6	7 8 9	10 11 12	13 14 15
Irradiated agent (B)	1 2 3	4 5 6	7 8 9	10 11 12	13 14 15
Without agent (C)	1 2 3	4 5 6	7 8 9	10 11 12	13 14 15

5.2.2 Experimental layout for Detailed Study

This study investigated the mechanisms involved in the progressive loss of photosynthetic capacity during a ten-day period of treatment with contaminated soil. The experiment consisted of contaminated growth medium (100% contaminated), contaminated growth medium that was diluted in a 1:3 ratio with silica sand (25% contaminated) and silica sand (control) as listed in Table 2.

Table 2: Detailed study layout conducted to evaluate the inhibition of photosynthesis within a ten-day period. Contaminated soil was diluted (3:1) with silica sand and all tests were performed in triplicate with bioremediation agent (X), irradiated agent (Y) and without the addition agent (Z).

Treatments	100% Contaminated	25% Contaminated	Silica sand
Agent (X)	1 2 3	4 5 6	7 8 9
Irradiated agent (Y)	1 2 3	4 5 6	7 8 9
Without agent (Z)	1 2 3	4 5 6	7 8 9

5.2.2 Non-destructive Measurements

5.2.2.1 Chlorophyll *a* Fluorescence

5.2.2.1.1 The Polyphasic Chlorophyll *a* Fluorescence Transient O-J-I-P

Illuminating a dark-adapted leaf results in characteristic changes in the intensity of chlorophyll *a* fluorescence, known as the Kautsky effect (Kautsky and Hirsch, 1931). The Kautsky transient shows a fast rise completed in less than 1s, with a subsequent slower decline towards a steady state. It has been proposed that the rising phase of the transient

reflects the primary reactions of photosynthesis (Krause & Weis, 1991). With the development of fluorimeters with high time resolution, additional and more accurate information about the kinetics of these transients was obtained (Schreiber & Neubauer, 1987; Strasser & Govindjee, 1992; Strasser et al., 1995). Strasser and Govindjee, (1992) demonstrated that the fluorescence rise kinetics of the Kautsky transient is polyphasic when plotted on a logarithmic time scale (Figure 1), which clearly exhibits the steps J and I, or I_1 and I_2 (Schreiber & Neubauer, 1987) between the initial O (F_0) and maximum P level ($F_P = F_M$).

Upon excitation with a saturated light pulse, there is a rapid initial rise in fluorescence intensity from O to the first intermediate step J within ca. 2 ms. This phase is followed by a further rise to the second intermediate step I within ca. 30 ms and to the final peak P in ca. 200 ms. The O-J-I-P fluorescence transient reflects the filling up of the electron acceptor side of PS II (Q_A, Q_B and PQ pool) with electrons from the donor side of PS II (Strasser & Govindjee, 1992). The relationship of these events to the O-J-I-P fluorescence transient was suggested by Strasser et al., (1995) to be the following: O, minimal Chl *a* fluorescence yield (highest yield of photochemistry); O to J, reduction of Q_A to Q_A^- (photochemical phase, light intensity dependent); J to I to P, reduction of the PQ pool (non-photochemical phase). Since the O-J-I-P fluorescence transient reflects the kinetics and heterogeneity involved in the filling up of the PQ pool with electrons, it can be used as a sensitive tool to investigate the photosynthetic apparatus *in vivo* (Strasser et al., 1995). The shape of the O-J-I-P fluorescence transient has been found to be very sensitive to various types of stress (Krüger et al., 1997; Tsimilli-Michael et al., 1999; Strauss et al 2005).

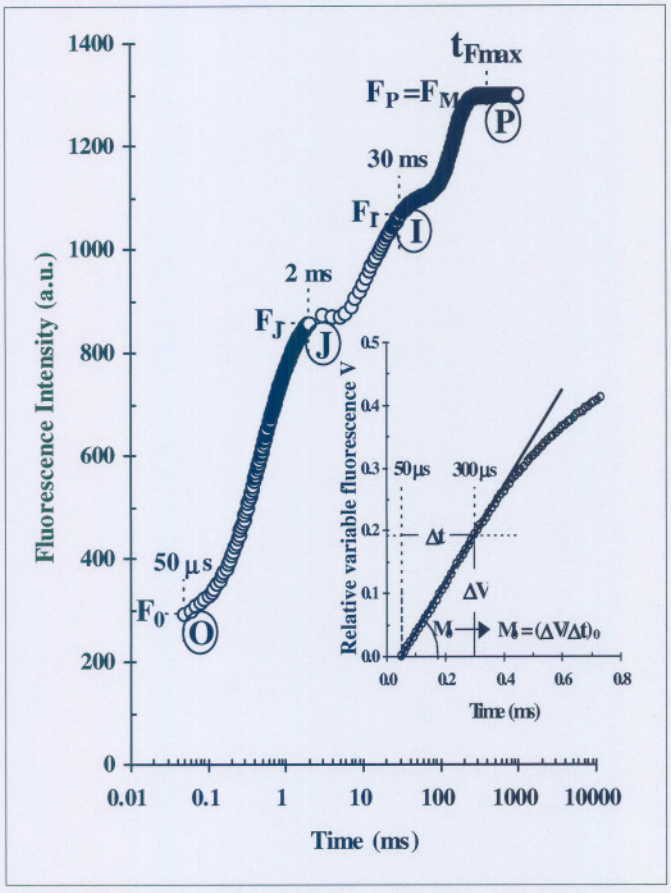


Figure 1. An example of a typical polyphasic chlorophyll *a* fluorescence transient O-J-I-P emitted by higher plants. A logarithmic time scale from 50 µs to 1 s is used to plot the transient. The labels refer to the fluorescence data used by the JIP-test for the calculation of various parameters quantifying PS II structure and function. The labels are: the fluorescence intensity F_0 (at 50 µs); the fluorescence intensity F_J (at 2 ms); the fluorescence intensity F_I (at 30 ms) and the maximal fluorescence intensity $F_P = F_M$. The figure insert shows the transient expressed as the relative variable fluorescence, $V = (F - F_0)/(F_M - F_0)$, on a linear time-scale and demonstrates how the initial slope (M_0) is calculated: $M_0 = (D_V/D_t)_0 = (V_{300\mu s})/(0.25\ ms)$. (From Tsimilli-Michael et al., 2000).

5.2.2.1.2 Analysis of the Chlorophyll *a* Fluorescence Transient by the JIP-Test

Strasser and Strasser (1995) have developed the JIP-test, which is used to translate the original fluorescence measurements of these O-J-I-P transients into several phenomenological and biophysical expressions that quantify PS II function. The O-J-I-P fluorescence transient is rich in information and can be used to derive a number of parameters (Table 3). The following data from the original measurements are used by the JIP-test: maximal fluorescence intensity (F_M); fluorescence intensity at 50 µs (considered as F_0); fluorescence intensity at 300 µs ($F_{300\mu s}$) required for calculation of the initial slope

(M_0) of the relative variable fluorescence (V) kinetics; and the fluorescence intensity at 2 ms (the J step) denoted as F_J.

The JIP-test represents a translation of the original fluorescence data to biophysical parameters that quantify the stepwise flow of energy through PSII at the reaction center (RC) as well as excited cross-section (CS) level (Strasser & Strasser, 1995). The parameters which all refer to time zero (onset of fluorescence induction) are: (i) the specific energy fluxes (per reaction centre) for absorption (ABS/RC), trapping (TR_0/RC), dissipation at the level of the antenna chlorophylls (DI_0/RC) and electron transport (ET_0/RC); (ii) the flux ratios or yields, i.e. the maximum quantum yield of primary photochemistry ($\varphi_{Po} = TR_0/ABS = F_V/F_M$), the efficiency ($\psi_0 = ET_0/TR_0$) with which a trapped exciton can move an electron into the electron transport chain further than Q_A^-, the quantum yield of electron transport ($\varphi_{Eo} = ET_0/ABS = \varphi_{Po} \bullet \psi_0$); (iii) the phenomenological energy fluxes (per excited cross section, CS) for absorption (ABS/CS), trapping (TR_0/CS), dissipation (DI_0/CS) and electron transport (ET_0/CS). The fraction of active PSII reaction centres per excited cross section (RC/CS) is also calculated. The formulae in Table 3 illustrate how each of the above-mentioned biophysical parameters can be calculated from the original fluorescence measurements.

The JIP-test was conducted at weekly intervals with a Plant Efficiency Analyser (PEA, Hansatech, Kingslynn, UK), which has a high time resolution and a large data acquisition capacity (Strasser et al., 1995). The O-J-I-P fluorescence transients were induced by a homogenous red light (peak at 650nm) of 3200 μmol m^{-2} s^{-1} provided by an array of six light-emitting diodes. The O-J-I-P fluorescence transient is influenced by various environmental conditions (Strasser et al., 1995). Fluorescence induction patterns and derived indices have been used frequently as empirical diagnostic tools in stress physiology (Strasser et al., 2000). Therefore chlorophyll *a* fluorescence can be regarded as a biosensing device for stress detection in plants.

Table 3. Summary of the JIP-test formulae using data from the fast fluorescence transient O-J-I-P.

Extracted and technical fluorescence parameters	
F_0	= $F_{50\mu s}$, fluorescence intensity at 50μs
$F_{100\mu s}$	= fluorescence intensity at 100μs
$F_{300\mu s}$	= fluorescence intensity at 300μs
F_J	= fluorescence intensity at the J-step (at 2ms)
F_I	= fluorescence intensity at the I-step (at 30ms)
F_M	= maximal fluorescence intensity
tF_M	= time to reach F_M, in ms
V_J	= $(F_{2ms} - F_0) / (F_M - F_0)$
$(dV/dt)_0 = M_0$	= $4 \cdot (F_{300} - F_0)/(F_M - F_0)$
Quantum efficiencies or flux ratios	
$\varphi_{P0} = TR_0/ABS$	= $[1-(F_0/F_M)] = F_v/F_M$
$\varphi_{E0} = ET_0/ABS$	= $[1-(F_0/F_M)] \cdot \psi_0$
$\Psi_0 = ET_0/TR_0$	= $(1-V_J)$
Specific fluxes or specific activities	
ABS/RC	= $M_0 \cdot (1/V_J) \cdot (1/\varphi_{P0})$
TR_0/RC	= $M_0 \cdot (1/V_J)$
ET_0/RC	= $M_0 \cdot (1/V_J) \cdot \psi_0$
DI_0/RC	(ABS/RC) – (TR_0/RC)
Phenomenological fluxes or phenomenological activities	
ABS/CS	= ABS/CS_{Chl} =Chl/CS or $ABS/CS_0=F_0$ or $ABS/CS_M= F_M$
TR_0/CS	= $\varphi_{P0} \cdot$ (ABS/CS)
ET_0/CS	= $\varphi_{P0} \cdot \Psi_0 \cdot$ (ABS/CS)
DI_0/CS	= (ABS/CS) – (TR_0/CS)
Density of reaction centres	
RC/CS	= $\varphi_{P0} \cdot (V_J/M_0) \cdot$ (ABS/CS)

ABS, absorption energy flux; CS, excited cross section of leaf sample; DI, dissipation energy flux at the level of the antenna chlorophylls; ET, flux of electrons from Q_A^- into the electron transport chain; φ_{D0}, quantum yield of dissipation; φ_{E0} probability that an absorbed photon will move an electron into electron transport further than Q_A^-; φ_{P0} maximum quantum yield of primary photochemistry; ψ_0, efficiency by which a trapped excitation, having triggered the reduction of Q_A to Q_A^- can move an electron further than Q_A^- into the electron transport chain, RC, reaction centre of PSII; RC/CS, concentration of reaction centres per excited cross section of a given leaf area; TR, excitation energy flux trapped by a RC and utilised for the reduction of Q_A to Q_A^-.

5.2.2.2 Measurement of CO_2 assimilation

Carbon dioxide gas exchange was measured after three weeks with an open-circuit infrared gas analysis system (CIRAS-1). A 2.5cm^2 portion of leaf of each plant was clamped into a photosynthetic leaf chamber (PLC). Measurements were conducted at a leaf temperature of 26°C and a light intensity of 1200 μmol m^{-2} s^{-1}.

5.2.2.2.1 Overview of CO_2 assimilation kinetics

A number of measurements, terms and units are employed when studying carbon dioxide assimilation (Von Caemmerer & Farquhar, 1981). The CO_2 assimilation rate (A) is expressed as the amount of CO_2 assimilated per unit leaf area and time (μmol CO_2 $m^{-2}s^{-1}$). The stomatal conductance (g_s) represents the flux of CO_2 through the stomata (μmol CO_2 $m^{-2}s^{-1}$).

A portable open-circuit photosynthesis system (CIRAS-1) was used to measure CO_2 assimilation by pumping air from the photosynthetic leaf chamber (PLC) enclosing the leaf into an infrared gas analyser that continuously measures the CO_2 concentration of the air stream. The CO_2 concentration of the air stream will decrease if the leaf inside the PLC is assimilating CO_2. The CO_2 assimilation rate equals the change in the amount of CO_2 in the air stream per unit time. Changes in temperature and pressure are compensated for in the calculation of the CO_2 assimilation rate, but humidity has to be controlled, since a rise in transpiration will cause an increase in the amount of water vapour, resulting in dilution of CO_2 in the air stream (Long & Hällgren, 1993).

Carbon dioxide must first diffuse through the boundary layer on the leaf surface before it is able to enter the leaf. The boundary layer conductance will be an order of a magnitude greater than the highest possible g_s under field conditions (Long, 1985). In the Parkinson-type PLC (used in the CIRAS-1 system) the boundary layer resistance is minimised and kept constant by the fan of the PLC, which keeps the turbulence high.

The intercellular CO_2 concentration (c_i) can be determined by:

$$c_i = c_a - A/g_t$$

If only diffusion is considered, total conductance, specifically boundary layer, as well as stomatal conductance, is represented by g_t, and c_a is the atmospheric CO_2 concentration. Carbon dioxide ($A:c_i$) response curves can be constructed by plotting A against c_i at a range of different c_a values for leaves enclosed in the PLC. An example of a typical $A:c_i$ curve is presented in Figure 2.

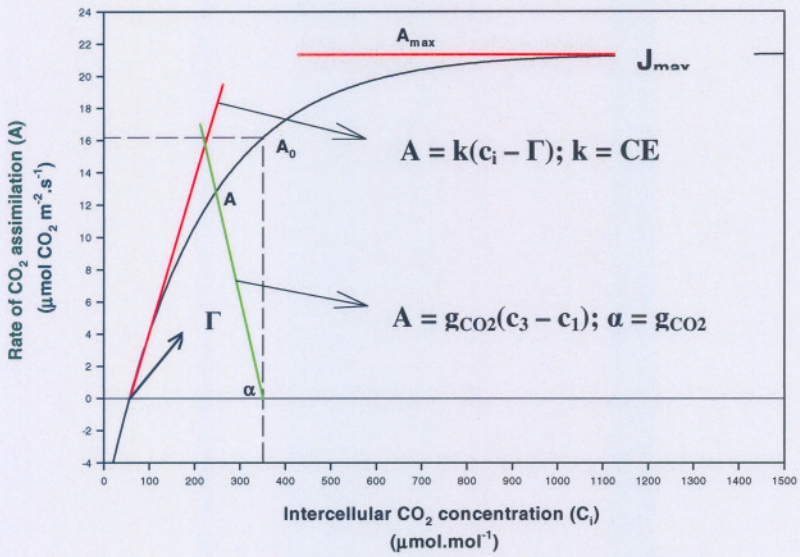


Figure 2. The response of carbon dioxide assimilation rate (A) against intercellular CO_2 concentration (c_i). A is the CO_2 assimilation rate under the given conditions (the point of simultaneous solution of the demand and supply functions). Carboxylation efficiency (CE) is represented by the initial slope of the demand function, the CO_2 compensation concentration (Γ) is the intercellular CO_2 level where the net usage of CO_2 equals zero, the maximum rate of assimilation (J_{max}) represents the rate of CO_2 assimilation at saturated levels of CO_2, and A_0 is the rate of assimilation that occurs when no stomatal limitations occur (at c_i > 350 µmol mol^{-1}) (Hall et al., 1995).

It is necessary to distinguish stomatal and mesophyll limitation of photosynthesis, therefore the following equation is employed:

$$l = (A_0 - A)/A_0$$

where, A = CO_2 assimilation rate at ambient CO_2 concentration (c_a = 350 µmol mol^{-1}) and A_0 = CO_2 assimilation rate where no stomatal limitations is present (at a c_i > 350 µmol mol^{-1}) (Farquhar & Sharkey, 1982). Stomatal limitation (l) represents the proportionate decrease in CO_2 assimilation that may be attributed to stomatal restrictions.

The carboxylation efficiency of photosynthesis can be deduced from the initial linear response ($\delta A/\delta c_i$) of the A:c_i response curve which is an *in vivo* measure of Rubisco activity (Bolhàr-Nordenkampf & Öquist, 1993). The maximal CO_2 assimilation rate at saturating c_i (J_{max}) can be regarded as a reliable indicator of the RuBP regeneration capacity of the leaf.

5.2.3 Destructive Measurements

5.2.3.1 Pigment Concentrations

Leaf samples were harvested weekly under full illumination, weighed, frozen in liquid N_2 and stored at -80°C. Special care was taken not to shade the leaves during harvesting, thereby preventing deactivation of light-activated enzymes and changes in the ratios of xanthophyll cycle pigments.

Leaf samples were grounded in liquid nitrogen and extracted with 1.5 ml acetone (85 %). The crude extracts were transferred into micro-centrifuge tubes and the extracts centrifuged at 10 000 x g for 5 min at 4°C. A green supernatant containing all pigments was obtained.

Pigments were separated using a 250 x 4.6 nm RP C18 (5 μm) Zorbax column together with a guard column. A gradient of two solvents were used. Solvent A consisted of 85% acetonitrile and 15% methanol. Solvent B consisted of 68% methanol and 32% ethyl acetate. The flow rate was 1.0 ml min^{-1} and the column temperature kept at 35 °C. The HPLC system used was an Agilent 1100 with multi-wavelength detector. Pigments were detected at 445 nm and identified by retention time compared with pure (> 95%) standards. Quantification was performed using the Agilent software comparing peak areas to standard reference curves. All solvents used were HPLC grade.

5.2.3.2 Protein Extractions

Three leaf discs collected from plants of each treatment were ground to a fine powder with liquid nitrogen in a pre-cooled mortar and extracted with 3 ml ice-cold extraction buffer containing 50 mM Tris-HCL, 1 mM EDTA (metallo-proteinase inhibitor), 3 mM DTT, 1 mM PMSF (serine proteinase inhibitor), 10 μM E-64 (cysteine proteinase inhibitor), 1 μM Pepstatin-A (aspartate proteinase inhibitor), 2 mM Benzamidine (trypsin and trypsin-like enzyme inhibitor) 2 mM Aminocarprioc acid (carboxypeptidase B inhibitor), and 30 mg of insoluble PVPP. The crude extract was transferred to a pre-cooled micro centrifuge tube and centrifuged at 10 000 x g at 4°C for 15 minutes. The soluble protein content of the supernatant was determined according to the method of Bradford (1976).

Two hundred and fifty microliters SDS-PAGE sample buffer containing 250 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 40 µl β-mercapto-ethanol, 0.015 mM bromophenolblue and 40% (w/v) glycerol was added to 750 µl plant extract, boiled for 5 minutes at 95°C and used for SDS-PAGE after brief cooling on ice.

5.2.3.2.1 Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for the separation of proteins and protein subunits. Proteins were separated by SDS-PAGE with a mini-gel system (Biorad). The resolving gel solution contained 2.5 ml 40% (w/v) acrylamide/bisacrylamide mixture and 2.5 ml 1.5 M Tris-HCl (pH 8.8) and was degassed prior to casting. The gel was casted between the glass plates after addition of 100 µl 10% (w/v) SDS, 100 µl 10% (w/v) ammonium persulphate and 10 µl TEMED. The stacking gel solution contained 200 µl 40% (w/v) acrylamide/bisacrylamide, 500 µl 0.5 M Tris-HCl (pH 6.8) and was degassed prior to casting. The gel was casted on top of the polymerised resolving gel after addition of 20 µl 10% (w/v) SDS, 12.5 µl 10% (w/v) ammonium persulphate and 2.5 µl TEMED. A plastic gel comb was immediately inserted into the stacking gel solution to form 10 sample wells in which the protein extractions and marker proteins were loaded. After the stacking gel has polymerised, the gel comb was carefully removed and the sample wells rinsed thoroughly with SDS running buffer containing 38.4 mM Glycine, 5 mM Tris, and 0.1% (w/v) SDS. Marker proteins were loaded into the first well with sample proteins loaded into wells 2-10 on an equal protein basis (20 µg per well) and the gel was run at room temperature for 1h30 – 1h45 at 100 volts.

5.2.3.2.2 Immunodectection of Rubisco

Separated proteins were transferred from the resolving gel to a nitrocellulose membrane (Hybond C-extra, Amersham Pharmacia Biotech, UK) in transfer buffer containing 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS and 20% (v/v) methanol. Proteins were transferred from the resolving gel to the membrane for 30 – 40 min at 100 V with cooling.

Primary anti-bodies directed against Rubisco were used for protein detection. Membranes were incubated for 12 h in the presence of the primary antibody followed by incubation with anti-rabbit IgG secondary HRP antibody (Horse Radish Peroxidase-linked) for 2 h.

Labeled proteins were detected on the membrane using a detection reagent containing 40 mg chloronapthol and 50 µl 30% H_2O_2 in 100 ml 50 mM Tris-HCl buffer (pH 7.6).

5.3. Statistical Analysis

Parametric and non-parametric statistical analyses were performed on all data obtained using STATISTICA 6 (StaSoft, Inc ©). The data was tested for normality using the Shapiro-Wilk's test. In the case of the data being normally distributed (parametric) a breakdown and one-way ANOVA was performed and the Tukey's honest significant difference (HSD) test was used to determine statistical significance between the various treatments.

5.4 Results and Discussion

The pilot trail was conducted for a period of 6 weeks not only for assessment of plant physiological changes but also for soil microbial properties which included chemical and physical properties of soil, potential soil enzymatic activity (dehydrogenase, β-glucosidase, acid- and alkaline- phosphatase, arylsulphatase and urease) and soil microbial community structure. Results from soil microbial properties revealed no significant differences between treatments with bioremediation agent, irradiated agent and without the addition of the specific agent. Measurement of chlorophyll *a* fluorescence and CO_2 assimilation rate (results not shown) also showed no significant differences between the effects induced by these treatments. In all cases, however, the inhibition of photosynthesis after three weeks of treatment was extreme, with decreases in CO_2 saturated rates of photosynthesis (J_{max}) of up to 95%. Thus a detailed study regarding the precise effects of the treatments on photosynthesis was conducted (Table 2).

All physiological procedures performed in the pilot study were on both *Z. mays* and *C. dactylon* and showed no significant differences between the two species regarding phytotoxic effects induced by the herbicides. Therefore, only the results of *Z. mays* will be shown. Furthermore, radiation of soil (contaminated and uncontaminated) revealed that on a plant physiological level there were no differences in plants growing in non-irradiated soil. Thus only results from contaminated and uncontaminated growth mediums will be shown.

5.4.1 Pilot Study

5.4.1.1Effects on PS II function and CO_2 assimilation rates

Fluorescence measurements were conducted at weekly intervals on *Z. mays* plants and revealed that the maximum quantum yield of primary photochemistry (F_V/F_M ratio) was reduced on average by 40% for plants growing in the contaminated growth medium irrespective of the inclusion/exclusion of irradiated/non-irradiated bioremediation agent (Figures 3i, 3ii and 3iii).

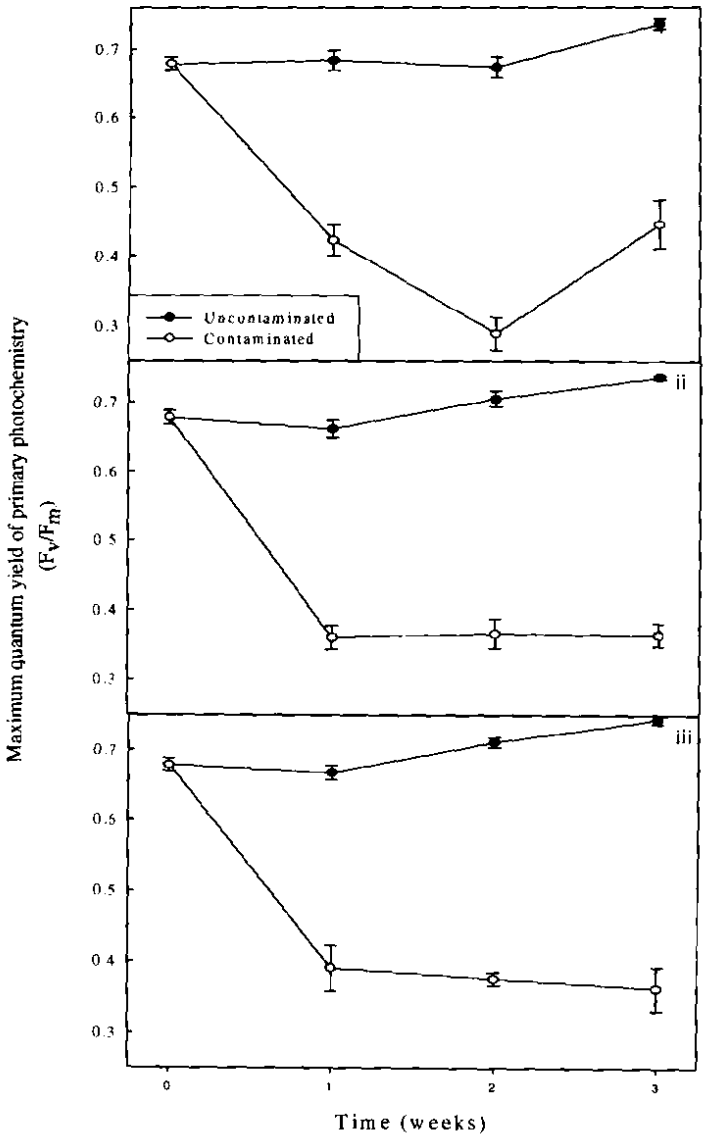


Figure 3: The maximum quantum yield of primary photochemistry (F_V/F_M) of maize leaves in treatments where plants were grown for three weeks in the presence of agent (i), irradiated agent (ii) and without agent (iii). Solid and open circles represent the mean values obtained in plants grown in uncontaminated and contaminated soil respectively. Values indicate the mean of three replicates.

The effect of the various treatments on the photosynthetic capacity of the plants was also assessed following three weeks of treatment. Figure 4 shows a typical A:c_i response curve obtained for all treatments. Extreme inhibition (up to 95%) of carboxylation efficiency (CE) and J_{max} was observed in the plants growing in contaminated growth medium. The decrease in J_{max} is indicative of a large inhibition of ribulose-1,5-bisphosphate (RuBP) regeneration capacity by the photosynthetic carbon reduction (PCR) cycle (Leegood, 1993). The two stromal bisphosphatases, Fructose-1,6-bisphosphatase and Sedoheptulose-1,7-bisphosphatase, are responsible for the production of RuBP in the regeneration phase of the PCR cycle. These enzymatic reactions are dependant on reducing equivalents (ATP and NADPH) supplied by PS II and PS I electron transport, firstly, for light activation of the two stromal bisphosphatases, and secondly, for the reactions *per se*. The large decrease in the F_V/F_M ratio (Figure 3) and J_{max} (Figure 4) strongly points towards severe disruption of electron transport with subsequent interference in the PCR cycle reactions responsible for RuBP regeneration. The decrease in CE, on the other hand, points towards inhibition of Rubisco activity and activation state (Taiz and Zeiger, 1998; Blackenship, 2002). Rubisco is the primary enzyme involved in the fixation of CO_2 in the reduction phase of the PCR cycle (Blankenship, 2002)

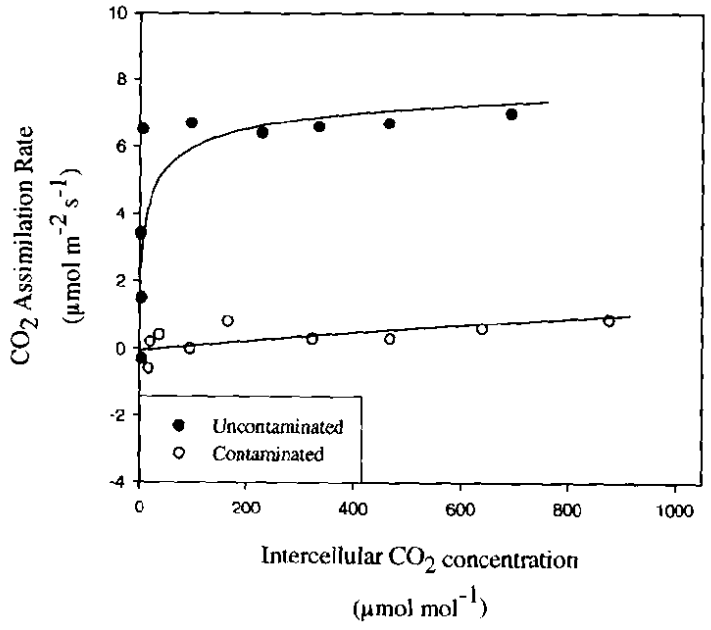


Figure 4: A:c_i curves for maize plants grown for three weeks in contaminated and uncontaminated growth medium.

The observed inhibition of electron transport was expected since the herbicides present in the soil are all PS II inhibitors, more specifically they bind to plastoquinone, a thylakoid membrane protein that allows the transfer of electrons, thereby inhibiting photosynthesis, or more precisely, inhibition of the Hill reaction (Pfister and Schreiber, 1983; Camillieri, 1987).

5.4.2 Detailed study

5.4.2.1 Effects on PS II function and CO_2 assimilation rates

As was seen in the pilot study, the F_V/F_M ratio was reduced in the leaves of plants grown in both the 25% and 100% contaminated growth mediums, whereas the ratio remained relative stable (c.a. 0.73) in the control plants (Figure 5). The decrease in the F_V/F_M ratio developed gradually during the eight days of growth in contaminated soil. There were no consistent significant differences between the 100% or 25% contaminated growth medium treatments regarding the reduction in the F_V/F_M ratio. The average decrease in the F_V/F_M ratio during the experimental period was 9.5% and 8.5% for the 100% contaminated and 25% contaminated soils respectively.

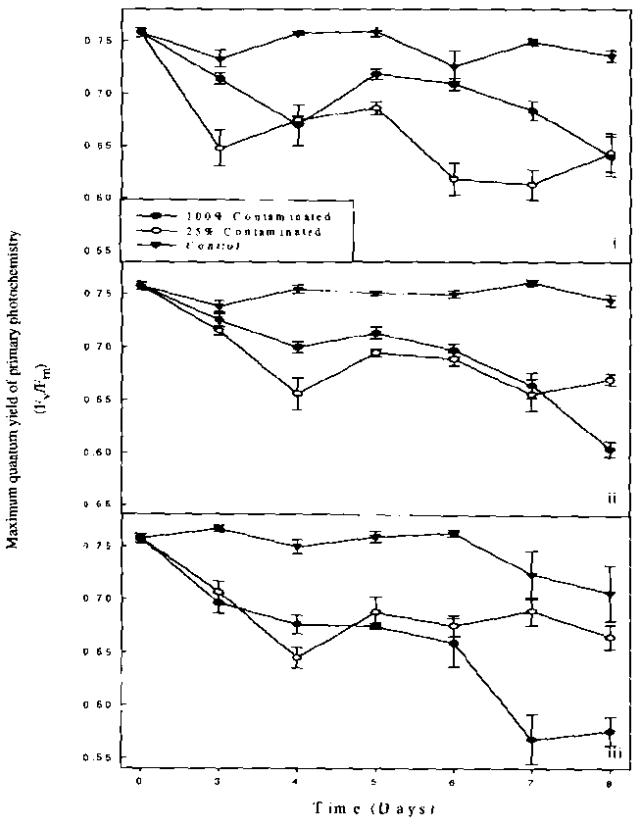


Figure 5: The maximum quantum yield of primary photochemistry (F_V/F_M) of maize leaves in treatments where plants were grown for eight days in the presence of agent (i), irradiated agent (ii) and without agent

(iii). Solid and open circles represent the mean values obtained in plants grown in 100% and 25% contaminated soil respectively. The inverted tri-angles represents the controls grown in silica sand Values indicate the mean of three replicates ± standard error.

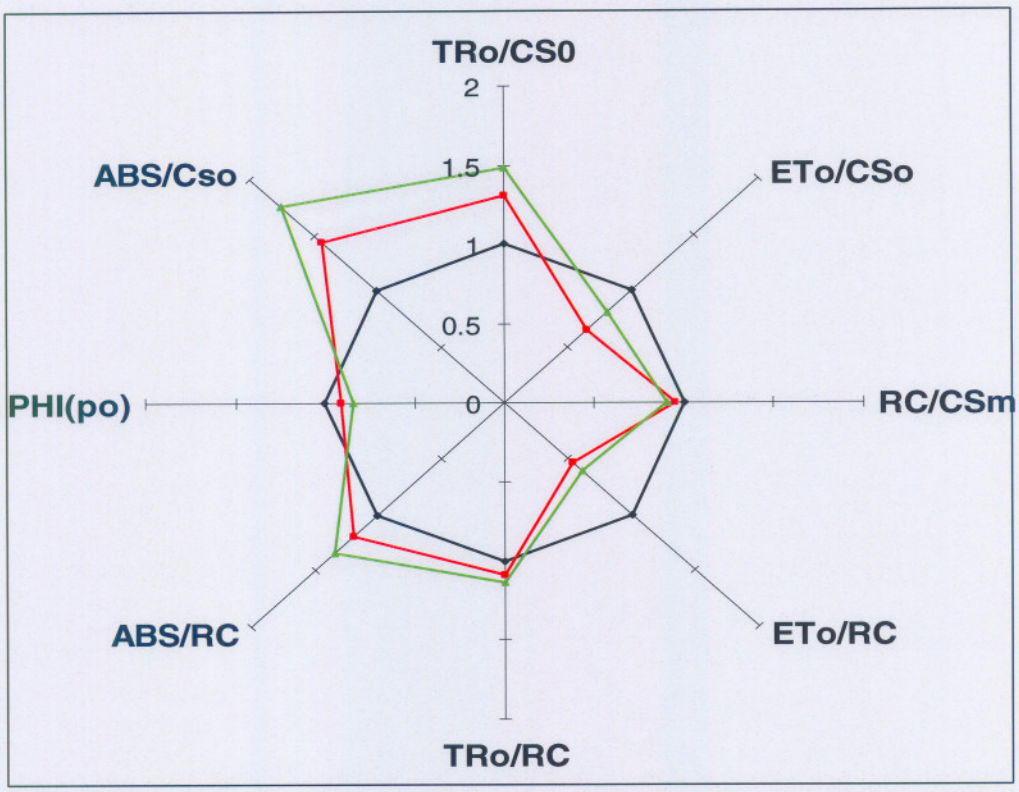


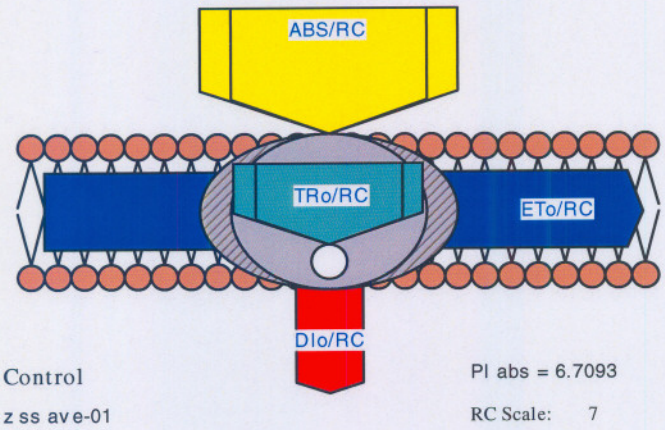
Figure 6. Changes in functional parameters of primary photochemistry in *Zea mays* after eight days of growth in contaminated soil. The black line represents the normalised control values, the green line shows the values obtained in the 25% contaminated soil and the red line the values obtained in the 100% contaminated soil. All values are expressed relative to the normalised control values. Parameters shown are: electron transport per cross section of leaf (ET_0/CS_0), the fraction of active PS II reaction centres per cross section of leaf (RC/CS_M), electron transport per PS II reaction centre (ET_0/RC), excitation energy trapping per PS II reaction centre (TR_0/RC), light absorption per PS II reaction centre (ABS/RC), maximum quantum yield of the primary photochemistry ($\varphi_{Po} = F_V/F_M$), light absorption per cross section of leaf (ABS/CS_0) and excitation energy trapping per cross section of leaf (TR_0/CS_0).

The results shown in Figure 6 revealed that electron transport has been negatively influenced (inward deviation of red and green lines away from the black control line) on both the PS II reaction centre (ET_0/RC) and leaf cross section (ET_0/CS_0) level. This was expected, as mentioned previously, because all three herbicides are PS II inhibitors. Furthermore, the parameters ABS/RC (chlorophyll antenna size of each PS II reaction centre), ABS/CS_0 and TR_0/CS_0 showed large increases (outward deviation of red and green lines) relative to the control. The results therefore demonstrate that a substantial

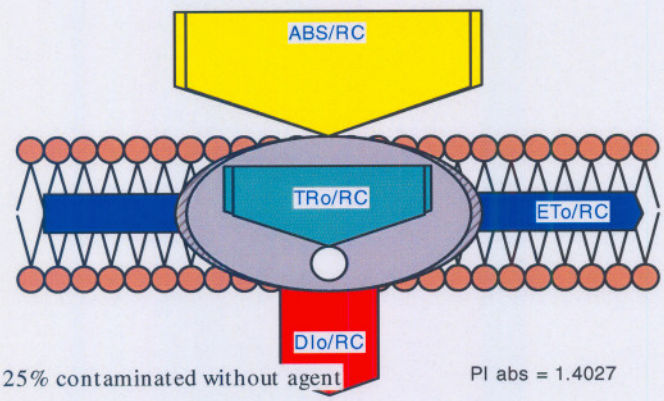
increase in light absorption and excitation energy trapping occurred in parallel with the loss in electron transport capacity (indicated by the decrease in ET_0/RC and ET_0/CS_0). This compensatory response to the loss of electron transport capacity resulted in a large increases in heat dissipation (non-photochemical quenching) by reaction centres (also see Figure 7) in order to remove excess excitation energy not utilised for electron transport (photochemical quenching).

In Figures 7a, b and c energy-flow models are shown illustrating the effect of the herbicides on overall PS II function. These models illustrate how light absorption (ABS/RC), excitation energy trapping (TR_0/RC), electron transport (ET_0/RC) and heat dissipation (DI_0/RC) is affected in the plants grown for eight days in contaminated soil relative to the control (Fig. 7a) The severe decrease in electron transport (ET_0/RC, dark blue arrows) in the 25% (Figure 7b) and 100% (Figure 7c) contaminated growth mediums are clearly shown. The large increases in light absorption (ABS/RC, yellow arrows), excitation energy trapping (TR_0/RC, light blue arrows) and heat dissipation (DI_0/RC, red arrows) are also shown. In general, the increasing effect on heat dissipation was the largest in the plants grown in 100% contaminated soil, mainly because of the larger increase in ABS/RC in this treatment.

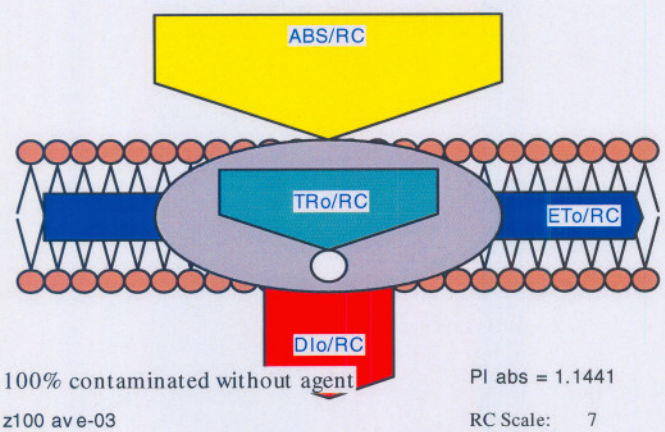
A major consequence of increased light absorption under conditions of very limited electron transport capacities (as induced by the herbicides) is the possibility of damaging effects on PS II reaction centre proteins. Under these conditions an increase in charge recombination events of the PS II reaction centre primary radical pair $P^{+}_{680}/Pheo^{-}$ becomes highly likely. This will promote the formation of $^3P_{680}$, which may interact with molecular oxygen and generate harmful singlet oxygen (Aro et al., 1993). Singlet oxygen is highly reactive and destructive and may cause direct damage to the PS II reaction centre proteins. Thus, inhibition of electron transport is possibly not the only negative effect induced by the herbicides.



A



B



C

Figure 7 A, B and C. Energy-flow models illustrating the herbicide-induced changes in ABS/RC, TR_0/RC, ET_0/RC and DI_0/RC after 8 days of growth in silica sand (Control, A) and the 25% (B) and 100% (C) contaminated growth medium.

The photosynthetic capacity of the plants was also assessed during the eight-day period to evaluate the effect of the various treatments. The respective A:c_i response curves are shown in Figure 8. Clear decreases in CE and J_{max} can be seen in both the 25% and 100% treatments. However, the plants grown in 25% contaminated growth medium maintained higher CO_2 assimilation rates for longer periods than the plants grown in the 100% contaminated growth medium. Once again, there were no consistent significant differences in the response induced by the various treatments, with agent, with irradiated agent and without the addition of the specific bioremediation agent.

Table 4 shows the values of J_{max} and CE measured over time in the various treatments. The above-mentioned decreases in J_{max} (up to 69%) and CE (up to 38%) are indicated for both treatments (25% and 100% contaminated growth media). As mentioned in the previous section the decrease in J_{max} strongly points towards a decrease in RuBP regeneration capacity by the PCR cycle, mainly because of the inhibitory effects of the herbicides on PS II electron transport.

Table 4. The CO_2-saturated rate of photosynthesis (J_{max}) and carboxylation efficiency (CE) measured over a 9-day period in maize plants grown in 25% and 100% contaminated soil with agent (X), irradiated agent (Y) and without the addition of the specific bioremediation agent (Z). Values indicate the mean of three replicates ± standard error.

Day		X100%	Y100%	Z100%
0	J_{max}	20.92 ± 2.65	20.92 ± 2.65	20.92 ± 2.65
5		16.28 ± 2.30	17.84 ± 0.98	17.23 ± 0.60
9		13.14 ± 5.00	4.28 ± 1.59	5.89 ± 1.04
0	CE	0.68 ± 0.27	0.68 ± 0.27	0.68 ± 0.27
5		0.60 ± 0.29	0.78 ± 0.18	0.69 ± 0.26
9		0.47 ± 0.09	0.19 ± 0.05	0.63 ± 0.26
Day		**X25%**	**Y25%**	**Z25%**
0	J_{max}	20.92 ± 2.65	20.92 ± 2.65	20.92 ± 2.65
4		22.37 ± 3.51	18.53 ± 0.49	19.21 ± 3.74
8		8.44 ± 3.01	12.40 ± 1.82	11.70 ± 1.56
0	CE	0.68 ± 0.27	0.68 ± 0.27	0.68 ± 0.27
4		0.68 ± 0.09	0.47 ± 0.01	0.67 ± 0.23
8		0.60 ± 0.06	0.32 ± 0.16	0.33 ± 0.16

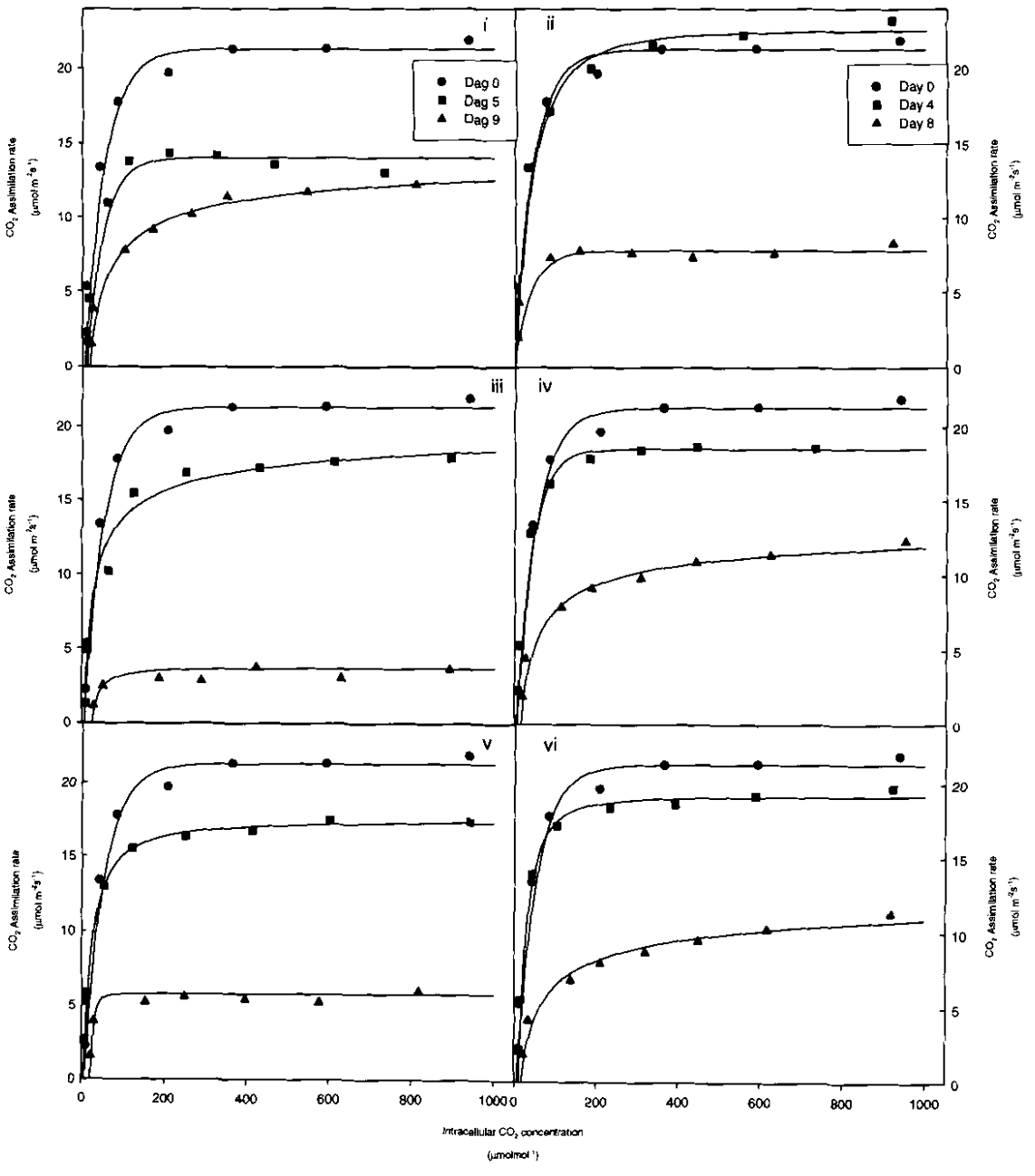


Figure 8: A:ci curves for plants growing in 100% contaminated (i, iii, v) and 25% contaminated (ii, iv, vi) growth medium with the addition of agent (i, ii), irradiated agent (iii, iv) and without agent (v, vi), respectively.

5.4.2.2 Effect on leaf soluble protein profiles

Because of the observed decrease in carboxylation efficiency (estimate of Rubisco activity obtained from A:c_i curves), an investigation was conducted to determine possible direct effects on Rubisco protein content. To determine to what degree Rubisco was affected, soluble proteins were extracted from leaves and separated by SDS-PAGE. The

levels of Rubisco were determined by using specific antibodies and Western immunodetection techniques. Figure 9A shows that after 11 days, leaf soluble protein profiles were not altered substantially by the herbicides. The position of phosphoenolpyruvate carboxylase (PEP), with a molecular weight of ca. 90 kDa, and the Rubisco large subunit, with a molecular weight of ca. 50 kDa, is indicated by the arrows. Both these enzymes are intimately involved in the C_4 photosynthetic pathway in maize. The levels of both these enzymes were not affected by the herbicides in any consistent fashion and appeared to be largely unaltered. This observation was further supported by western blot analysis that showed no loss (all bands of similar intensity) of protein in treated plants (Figure 9B). Therefore, changes in the levels of photosynthetic proteins was not involved in the loss of photosynthetic activity.

Although the steady-state levels of Rubisco were unaffected, herbicide-induced effects on its activity *per se* cannot be excluded. Rubisco activity can be influenced indirectly by the inhibition of PS II electron transport, more specifically through the inhibition of the enzyme Rubisco activase. This enzyme, similar to FBPase en SBPase, is light-activated via photosynthetic electron transport and is responsible for the activation of Rubisco during dark-light transitions. The inhibition of PS II electron transport by the herbicides could therefore inhibit Rubisco activity indirectly through disruption of this activation mechanism.

A

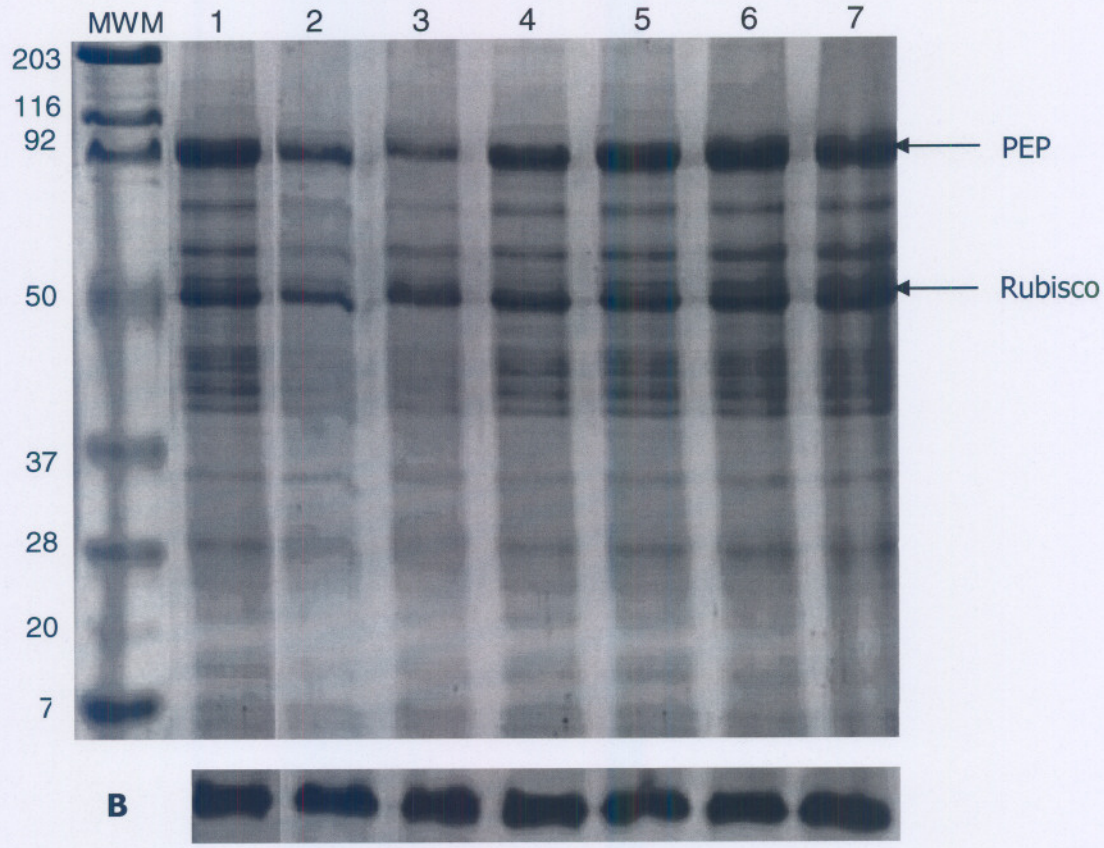


Figure 9. Protein profiles in control and herbicide-treated maize leaves after 11 days of treatment (A). The sizes of the molecular weight marker proteins (MWM) are indicated on the left. Control (1) and treatments 100% contaminated with agent (2), 25% contaminated with agent (3), 100% contaminated with irradiated agent (4), 25% contaminated with irradiated agent (5), 100% contaminated without agent (6), 25% contaminated without agent (7). Western blot showing the levels of Rubisco protein (B).

5.4.2.3 Pigment Concentrations

The energy-flow models (Figure 7) showed that there was an increase in heat dissipation, which is a form of non-photochemical quenching of excess absorbed light energy. This is a clear response to the decrease in electron transport (photochemical quenching) induced by the herbicides. Non-photochemical quenching also involve the conversion of violaxanthin via antheraxanthin into zeaxanthin by means of the enzymes epoxidase and de-epoxidase, better known as the xantophyll cycle. The carotenoids (zeaxanthin, antheraxanthin and violaxanthin) is necessary for the dissipation of excess energy absorbed by chlorophylls (Adams et al., 1994). The results in Figure 10(i) show that after 9 days of treatment there were only small changes in the chlorophyll a and b ratio. In addition, the chlorophyll a and b content was not decreased by the herbicide treatments

(results not shown). The process by which de-epoxidase transforms violaxanthin via antheraxanthin into zeaxanthin, and again into violaxanthin by epoxidase, can be influenced in several ways. Firstly, a pH decrease in the lumen of the membrane induces the biochemical conversion of violaxanthin to antheraxanthin and zeaxanthin. Secondly, the protonation of proteins of the antenna system has been proposed to cause structural changes of these proteins necessary to induce energy dissipation (Demmig-Adams and Adams, 1996). Both these are control mechanisms for the xanthophyll cycle. A surprising finding was that xanthophyll cycle activity was not stimulated in the treatment plants. This is indicated by the absence of any increase in the VAZ/V ratios (Figure 10ii). Taken together, these results show that the xanthopyll cycle did not convey any additional protection against the absorption of excess light energy under conditions of very low electron transport capacity, as measured in the treatment plants. This could have substantially aggravated the negative effects of the herbicides on photosynthesis.

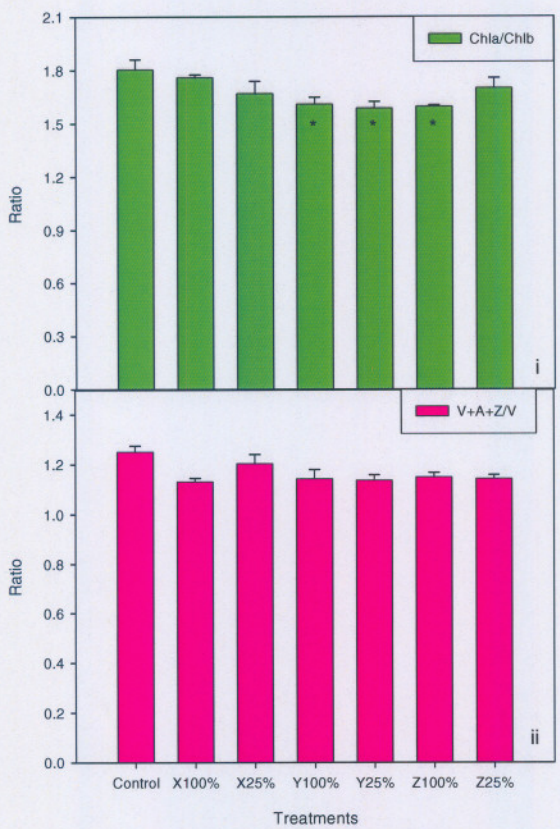


Figure 10. Ratios of chlorophyll a / chlorophyll b (i) and (Violaxanthin + Antheraxanthin + Zeaxanthin) / Violaxanthin (ii) for all treatments, with the addition of agent (X), with the addition of irradiated agent (Y) and without the addition of the specific bioremediation agent (Z) after 11 days of growth. (* represents significant differences at $p < 0.05$).

5.5 Conclusion

Over the duration of the study there were no significant difference between treatments with bioremediation agent, irradiated agent and without the addition of the specific agent. Growth of maize plants in soil contaminated with herbicides led to very large decreases in PS II electron transport capacity. Results demonstrated that a substantial increase in light absorption and excitation energy trapping occurred in parallel with the loss in electron transport. This resulted in large increases in heat dissipation (non-photochemical quenching) by reaction centres. A major consequence of increased light absorption under conditions of very limited electron transport capacities (as induced by the herbicides) is the possible damage to PS II reaction centre proteins by generation of harmful singlet oxygen. The decrease in J_{max} strongly points towards a decrease in RuBP regeneration capacity by the PCR cycle, mainly because of the inhibitory effects of the herbicides on PS II electron transport. The levels of Rubisco and PEPcase was not affected by the herbicides in any consistent fashion and appeared to be largely unaltered. Therefore, changes in the levels of photosynthetic proteins do not appear to be involved in the loss of photosynthetic activity. Xanthophyll cycle activity was not stimulated as a protective response against excess light energy under conditions of very low electron transport capacity, which could have substantially aggravated the negative effects of the herbicides on photosynthesis.

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Chapter 6

General Discussion and Conclusions

6.1 Background

The development and modernisation of the world resulted in an increase of agricultural practices due to alarming growth of the world population (Dikshith, 1991). Increased levels of pesticide production and application of pesticides for agriculture, plant protection and animal health has resulted in soil, water and air pollution and consequently relating a serious risk to the environment and also to human health (Gonzalez-Pradas, 2005). Pesticides include several groups of compounds, herbicides, insecticides, rodenticides and fumigants consisting of several hundred individual chemicals (Voutsas et al., 2004).

Herbicides are an integral part of modern agriculture and for industries requiring total vegetation control with a 47% of the total pesticide sales in 1997 in South Africa alone (Goszszynska, 2001). Most herbicides are soil applied and more and more concern is raised that herbicides not only affect target organisms but also the microbial community present in soil.

For many years ESKOM (South Africa) has used herbicides for industrial weed control in and around the power stations, under power lines, servitude's and at substations. Treatments were usually applied to the strip between security fences and consisted of Bromacil, Ethidimuron and Tebuthiuron. The herbicides belong to the uracil and phenylurea families, respectively. The urea family is one of the most important herbicide classes used worldwide and can be used selectively for germinating grass and broad leaved weeds in many crops, especially cereals or non-selectively for total weed control as part of the maintenance of roads, railways, parks, etc. These three herbicides are all photosynthesis inhibitors, more specifically, they disrupt the b-quinone protein during electron transport at photosystem II (PSII) (Camillieri et al., 1987). Other processes also occur that are most commonly responsible for plant death (Herbicide Handbook, 1994). The herbicides are designed to be stable in soil and are not prone to chemical, hydrolytic or photolytic degradation. Therefore, they are ideal for use as encroachment control agents, since they will persist in soil for long periods of time (Goszszynska 2001).

Soil persistence is the length of time a herbicide remains active in soil and herbicides vary in their potential to persist in soil. Several factors determine the length of time herbicides persist, including soil factors (soil composition, soil chemistry and microbial activity), climatic conditions (moisture, temperature and sunlight) and herbicidal properties (water solubility, vapor pressure and the compound's susceptibility to chemical and microbial breakdown) (Curran, 1998).

The quality of soil plays an important role in the well-being and productivity of a given ecosystem and the environments related to it. Soil is a critically important component of the earth's biosphere, functioning not only in the production of food and fiber but also in the maintenance of local, regional and worldwide environmental quality (Doran and Parkin, 1994). Soil quality is 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). To assess soil quality, indicators representing the physical, chemical and biological controlling components of soil are necessary (Kennedy and Papendick, 1995).

Bioremediation allows natural processes to clean up harmful contaminants in the environment. It uses microorganisms, such as bacteria, protozoa and fungi to degrade contaminants by optimising conditions for biodegradation through aeration, addition of nutrients and control of pH and temperature (Atlas and Bartha, 1992; Morgan and Watkinson, 1989; Norris, 1994). The most important aspect of bioremediation is the fact that it involves the management of a contaminated site using biologically based technologies to prevent, minimise or mitigate damage to human health or the environment. Bioremediation provides a technique for cleaning up pollution by enhancing some biodegradation processes that occur in nature (Pankhurst et al., 1995).

6.2 Discussion

In this study several factors were investigated including the chemical and physical properties of soil, potential soil microbial activities, soil microbial community structure and plant stress physiology of *Zea mays* and *Cynodon dactylon*. These parameters were measured and evaluated to determined their inter-relationship with each other. Analyses

were conducted on three time intervals, before, after three and six weeks of adding the specific bioremediation agent (B350).

The chemical and physical properties of soil, uncontaminated, contaminated, irradiated contaminated, irradiated uncontaminated and silica sand was characterised before the trial started as to indicate the composition, deficiencies, etc. of soil. Particle size and distribution analysis (Table 2, chapter 3 and 4) revealed that the soil used in this study was typified by a large clay fraction with a relatively high silt fraction. Thus, any residual herbicide would absorb to the clay fraction and leaching of herbicides will be restricted since soil will compact and infiltration of water would be limited. Furthermore, soil had a potassium (K) deficiency and percentage organic matter (Table 5, chapter 3 and 4) was less within the contaminated medium than uncontaminated medium, which could be as a result of the residual herbicide concentration in soil, limiting the growth of vegetation and microorganisms which is indicated by the percentage organic matter.

The inter-relationship of soil properties, potential microbial activity and soil microbial community structure was investigated by means of multivariate statistical analysis, specifically Redundancy Analysis (RDA). Statistically, the potential microbial activity, community structure and plant physiology showed that there were no significant differences ($p < 0.05$) between the different treatments with agent, irradiated agent and without the addition of the specific bioremediation (B350) agent over the whole study.

Before the trial started the uncontaminated medium was characterised by a high dehydrogenase, urease and arylsulphatase activity and a low β-glucosidase activity (Table 7 and Figure1, Chapter 3). Furthermore this medium was characterised by a high viable biomass which included polyunsaturated fatty acids (Polys). Before the addition of the agent, a higher percentage organic carbon (%C) and nitrate (NO_3) than contaminated mediums was detected. Contaminated soil and irradiated contaminated soil was characterised by lower enzymatic activity (Table 7, Chapter 3) and by the phospholipid fatty acid (PLFA) groups, mid-branched saturated fatty acids (MBSats), terminally branched saturated fatty acids (TBSats) and normal saturated fatty acids (NSats) (Table 7 and Figure 4, Chapter 4). The harsh environment, lack of organic carbon, radiation of soil and high clay content could explain the higher mole percentage (mole %) MBSats, which is indicative of Actinomycete bacteria that can survive better in harsh environments

(Olson, 1999). Silica sand was also characterised by lower enzymatic activity than uncontaminated soil and showed a higher mole % monounsaturated fatty acids (Monos) than all other treatments (Table 7, Chapter 3; Table 7, Chapter 4). Monosaturated fatty acids are indicative of Gram-negative bacteria that grow rapid and are able to adapt to many environments (White et al., 1996;. Ponder and Tadros, 2002; Rütters et al., 2002).

Three weeks after the addition of the specific bioremediation agent, the uncontaminated medium was still characterised by a high dehydrogenase activity but β-glucosidase activity declined (Table 8 and Figure 2, chapter 3). This medium was also characterised by a higher mole % of MBSats and TBSats. The activity of urease however, decreased from that of previous results. Contaminated and irradiated contaminated soil was still characterised by a high mole % NSats. Once again silica sand was characterised by a high mole % Monos and Polys (Table 9 and Figure 5, Chapter 4). Both of these PLFA groups are indicative of gram negative actinomycete bacteria and fungi, that can adapt and withstand harsh environments (Olsson, 1999; Ponder and Tadros, 2002; Rütters et al., 2002; White et al., 1996) Silica sand had a very low microbial activity compared to uncontaminated mediums.

Plant stress physiology were monitored and after three weeks it was evident that plants growing in the contaminated mediums experienced extreme inhibition of photosynthesis capacity (Figure 3A, Chapter 6) with a rate of nearly zero. Indications that the plants were unable to photosynthesise and the decrease in the maximum photosynthetic rate (J_{max}) points to an effect on electron transport. The maximum quantum yield of primary photochemistry (F_v/F_M) confirmed that photosystem II (PS II) function was inhibited within a weeks time (Figure 3B, Chapter 6). The uncontaminated medium showed a higher rate of photosynthesis and a constant tendency as to its PS II function, suggesting that there was no change in electron transport. However, the average rate of photosynthesis for *Zea Mays* is approximately 20 ($\mu mol^{-2}s^{-1}$) whereas results showed a J_{max} of 6.7 ($\mu mol^{-2}s^{-1}$), which could be as a result of stress experienced by plants. Results of *Z. mays* and *Cynodon dactylon* showed the same extent of inhibition in photosynthesis and PS II function. Furthermore, no significant differences were observed between the treatments with agent, irradiated agent and without the addition of the specific agent. As a result of the fast and extreme inhibition of photosynthetic capacity experienced by plants

a further study was conducted to determine the precise mechanism of inhibition seen in the first trail.

This detailed study included a 3:1 dilution of the contaminated medium with silica sand, and the addition of agent, irradiated agent and without the addition of the agent, (Table 3, Chapter 5). The decrease in the F_V/F_M ratio developed gradually during the eight days of growth in contaminated soil (Figure 5, Chapter 5). Further fluorescence results revealed that electron transport had been negatively influenced (Figure 6, Chapter 5) on both the PS II reaction centre (ET_0/ RC) and leaf cross section (ET_0/ CS_0) levels. Furthermore, the parameters ABS/RC (chlorophyll antenna size of each PS II reaction centre), ABS/CS_0 and TR_0/CS_0 showed large increases relative to the control. The results demonstrate that a substantial increase in light absorption and excitation energy trapping occurred in parallel with the loss in electron transport capacity. This compensatory response to the loss of electron transport capacity resulted in a large increase in heat dissipation by reaction centres (Figure 7, Chapter 5). A major consequence of increased light absorption under conditions of very limited electron transport capacities, is the possibility of damaging effects on PS II reaction centre proteins since harmful singlet oxygen will be generated (Aro et al., 1993; Hideg et al., 1994) which is highly reactive and destructive and may cause direct damage to the PS II reaction centre proteins. Thus, inhibition of electron transport is possibly not the only negative effect induced by the herbicides.

The photosynthetic capacity of plants showed a clear decrease in CE and J_{max} for both the 25% and 100% treatments (Figure 8 and Table 4, Chapter 5). The decrease in J_{max} strongly points towards a decrease in RuBP regeneration capacity by the PCR cycle, mainly because of the inhibitory effects of the herbicides on PS II electron transport. The levels of Rubisco and PEP enzymes were not affected by the herbicides in any consistent fashion and appeared to be largely unaltered (Figure 9A and B, Chapter 5). Therefore, changes in the levels of photosynthetic proteins was not involved in the loss of photosynthetic activity. Rubisco activity could however be influenced indirectly by the inhibition of PS II electron transport, more specifically through the inhibition of the enzyme Rubisco activase. This enzyme is light-activated via photosynthetic electron transport and is responsible for the activation of Rubisco during dark-light transitions. The inhibition of PS II electron transport by the herbicides could therefore inhibit Rubisco activity indirectly through disruption of this activation mechanism. There were

only small changes in the chlorophyll a and b ratio (Figure 10(i), Chapter 5). In addition, the chlorophyll a and b content was not decreased by the residual herbicide concentrations. It was also found that xanthophyll cycle activity was not stimulated in the plants growing in contaminated soil. This is indicated by the absence of any increase in the VAZ/V ratios (Figure 10ii, Chapter 5). These results show that the xanthopyll cycle did not convey any additional protection against the absorption of excess light energy under conditions of very low electron transport capacity, as measured in plants growing in contaminated growth medium.

After six weeks there was a clear decrease in the estimated viable microbial biomass for all growth mediums. Also uncontaminated medium was no longer characterised by a high dehydrogenase and β-glucosidase activity (Table 11 and Figure 3, Chapter 3). As for the other mediums, not one showed an increase in microbial activity. Contaminated and silica sand was characterised by a low mole % of major PLFA groups (Table 11 and Figure 6, Chapter 4). Irradiated contaminated soil was the only medium that showed a shift in microbial community structure since it was the only medium characterised by a high mole % Polys (Table 11, Chapter 4). Results of the *trans-* to *cis-* monoenoic fatty acids and cyclopropyl fatty acids to their monoenoic precursors ratios, indicated that the soil microbial community experienced nutritional stress throughout this trail.(Table 8, 10 and 12, Chapter 4).

6.3 Conclusion

In this study a definite decline could be seen in the microbial enzymatic activity and viable microbial biomass. On commencement of the study, the microbial activity within the uncontaminated treatments were lower than three weeks later, but this could be due to the fact that soil was stored prior to study. Almost all the treatments showed that the microbial enzymatic activity did not increase as the study progressed. There was also a decline in microbial viable biomass throughout the study. Contaminated mediums were characterised by PLFA groups that can survive in nutrient depleted and harsh environments and showed a very low microbial activity. Furthermore, the *trans-* to *cis-* monoenoic fatty acids and cyclopropyl fatty acids to their monoenoic precursors ratios indicated that all treatments experienced nutritional stress throughout the study. Plant physiology confirmed that concentration of residual herbicides was so high that plant

growth was influenced within a weeks time. The inhibition of the electron transport of PS II as a result of herbicide action results in several photo-toxic effects. The fact that all experimental data showed no significant differences between treatments with agent, irradiated agent and without the addition of the agent, reveals that this specific agent, B350, had no effect on the residual herbicides and that the residual concentration of herbicides is to such an extent that it will limit the growth of plants.

6.4 Future Recommendations

It is recommended that any remediation agent considered for use by Eskom, either be evaluated for their activity and specific application (target compounds), or that a guarantee of effectiveness is obtained from the supplier. According to the supplier, the specific bioremediation agent (B350) under investigation is effective for the remediation of a broad range of compounds (hydrocarbons). Furthermore, it is recommended that all areas that have been treated in the past, as well as future sites to be treated with herbicides, be evaluated in terms of the biological components.

Alternative remediation practices could be used in future and should include the selection and identification of various types of phyto-remediation techniques. These techniques should be evaluated according to their respective abilities to decrease herbicide levels in varying soil conditions (soil type, organic content etc.) to provide at least 80% ground cover. Other successful substitutes may be used as communal seeding treatments under varying soil types and conditions.

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