Faculty of Natural and Agricultural Sciences

Academic Year 2004

Microbial Ecology and Bio-monitoring of Total Petroleum Contaminated Soil Environments

by

MP Maila

Thesis submitted in fulfilment of the requirement for the degree of Doctor (PhD) in Applied Biological Science specialisation in Biotechnology

Rector: **Prof CW Pistorius**

Dean: **Prof A Ströh**

Promotor: **Prof TE Cloete**

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The promoter: The author:

Prof TE Cloete MP Maila

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In memory to my parents

SL Maila (1945-1996)

PR Maila (1952-1996)

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‘So little is known about most of the microbial world that no one has ever documented the extinction of a bacterium’.

New Scientist, “Save a bug for Biotechnology”, 1 August 1992 p. 7.

Acknowledgements

I express my gratitude to Prof T E Cloete (University of Pretoria), Prof V Torsvik (University of Bergen, Norway) and Dr J De Beer of the CSIR for giving me the opportunity to carry out my PhD in their laboratories.

I'm also grateful to Dr EM Top (University of Idaho, US), Dr K Drønen (University of Bergen), Dr P Wade (Phokus Technologies, SA) and Prof W Verstraete (University of Gent, Belgium) for their spontaneous and free of charge co-operation in reviewing the work and in microbiological investigations.

I thank my family (especially my brothers Chipu and Motinyane Maila) for the support and understanding they have given me throughout the duration of the studies.

I would also like to thank all my colleagues at the CSIR and the University of Pretoria for their support and assistance in microbiological investigations and preparing the thesis.

Finally, yet importantly, I thank God for giving me the wisdom, strength and perseverance, which enabled me to reach this stage of my career.

Summary

The contamination of environmental media by total petroleum hydrocarbons (TPH) is a concern in many parts of the world; particularly as most petroleum components like polycyclic aromatic hydrocarbons (PAHs) are either toxic or carcinogens. In South Africa, the sale of major petroleum products by the South African Petroleum Industry Association (SAPIA) reveals that about 21 billion litres of petroleum products are sold per year. These products include bitumen, diesel, fuel oil, illum paraffin, jet fuel and petrol. In addition, 19.5 million tonnes of crude oil are brought into South Africa annually to feed the country's four refineries. The production of oily sludges at refineries, transportation, storage, and handling of petroleum products by end users, results in environmental contamination. The soil environment is particularly vulnerable to hydrocarbon contamination as most of the accidental spillages by trucks, rail locomotives and pipelines have a direct impact on the soil medium. As most of the petroleum compounds are either toxic or carcinogenic, their removal from the soil is necessary.

The literature reveals that biological treatment of hydrocarbons is cost effective compared to other treatment options. However, in order to improve the efficiency of biological treatments, there is a need to understand the microbial diversity of TPH stressed environments and how simple biomonitoring 'instruments' can be used to evaluate the removal of hydrocarbons from the soil. The message from the literature indicates some potential solutions to the existing problems associated with soil microbial diversity and biotreatment of hydrocarbon contaminated soil, which must be investigated.

The main aim of this work was to evaluate the microbial diversity of the different soil environments disturbed by Total Petroleum Hydrocarbons (TPHs) and the potential use of plants and microorganisms in monitoring and removing hydrocarbons from the soil. In addition, the potential of the culture-independent methods in complementing, the culture-dependent methods when evaluating soil microbial diversity were also evaluated.

The polyphasic approach was successfully used in evaluating microbial diversity in both hydrocarbon-contaminated and uncontaminated soils. The approach involved the use of community level physiological profiles (CLPP) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) to evaluate the effects of hydrocarbons on the soil microbial communities of both the contaminated and non-contaminated soil layers at a diesel contaminated site. Because of the ability of the molecular methods (PCR-DGGE) to complement the CLPP, the polyphasic approach is recommended when evaluating soil microbial diversity and the effect of pollutants on microbial community structure as the approach appears to compensate for the limitations of each of the methods of evaluating microbial diversity. However, further work is needed to improve the recovery of bacteria from the soil, particularly where the interest is to evaluate the availability of the indigenous microbial populations for bioremediation.

The substrate utilisation pattern and 16S DNA fragments of the soil microbial communites in different soil layers at a diesel contaminated site were different. The substrate utilisation pattern of the topsoil was different from the substrate utilisation pattern of the soil layers below 1m. In addition, the substrate utilisation pattern of the contaminated and uncontaminated soil layers were different. 16S DNA fragments of the different soil layers were also different. While the metabolic activities of different samples as reflected by CLPP does not necessarily imply the difference in community structure of

the samples, PCR-DGGE revealed differences in 16S DNA fragments and this complemented the results of the culture based methods. The results suggest that the use of functional and genetic approaches (in combination) have a better chance of revealing a 'clearer' picture of soil microbial diversity.

The distribution of hydrocarbon-utilising bacteria and the efficiency of biodegradation of hydrocarbons vary with soil depth. The biodegradation rate of hydrocarbon was highest in the topsoil compared to other soil layers and this was supported by the high number of hydrocarbon-degrading bacteria in the topsoil compared to soil layers at and below 1m. The results suggest that the biological removal of hydrocarbons varies in different soil layers and that microbial diversity as measured by CLPP and PCR-DGGE varies with depth in hydrocarbon-contaminated soil. The information about metabolic activities of different soil layers is important when assessing the footprints of degradation processes during monitored natural attenuation (MNA). However, further studies are required to understand the effect of (not only) other pollutants, but the influence of soil components (pore volume, level of adsorbents and other environmental factors) on the microbial diversity of different soil layers in both 'shallow' and deep aquifers.

The microbial diversity of different environments contaminated by hydrocarbons has different community level physiological profiles. At diesel depots where similar hydrocarbons are used for maintenance of locomotives, the number of bacteria (both total culturable heterotrophic bacteria and hydrocarbon-degrading bacteria) was proportional to the level of hydrocarbon contamination. However, there was no significant difference in the level of total culturable heterotrophs (TCHs) and the hydrocarbon degrading bacteria. In addition, the biological activities as evaluated by CO_2 production were higher in nutrient amended treatments in which high numbers of TCHs

were present. Microbial diversity of polluted surfaces needs to be studied further to investigate the concentration or the thickness of the hydrocarbons layer on the rock surfaces that encourages the attachment or colonization of the TCHs and the hydrocarbon-degrading bacteria.

The hydrocarbons rather than the geographical origin of the soil sample appear to be more important in determining functional or species diversity within the bacterial communities. The samples from different locations were as different as samples from the same location but from contaminated versus uncontaminated soil. The results of the soils from different locations artificially contaminated by different hydrocarbons also reached the same conclusion. However, further work is required to investigate the importance of soil heterogeneity in community studies of soil environments contaminated by similar hydrocarbons.

The removal of Polycyclic Aromatic Hydrocarbons (PAHs) in multi-planted soil microcosm was higher compared to PAHs removal in monoculture soil microcosms. In addition, the PAH removal was higher in the vegetated soil microcosms compared to the non-vegetated microcosms. There was however, no significant difference in the PAH removal in the soil microcosms planted with *Branchiaria serrata* and the microcosm with *Eulisine corocana*. The Principle Component Analysis (PCA) and Cluster analysis used to analyse the functional diversity of the different treatments revealed differences in the metabolic fingerprints of the PAH contaminated and non-contaminated soils. However the differences in metabolic diversity between the multi-planted and mono-planted treatments were not clearly revealed. The results suggest that multi-plant rhizoremediation using tolerant plant species rather than monoculture rhizoremediation have the potential to enhance pollutant removal in moderately contaminated soils.

Lepidium sativum, a plant with short germination period, was successfully used to monitor, the removal of Polycyclic Aromatic Hydrocarbons (PAHs) from the soil. The sensitivity of *L. sativum* decreased with increasing concentration of the polycyclic aromatic hydrocarbons in the artificially contaminated soil while no germination occurred in the historically polluted soil. When used during phytoremediation of PAH, the germination level of *L. sativum* was inhibited during the first weeks, after which germination increased, possibly due to PAH dissipation from the soil. The methodology based on the sensitivity of *L. sativum* to PAH can be used as a monitoring tool in bioremediation of soil contaminated with PAH. However, the methodology should be developed further to gain more knowledge on aspects of bioavailability of PAH in both the aged as well as the freshly spiked soil. Also critical is the sensitivity of the seeds to other pollutants (e.g. heavy metals), which are most likely to occur in the presence of the PAHs. Although the biological activities have the potential to monitor the removal of hydrocarbons from the soil, the methodologies have not been developed sufficiently to cater for the heterogeneity of the soil and to differentiate toxicity by the parent compound and the metabolites. At present, it is best that they be used to complement existing conventional monitoring instruments.

Finally, the biological removal of hydrocarbons is cost-effective compared to other treatments. However, inherent physical, chemical and biological limitation hampers the efficient utilisation of the bioremediation technologies. Biostimulation approaches involving the stimulation of indigenous pollutant-degrading bacteria should be preferred ahead of bioaugumentation. The latter approach should be considered when the contaminated site does not have the indigenous pollutant-degrading bacteria. Even in this case, the aim should be to 'seed' the biodegradation knowledge to the indigenous microbial populations due to poor survival of the added strains.

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Chapter 1

GENERAL INTRODUCTION

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1. SOIL MICROBIAL DIVERSITY- AN OVERVIEW

Microbial diversity can be defined as "the variety of bacterial species in ecosystems, as well as the genetic variability within each species" (Conservation, 1987). Theoretically, microbial diversity can also be regarded as the amount and distribution of genetic information in a natural community. A representative estimate of microbial diversity is therefore a prerequisite for understanding the phenotypic diversity of microorganisms in ecosystems (Garland and Mills, 1994; Zak *et al.*, 1994).

In the soil, the number of different species can be 10^4 or higher per gram of soil (Torsvik *et al.*, 1990; Klug and Tiedje, 1994). Because of the immense genotypic and phenotypic diversity, soil microbial communities remain some of the most difficult to characterise. Two approaches generally used to characterise microbial diversity includes the culture-dependent (or phenotypic) and the culture independent (genomic) based methods. However, due to the heterogeneity of the soil and the inherent limitations of the existing methodologies, most of the bacterial species in the soil remains unidentified.

Microbial diversity can further be considered from a variety of perspectives. It has been suggested that 'trophic, physiological or functional diversity, intraspecific genetic diversity, or phylogenetic diversity of species or higher taxa' are all levels of diversity of concern to the microbial ecologist (Delong, 1996). Kawanabe (1996), proposed that the diversity of ecological relationships among life forms (e.g. competition, cooperation, etc) is a more important part of biodiversity than 'simply the diversities among creatures'. Thus, synergistic and antagonistic interactions play a critical role in community functional diversity (Atlas, 1984).

It is generally assumed that in the soil ecosystem, in which most of the niches or tasks are fulfilled, microbial diversity is high. However, the microbial community structure changes and diversity decreases due to environmental stress or disturbances (Atlas *et al.*, 1991). The change in community structure results in the emergence of dominant populations within disturbed communities, which have enhanced physiological tolerances and substrate utilisation capabilities (Atlas *et al.*, 1991; Wünsche *et al.*, 1995).

The soil microbial diversity is critical to the maintenance of good soil health, because microorganisms are involved in many important functions such as soil formation, toxin removal, and elemental cycles of carbon, nitrogen, phosphorus and others (Brock *et al.*, 1984; Fredrickson and Hagedorn, 1992; Leung *et al.*, 1994). However, environmental stresses, can alter microbial populations and therefore endanger soil health.

Environmental stresses caused by Total Petroleum Hydrocarbons (TPH), like other pollutants, can cause microbial community structure changes and a decrease in microbial diversity. However, as most of the low molecular weight hydrocarbons are volatile and most of the high molecular weight alkanes are biodegradable, the impact of hydrocarbons on microbial community structure can be mitigated by treating the contaminated soil. It is necessary to understand microbial diversity in soil as existing bioremediation technologies can be optimised if the current knowledge of both functional and genetic microbial diversity can be improved.

2. RESEARCH IN MICROBIAL DIVERSITY

In the field of microbial ecology, more specifically soil microbial diversity, slow progress has been made during the last two decades since the discovery that culture-dependent methods are only capable of enumerating 1% of the bacteria and that the microbial diversity in soil can be as high as 10^4 species per gram of soil (Alexander, 1977; Torsvik *et al.*, 1990; Klug and Tiedje, 1994; Borneman *et al.*, 1996). Indeed, (99.5 to 99.9%) of the soil bacteria observed using the fluorescence microscope cannot be isolated and cultured on laboratory media (Torsvik *et al.*, 1990; Aman *et al.*, 1995). They will therefore be excluded when phenotypic diversity is estimated.

Previous studies on microbial diversity involved the use of culture-dependent methods or the phenotypic characterisation of isolated strains (Kaneko *et al.*, 1977; Bell *et al.*, 1982; Margulis *et al.*, 1986). These culture-dependent methods included the numerical taxonomic studies, which use either profiles of cellular constituents (Mallory and Saylor, 1984; Lambert *et al.*, 1990), or phenotic characteristics (Kaneko *et al.*, 1977) of isolates to define operational taxonomic units as defined by Sneath and Sokal (1973). However, the analysis of microbial diversity using culture-dependent methods alone limits insight into the ecological relevance of microbial community structure due to the inability of most bacteria to grow on laboratory media. As a result of this limitation other techniques are needed to study microbial diversity.

It was largely due to the discovery of the high diversity of DNA of soil bacteria (Torsvik *et al.*, 1990) and the limitations of culture-dependent methods that emphasis on microbial diversity included the use of molecular techniques to estimate microbial diversity in soil. Molecular biological techniques offer new opportunities for the analysis of the structure

and species composition of microbial communities. In particular, sequence variation in rRNA has been exploited for inferring phylogenetic relationships among microorganisms (Woese, 1987) and for designing specific nucleotide probes for the detection of individual microbial taxa in natural habitats (Giovannoni *et al.*, 1990; Amann *et al.*, 1992). However both culture-dependent and independent approaches have limitations, which must be considered when investigating microbial diversity in ecosystems.

3. EVALUATION OF DIFFERENT METHODS OF ESTIMATING MICROBIAL DIVERSITY IN SOIL

3.1 Community Level Physiological Profiles (CLPP)

Biolog is a redox-based technique that was originally developed for classification of bacterial isolates based on the ability of the isolates to oxidise 95 different carbon sources (Bouchner, 1989). Garland and Mills (1991) adapted the method and used it to characterise the functional potential of microbial communities. The data from the carbon utilisation patterns have been used in two ways (i) to quantify differences among specific environmental samples, or (ii) to assess the functional diversity of microbes in ecosystem (Zak *et al.*, 1994). Using Biolog in such a way is called Biolog-generated community level physiological profiles (CLPP) and is thus used to estimate the ex-situ metabolic potential of members of the microbial community from a variety of environments.

Community level physiological profiles provide an indication of the metabolic diversity present in an environment with respect to the number of defined substrates that can be oxidised. Table 1 summarises the advantages and disadvantages of the CLPP. Recent studies have suggested that the faster growing species such as *Pseudomonas* produce the generated patterns and not the numerically dominant members of the microbial

communities (Garland, 1997; Konopka *et al*., 1998). Smalla *et al.* (1998), reported that carbon source utilisation profiles obtained with Biolog GN plates do not necessarily reflect the functional potential of the numerically dominant members of the microbial community used as the inoculum.

Table 1: Advantages and disadvantages of CLLP

Advantages	Disadvantages
• Easy to use & generates large data set • Sensitive to soil management treatment effects on soil communities • Inoculation of plates is less labour intensive and less costly in time and materials • *SUP provides data set amenable to multivariate statistical analysis that can quantify sample differences	• Have the same limitations of culture methods • Present functional rather than structural information about microbial communities • Not known if changes in substrate utilisation pattern represent changes in community composition

*SUP = substrate utilisation pattern

3.2 Phospholipid Fatty Acid Analysis (PLFA)

Characterisation of phospholipid fatty acid (PLFA) profiles and substrate utilisation pattern are increasingly common in studies of microbial communities. PLFA is a valuable approach, because it is a biochemical method that provides direct information about the structure of the active microbial community, free of the limitations inherent in culturing

microorganisms (Bossio and Scow, 1998). Table 2 summarises the advantages and disadvantages of PLFA. PLFA can be used to interpret effects at several levels. The most general level is to use the entire profile of fatty acids as a fingerprint. This allows a more detailed assessment of changes at the community level than bulk measures. With the appropriate statistics, the degree of similarity among communities or environmental effects on a community can be quantified and tested for significance. Major taxonomic groups such as eukarya vs. bacteria, can be differentiated at the next level. A higher level of resolution can be achieved by focusing on the specific fatty acids that act as biomarkers of certain functional groups or species of microorganisms (Vestal and White, 1989).

Table 2: Advantages and disadvantages of PLFA

Advantages	Disadvantages
• Can differentiate taxonomic groups • Present structural information about microbial diversity • Free of limitations inherent in culturing microorganisms	• Database for fingerprinting is derived from information on pure cultures • Biomarker not always universal to a specific group • Difficulty in interpreting specific PLFA peaks

3.3 Molecular Techniques

The identification of organisms has traditionally been achieved by cultivation techniques such as plate counting. However, because typically >99% of naturally occurring microorganisms are not cultivated by standard techniques (Amann *et al.*, 1995), alternative methods are needed to describe community constituents. Molecular methods

used to study microbial divesity includes Polymerase Chain Reraction- Denaturing Gradient Gel Electrophoresis (PCR-DGGE), Automated method of ribosomal intergenic spacer analysis (ARISA), amplified rDNA restriction analysis (ARDRA), reassociation kinetics and many other modified molecular methods (Torsvik *et al.*, 1990; Øvreås and Torsvik, 1998; Nusslein and Tiedje, 1999; Fisher and Triplett, 1999 and many others).

During the last decade, methods based on direct Polymerase Chain Reaction (PCR) amplification and analysis of ribosomal RNA genes were developed and allowed a more comprehensive analysis of microbial communities in comparison with cultivation based techniques. The amplified fragments of 16S or 18S rRNA genes and especially the analysis of these genes by temperature or denaturing gradient gel electrophoresis (DGGE) have been frequently used to examine the microbial diversity of environmental samples and to monitor changes in microbial communities (Curtis and Craine, 1998; Muyzer and Smalla, 1998; van Elsas *et al.*, 1998; Eichner *et al.*, 1999).

In a DGGE gel, the number, precise position, and intensity of the bands in a gel track give an estimate of the number and relative abundance of numerically dominant ribotypes in the sample. This approach allows a comparison of different microbial communities but not without specific problems. The advantages and disadvantages of methods based on analysis of ribosomal RNA genes are summarised in table 3.

Table 3: Advantages and disadvantages of methods based on rRNA genes

Advantages	Disadvantages
• Characterise high portion of community constituents • Identifies rRNA genes (rDNA) in DNA extracted directly from the environment • Permits the detection and phylogenetic identification of fastidious and as yet uncultured organisms	• Banding patterns of high diverse microbial communities are usually very complex when bacterial primers are used. • Only major populations are represented on the DGGE patterns • Less abundant species may not be detected by the method • Lack of rDNA sequences from close relatives in the databases results in un-identification of 'new' species

4. THE USE OF BIOLOGICAL ACTIVITIES TO MONITOR THE REMOVAL OF TPH FROM THE SOIL

The increasing concern about the cost of soil remediation has necessitated the need to explore not only cost effective technologies but also alternative monitoring tools. Conventional chemical analytical instruments like GC-MS usually monitor the progress of remediation of hydrocarbon-contaminated soil, which can be expensive (Maila and Cloete, 2002). Due to the cost associated with traditional monitoring tools, focus is now shifting towards using biological activities for monitoring of bioremediation of hydrocarbon-polluted soil. The use of bioindicators to evaluate hazardous chemical

waste sites provides a direct, inexpensive and integrated estimate of bioavailability and contaminant toxicity (Mueller *et al.*, 1991; Wang and Freemark, 1995; Maila and Cloete, 2002). Table 4 summarises the advantages and disadvantages of bioindicators.

Many promising approaches using bioindicators as monitoring instruments have been reported (Athey *et al.*, 1989; Siciliano *et al.*, 1997; Dorn *et al.*, 1998; Marwood *et al.*, 1998; Margesin *et al.*, 1999; Maila and Cloete, 2002). These include the use of enzymes, earthworm survival, microbial bioluminescence and seed germination.

Table 4: Advantages and disadvantages of using bioindicators as monitoing instruments

Advantages	Disadvantages
• Can detect both toxicity of parent compounds and toxic metabolites; • Readily available materials are required to do the test; • The test can be performed ex or *in-situ*; • The test period in most cases is short; • Uncomplicated methodology is used to assess the extent of pollution reduction.	• Inability to distinguish toxicity resulting from parent compound and metabolites; • Bioindicator response don't always correspond with contaminant concentration; • Different tests respond differently to individual toxicants; • Sensitivity depends on the toxicant and soil (i.e. the test can be sensitive to other factors of the soil).

The use of bioindicators to monitor the removal of hydrocarbons in soil should be considered when planning the monitoring programmes for soil remediation as it has the potential to reduce soil cleanup costs.

5. BIOTREATMENT OF HYDROCARBONS IN SOIL

The technologies, which involve the biological removal of hydrocarbons from contaminated soil, are today well established and many are applied commercially in large scale. During the 1970's, when environmental concerns associated with uncontrolled disposal became apparent, and environmental regulations were established and applied in North America and Europe (aimed at minimising the risk of air and groundwater contamination), landfarming gained popularity. This 'low tech' biological treatment method involves the controlled application and spread-out of a more or less defined organic bio-available waste on the soil surface and the incorporation of the waste into the upper soil zone (Genou *et al.*, 1994). In 1983 it was estimated that at least one-third of all United States refineries operated full-scale or pilot scale landfarmers (American Petroleum Institute, 1983). The technology has been widely used as it is simple and cost effective to implement compared to other treatments (Genou *et al.*, 1994; Balba *et al.*, 1998; Marijke and van Vlerken, 1998; Picado *et al.*, 2001).

Other biological treatment technologies include phytoremediation, bioventing, biopiles, composting and bioslurping. Phytoremediation uses the plants and associated microorganisms to degrade or immobilise contaminants in soil and groundwater (Figure 1d). The technology is used to decontaminate moderately contaminated soil, as highly contaminated soil can be toxic to plants (Lin and Mendelssohn, 1998). Bioventing integrates physical removal (soil venting) and enhanced aerobic biodegradation of hydrocarbons. The technology is recommended at contaminated sites with soil gas permeability greater than 0.1 Darcy (1 Darcy = $1 * 10^{-8}$ cm^2) and a radius of influence or radius of remediation of between 8 and 49 m (Johnson *et al.*, 1990; Long, 1992; Li, 1995).

Biopiles refer to the piling of the material to be biotreated by adding nutrients and air into piles or windrows usually to a height of 2-4 m. Biopiles may be static with installed aeration piping or they may be turned or mixed by special devices for this purpose (Figure 1c). Biopiles may be amended with bulking agents, usually with straw, sawdust, bark or wood chips or some other organic material. If organic material is added, the technology is termed composting. The different biological treatments technologies used for removing hydrocarbons in soil are shown in Figure 1.

Bioslurping combines the two remedial approaches of bioventing and vacuum enhanced free product recovery. Bioventing stimulates the aerobic bioremediation of hydrocarbon-contaminated soil. Vacuum enhanced free product recovery extracts the light nonaqueos phase liquids (LNAPLs) from capillary fringe and the water table.

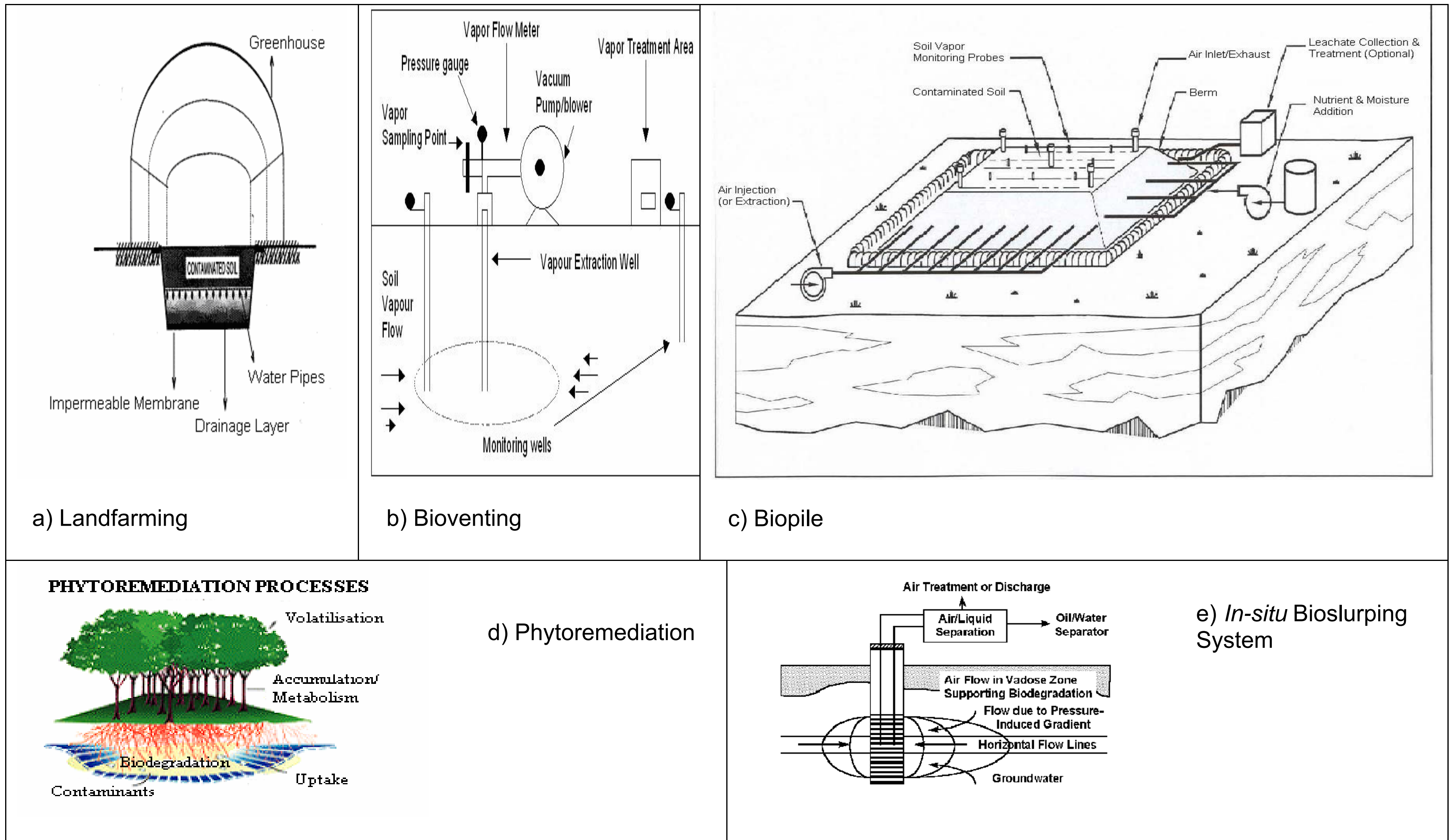

Figure 1. The biological treatment technologies used for treating organic compounds in the soil (Black, 1999; US EPA, 1994).

For efficient full-scale bioremediation of hydrocarbons, four important questions (adapted from Jørgsen *et al.*, 2000) that must be answered are:

- How low a concentration of the contaminant can one obtain (microbial activity and bioavailability)?
- What is the fate of the contaminant (volatilisation, biotransformation, build-up of microbial biomass, adsorption or incorporation to the bound residues)?
- How much time is needed to obtain the set goal (removal or degradation rate)?
- What are the costs?

Biological removal of hydrocarbons is cost effective compared to other remediation technologies and a large range of microbial genera have been reported to degrade hydrocarbons (Atlas, 1981; Rosenberg, 1992).

6. RESEARCH OBJECTIVES

Petroleum compounds are known environmental pollutants. They contaminate the soil through spillages of oil or diesel pipelines, maintenance activities at workshops, leakages from locomotives at diesel depots and underground storage tanks, spillages from oil trucks accidents, and many others.

The literature reveals that stresses exerted by hydrocarbons on soil microbial communities results in a decrease in microbial diversity and an emergence of the dominant populations, which have enhanced physiological tolerances and substrate utilisation patterns (Atlas *et al.*, 1991; Wünsche *et al.*, 1995). However, these studies only investigated the influence of hydrocarbons using mainly the topsoil. Information about the microbial diversity of different soil layers at a given site is lacking. Because oil

contamination normally penetrates deeper than the top layer, it is important to understand the distribution of degrading populations with soil depth and how the distribution patterns influence the efficiency of biodegradation. The biological removal of hydrocarbons in different soil layers is evaluated in Chapter 2.

Previously, microbial community structure was evaluated using culture dependent methods and most recently culture independent methods. In addition to the biological removal of hydrocarbons, Chapter 2 further evaluates microbial diversity in hydrocarbon-contaminated soil using both the community level physiological profiles (CLPP) and Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE). The objective was to assess the potential of the two methods to complement each other in assessing environmental disturbances brought by hydrocarbon contamination. As the contaminated soil is often poor in organic matter and can have a general low microbial activity, the hydrocarbon removal capacity and microbial diversity of different soil layers is also evaluated (Chapter 2).

The impact of environmental stresses on microbial diversity has been well documented (Atlas *et al.*, 1991). However, information about the importance of geographical origin of the soil samples and hydrocarbons is lacking. It is not known if the geographical origin of the samples or the pollutants is more important in determining functional or species diversity within bacterial communities. Chapter 4 assesses the importance of geographical origin of the soil contaminated by similar pollutants. In addition, the effect of different hydrocarbons on microbial diversity of similar soil environments was also investigated.

The heterotrophic bacteria and more specifically the hydrocarbon utilising bacteria play an important role in the restoration of hydrocarbon-contaminated soils. However, the distribution and heterotrophic diversity of these bacteria at different sites conducting similar anthropogenic activities is not known. Chapter 3 evaluates the heterotrophic diversity and microbial activities at different diesel depots contaminated by similar hydrocarbons.

Plant-mediated removal of organic pollutants has been reported with relative success (April and Sims, 1990; Lee and Banks, 1993; Walton *et al.*, 1994; Günther *et al.*, 1996; Reilly *et al.*, 1996). Laboratory and greenhouse experiments on plant-mediated dissipation of polycyclic aromatic hydrocarbons (PAHs) have concentrated mainly on the use of monoculture rhizoremediation of PAHs from the soil (April and Sims, 1990; Lee and Banks, 1993; Walton *et al.*, 1994; Günther *et al.*, 1996; Reilly *et al.*, 1996). The information about the effectiveness of multi-plant rhizoremediation of PAHs is lacking. The use of multi-plant microcosms or microcosms with mixed planted species has the potential to increase soil heterogeneity (Angers and Caron, 1998) and microbial diversity, which can improve the microbial competence of the soil bacteria for effective pollutant removal. Chapter 6 evaluates the effectiveness of multi-plant rhizoremediation compared to monoculture rhizoremediation of PAH contaminated soil.

Soil remediation is expensive. While biological removal of pollutants is often regarded as cost-effective compared to other non-biological technologies, there are inherent constraints to biological treatment technologies. Chapter 8 reviews the simplest of technology (landfarming) and how best to implement it (for effective pollutant removal) and the prevention of potential health and environmental problems.

The progress of soil remediation is usually monitored by conventional chemical analysis of pollutants using Gas Chromatography-Mass Spectrometer (GC-MS). However, these form of monitoring is not only expensive, it also requires a high degree of skill to use the analytical instruments. The use of bioindicators to monitor the removal of hydrocarbons is an alternative. In Chapter 6, we assess the potential of *Lepidium sativum* as a bioindicator of hydrocarbon removal from the soil. The review of potential bioindicators of hydrocarbon removal is also reviewed in Chapter 7.

Biological process cannot only be used for soil remediation only; they can also be used as potential bioindicators of biological removal of pollutants and can therefore act as monitoring 'instruments'.

The main aim of this work was to evaluate the microbial diversity of the different soil environments disturbed by Total Petroleum Hydrocarbons (TPHs) and the potential use of plants and microorganisms in monitoring and removing hydrocarbons from the soil. In addition, the potential of the culture-independent methods in complementing the culture-dependent methods when evaluating soil microbial diversity were also evaluated.

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University of Pretoria etd – Maila, M P (2005)

Chapter 2

EVALUATION OF MICROBIAL DIVERSITY OF DIFFERENT SOIL LAYERS AT A CONTAMINATED DIESEL SITE

A modified version of this text was accepted for publication as:

Mphekgo P. Maila, P Randima, K Drønen, K Surridge and Thomas E Cloete (2004) Evaluation Of Microbial Diversity Of Different Soil Layers At A Contaminated Diesel Site. International Biodegradation and Biodeterioration.

EVALUATION OF MICROBIAL DIVERSITY OF DIFFERENT SOIL LAYERS AT A CONTAMINATED DIESEL SITE

Abstract

In this study, we evaluated the hydrocarbon removal efficiency and microbial diversity of different soil layers. The soil layers with high counts of recoverable hydrocarbon degrading bacteria had the highest hydrocarbon removal rate compared to soil layers with low counts of hydrocarbon degrading bacteria. Removal efficiency was 48% in the topsoil compared to 31% and 11% in the 1.5 m and 1 m respectively. There was no significant difference between the Total Petroleum hydrocarbon (TPH) removal in the nutrient amended treatments and the controls at 1 m and 1.5 m soil layers. The respiration rate reflected the difference in the number of bacteria in each soil layer and the availability of nutrients. The high O_2 consumption rate corresponded positively with the high TPH removal rate. Analysis of the microbial diversity in the different soil layers using functional diversity (community level physiological profile using Biolog) and genetic diversity using Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) of 16 SrDNA revealed differences in substrate utilisation patterns and DGGE profiles of 16 SrDNA fragments respectively. The microbial diversity as revealed by DNA fragments was reduced in the highly contaminated soil layer (1.5 m) compared to the topsoil and the soil layer at 1 m.

Introduction

The effect of hydrocarbon contamination on soil microbial communities has been studied (Atlas *et al.*, 1991; Wünsche *et al.*, 1995; Lindstrom *et al.*, 1999; MacNaughton *et al.*, 1999; Stephen *et al.*, 1999; Juck *et al.*, 2000; Bundy *et al.*, 2002). However, these

studies only investigated the influence of hydrocarbons using mainly the topsoil. Information about the microbial diversity of different soil layers at a given site is lacking. Because oil contamination normally penetrates deeper than the top layer, it is important to understand the distribution of degrading populations with soil depth and how the distribution patterns influence the efficiency of biodegradation.

The subsurface soil environment, though devoid of sufficient nutrients, oxygen and other factors, harbors an array of soil microorganisms that plays an important role in decomposition and the recycling of nutrients (Krumholz, 1998). It is widely presumed that the number of heterotrophic bacteria changes with increasing depth. This can be attributed to spatial and resources factors which can influence the microbial diversity of the soil (Zhou *et al.*, 2002). Shallow subsurface micro-flora appears to be predominantly prokaryotic, appears to be specially adapted for growth and survival in nutrient poor conditions, includes strains that can function throughout a wide range of nutrient concentrations and may sometimes exert significant effect on groundwater chemistry (Ghiorse and Balkwill, 1983; Balkwill and Ghiorse, 1985; Bone and Balkwill, 1988; Ghiorse and Wilson, 1988; Balkwill *et al.*, 1989).

The availability of hydrocarbons in the vadose zone can alter the diversity of the heterotrophic community due to an increase in the carbon substrate. According to Atlas (1981), Leahy & Colwell (1990), the number of hydrocarbon bacteria and their relative abundance in the bacterial communities increases significantly in the presence of readily available hydrocarbons. Also the changes in hydrocarbon content in soil results in characteristic shifts of the substrate utilisation patterns by the microorganisms and that the altered pattern of substrate utilisation corresponds with similar changes in abundance of hydrocarbons in the soils (Wünsche *et al.*, 1995). This is not surprising,

and in accordance with the theories about gene accumulation and selection pressures, we can predict lower abundance of hydrocarbon degraders with depth as selection pressure and growth conditions in general lowers with depth.

In this study, we investigated the hydrocarbon removal capacity and the microbial diversity of different soil layers after diesel contamination. The capacity of the soil layers in removing hydrocarbons was evaluated (using simple microbial assays), while microbial diversity was evaluated using functional diversity (community level physiological profiles using Biolog micro plates) and genetic diversity (PCR-Denaturing Gradient Gel Electrophoresis of 16SrRNA).

Materials and Methods

Soil: The contaminated soil layers were collected in sterile bags from a diesel-contaminated site at Coalsbrook, in the Free State Province, South Africa. The soil collected was a loam soil with depth to ground water, 2m. The organic carbon of the uncontaminated topsoil was 0.9%. The electron acceptors were not measured. The soil layers were collected one month after contamination by a leaking diesel pipeline. Direct push drilling to 2 m was used to sample the contaminated soil layers at a depth of 1 m (CS1m) and 1.5 m (CS1.5m). The contaminated topsoil layer (CTS) was collected within 10 cm of the soil surface. Uncontaminated topsoil (UCTS) was also collected from the same site. Samples were kept at 4°C until analysis, which was completed within 24 h.

Microbiological analysis: 100 mℓ of 0.2% tetra-sodium pyrophosphate was added to 250 mℓ Erlenmeyer flask containing 10 g of the soil from each sample. The flasks were placed on a shaker (140 rpm) for 45 min. The mixtures in the flasks were allowed to settle for 5 min after mixing. Serial dilutions (with saline solution) were done using the samples before inoculating both the agar plate and the Biolog GN plates. The Total

Recoverable Heterotrophs (TRHs) were enumerated by spread plate technique using nutrient agar (Biolab Diagnostics). The hydrocarbon-degrading bacteria were isolated as described by Margesin and Schinner (1999a) with diesel being the only source of carbon and energy. Both agar plates were incubated at 28°C and counted after 24 h and 7 d respectively. The bacterial counts were not corrected for the dry mass of the soil. Bacterial counts were done in triplicates. Analysis of Variance (ANOVA) was used to determine the difference between the treatments.

Carbon source utilization pattern determination: Sample dilutions were done as described above. Heterotrophic plate count data were used to adjust the samples to similar cell density for Gram Negative Biolog plate inoculation. 100 μℓ of the each sample was added to each well. The Biolog plates were read at 600 nm using Bio-Tek Elx800 microreader (Bio-Tek Instruments Inc) before incubation at 28°C. The plates were further read after 24, 48 and 72 h. Readings of the micro plates were made in triplicate. Statistical analyses were done using STATISTICA for Windows release 5.1.

Respiration rate determination: The biological activity of the different samples was evaluated by monitoring oxygen consumption using a Micro-Oxymax Respirometer (Columbus Instruments). 100 g of each soil layer was added to a 250 mℓ bottle containing 10 mℓ nutrients (mineral salt medium) with no carbon and energy source. The nutrients were added to the soil layers to stimulate bioremediation in hydrocarbon contaminated layers. The treatments in which no nutrients were added to the soil, served as control. The O_2 consumption was measured over 5 d. The composition of the nutrient solution was (g ℓ^{-1} in the medium): 10 g ℓ^{-1} Na_2HPO_4, 10 g ℓ^{-1} KH_2PO_4, 2.5 g ℓ^{-1} $(NH_4)_2SO_4$, 0.4 g ℓ^{-1} $MgSO_4$, 0.05 g ℓ^{-1} $CaCl_2.2H_2O$, 0.0086 g ℓ^{-1} EDTA, 0.01 g ℓ^{-1}

$FeSO_4.7H_2O$, 0.004 g ℓ^{-1} $ZnSO_4.7H_2O$, 0.01 g ℓ^{-1} $MnSO_4.H_2O$, 0.0015 g ℓ^{-1} $CuSO_4.5H_2O$, 0.0008 g ℓ^{-1} $Co(NO_3)_2$ .6H_2O, 0.0001 g ℓ^{-1} $(NH_4)_6Mo_7O_{24}.4H_2O$.

Chemical Analysis: The contaminated soil layers were analysed using the Total Petroleum Hydrocarbons (TPH) method described in Margesin *et al.* (1999). 10 g of the contaminated soil were used for the analysis. The analyses were done in triplicates.

DNA extraction and purification: Total DNA was isolated from the soil using the Bio101 extraction kit (Bio Inc.)

PCR conditions: A 1 $\mu\ell$ volume of the extracted DNA was amplified by PCR with a 9600 thermal cycler (Perkin-Elmer/Cetus). The PCR mixture used contained 100 μm each primer, 100 mM each deoxy-nucleoside triphosphate, 5 $\mu\ell$ of 10x PCR buffer, 0.25 $\mu\ell$ (5 U/$\mu\ell$) of hot start polymerase, (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ), 2.5 $\mu\ell$ of 2% Bovine Serum Albumin, 40 $\mu\ell$ sterile water to a final volume of 50 $\mu\ell$. The 16S rRNA genes from soil microbial communities were amplified by PCR using the primers, pA8f-GC (5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3') and KPRUN518r (5'ATTACCGCGGCTGCTGG-3'). The primers were found to be useful for 16S rRNA gene amplification in ecological and systematic studies (Øvreås and Torsvik, 1998). Samples were amplified as follows: 95°C for 10 min, 30 cycles of denaturation (1 min at 94°C), annealing (30 sec at 51°C), and extension (1 min at 72°C) and a final extension at 72°C for 10 min. Amplified DNA was examined by horizontal electrophoresis in 1% agarose with 5 $\mu\ell$ aliquots of PCR product.

DGGE: DGGE was performed using Hoefer SE600 vertical dual cooler system (Hoefer Scientific, San Francisco, CA). PCR samples were loaded onto 8% (wt/vol) polyacrilamide gels in 0.5x TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na-EDTA, pH 7.4). The 8% (wt/vol) polyacrylamide gels (bisacrylamide gel stock solution, 37.55:1; BioRad Laboratories, Inc) were prepared with a 20 to 55% gradient of denaturant (urea and formamide) (and allowed to polymerise). The electrophoresis was run at 60°C, first for 10 min at 20 V, and subsequently for overnight at 70 V. After electrophoresis, the gels were stained for 15 min in SYBR Green I nucleic acid gel stain rinsed in distilled water for 1 min and photographed with a Polaroid MP4 Land camera. The gels were analysed using a software program developed by Svein Norland (Department of Microbiology, University of Bergen), where presence/absence of bands was recorded. Clustering was based on the simple matching algorithm, while the dendrogram was drawn applying the group average method.

Diversity Indices

The Shannon index, H' (Shannon, 1948), was calculated (log 2) on the basis of biotypes defined in the cluster analysis on data retrieved from PCR-DGGE. The equitability J (Pielou, 1966) index was also calculated (Watve and Gangal, 1996).

Results

Microbiological analysis

The number of Total Recoverable Heterotrophs (TRHs) decreased with soil depth (Figure 1). Similar results were obtained for the hydrocarbon-degrading bacteria. There was not much difference in the number of TRHs and hydrocarbon-degrading bacteria in the 1 m and 1.5 m samples.

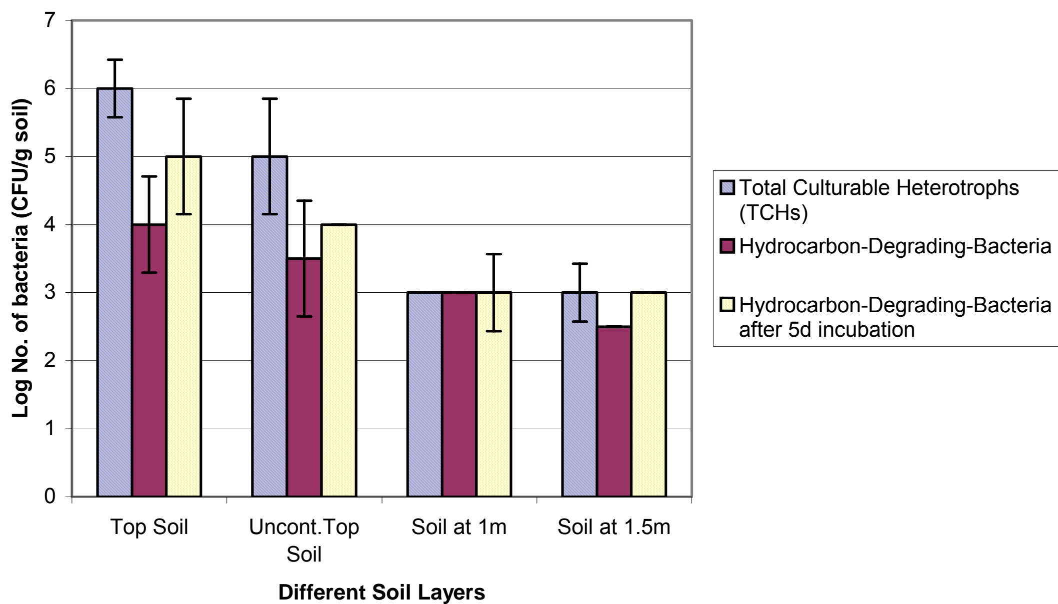


Figure 1. Bacterial counts of different soil layers at Coalsbrook diesel contaminated site

The Total Petroleum Hydrocarbon (TPH) concentration was highest in the soil layer at 1.5 m, followed by the topsoil and then at 1 m (Figure 2). However, TPH removal during the period of incubation reflected the difference in the number of bacteria in the samples as it was highest in the topsoil compared to the other soil layers (1 m and 1.5 m).

There was no significant difference ($p<0.01$) between the TPH removal in the nutrient amended treatments and the controls (at 1 m and 1.5 m soil layers) Figure 2. Removal efficiency was 48% in the topsoil compared to 31% and 11% in the 1.5 m and 1 m respectively. Soil layers with the high recoverable hydrocarbon degrading bacteria had the highest removal capacity compared to soil layers with low counts of hydrocarbon degrading bacteria.

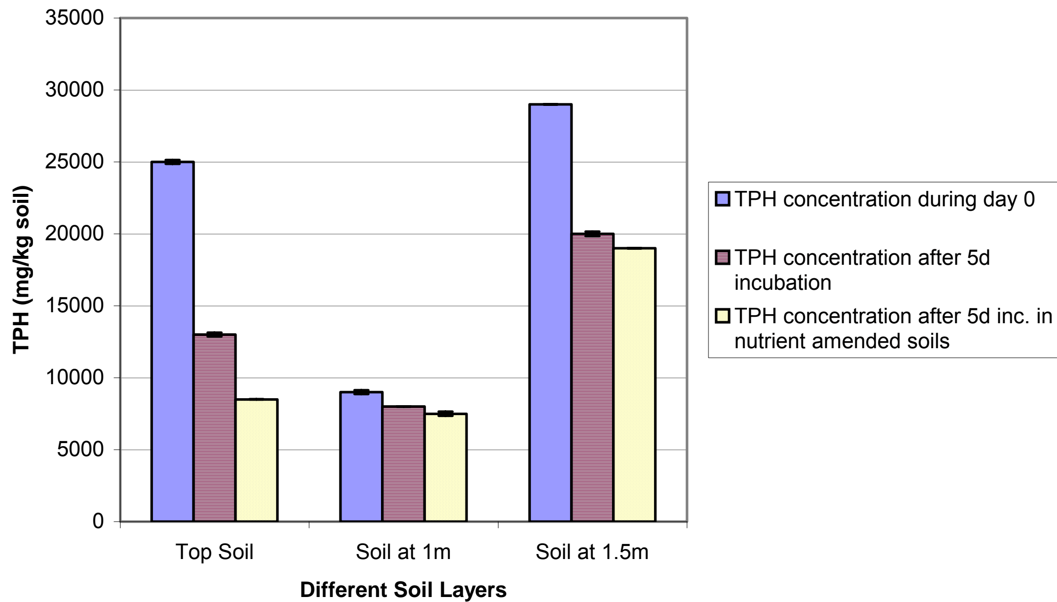
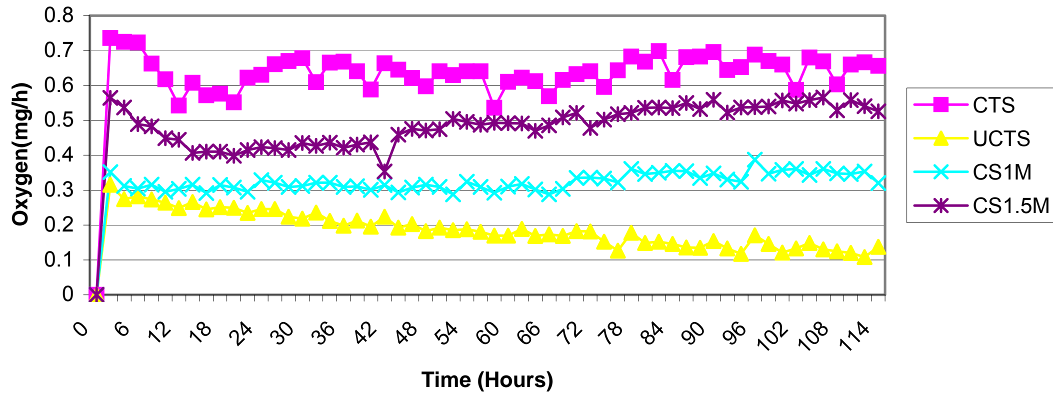


Figure 2. The concentration of hydrocarbons in Coalsbrook's different soil layers

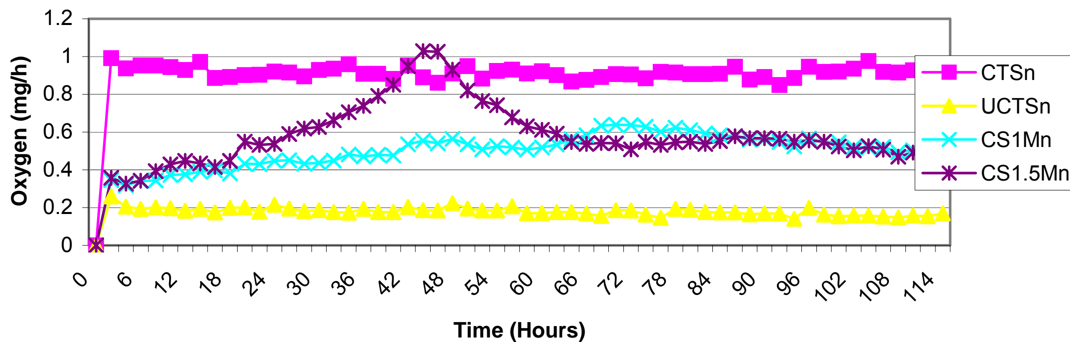
Respiration rate determination

The respiration rate reflected the difference in the number of bacteria in each soil layer and the availability of nutrients. The O_2 consumption rate was highest in the contaminated topsoil (CTS) compared to other soil layers (Figure 3a). Oxygen consumption rate was also highest in the nutrient amended treatments compared to the 'controls' (Figure 3a and b). The respiration rate in the uncontaminated topsoil was low

compared to the contaminated soil layers at the top, 1.5 and 1 m. The respiration rate corresponded positively with the high TPH removal rate (Figure 2).



a)



b)

Figure 3. Oxygen consumption by microorganisms in different diesel contaminated soil layers. a) Soil layers with no nutrients, b) Soil layers with nutrients. CTSn-Contaminated topsoil with nutrients, CTS-Contaminated topsoil with no nutrients, UCTS-Uncontaminated topsoil, UCTSn-Uncontaminated topsoil with nutrients, CS1Mn-Contaminated soil at 1m depth with nutrients, CS1 m-Contaminated soil at 1 m with no nutrients, CS1.5Mn-Contaminated soil at 1.5 m depth with nutrients, CS1.5 Mn-Contaminated soil at 1.5 m with no nutrients.

Biolog Analysis

The Principle Component Analysis (PCA) of the colour response data of the soil layers revealed different substrate utilisation pattern (Figure 4).

PCA was performed to characterize the associations amongst samples, taking into account the absorbance values for all 96-response wells at the different incubation times. Two principal factors were isolated from the individual UCTS, CTS, CS 1 m and CS 1.5 m patterns, which explained 58% of the variation. This low percentage of variation, explained by the two factors, can be a result of the few samples used in the analysis. The use of more samples would probably improve the variation explained by the two factors. For the four samples, factor one was related to the absorbance values for the wells, while factor 2 was related to the incubation time.

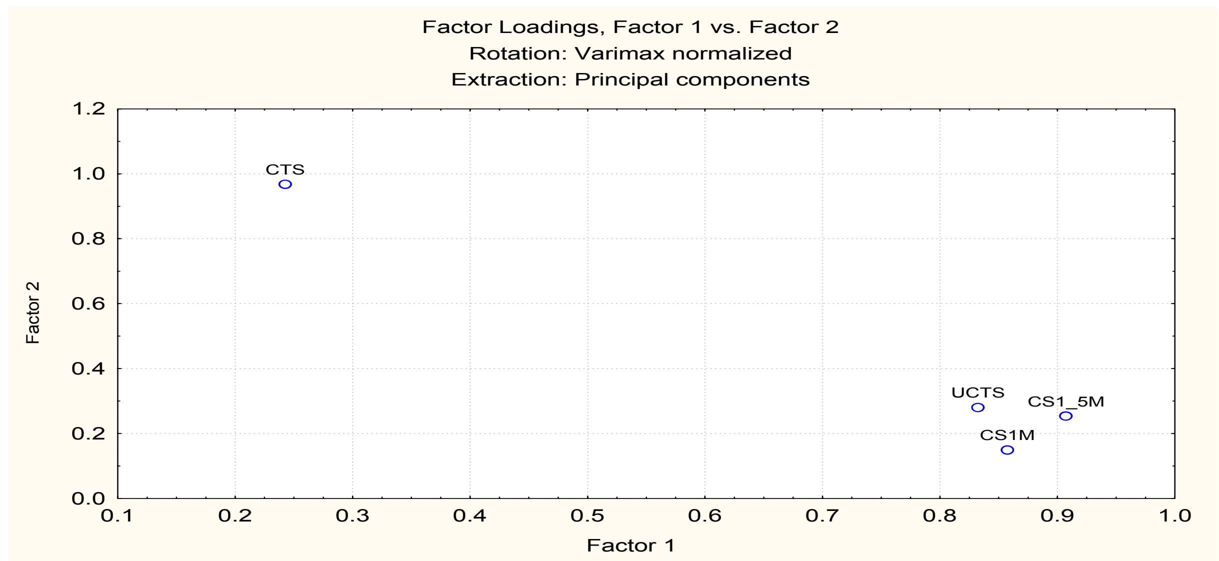


Figure 4. The substrate utilization pattern of different soil layers as depicted by Principle Component Analysis (PCA). CTS-Contaminated topsoil, UCTS-Uncontaminated topsoil, CS1 M-Contaminated soil at 1 m, CS1.5M-Contaminated soil at 1.5 m.

The relationship between the substrate utilization patterns was further analysed using hierarchical clustering. In a dendrogram (Figure 5), the results of cluster analysis also showed that the metabolic activities of CS1m were 'closely' related to CS1.5m than to UCTS and CTS. The uncontaminated topsoil (UCTS) was different from the hydrocarbon-contaminated topsoil (CTS).

Both the dendrogram and the PCA illustrate that the substrate utilization pattern of the microbial communities in different soil layers are different.

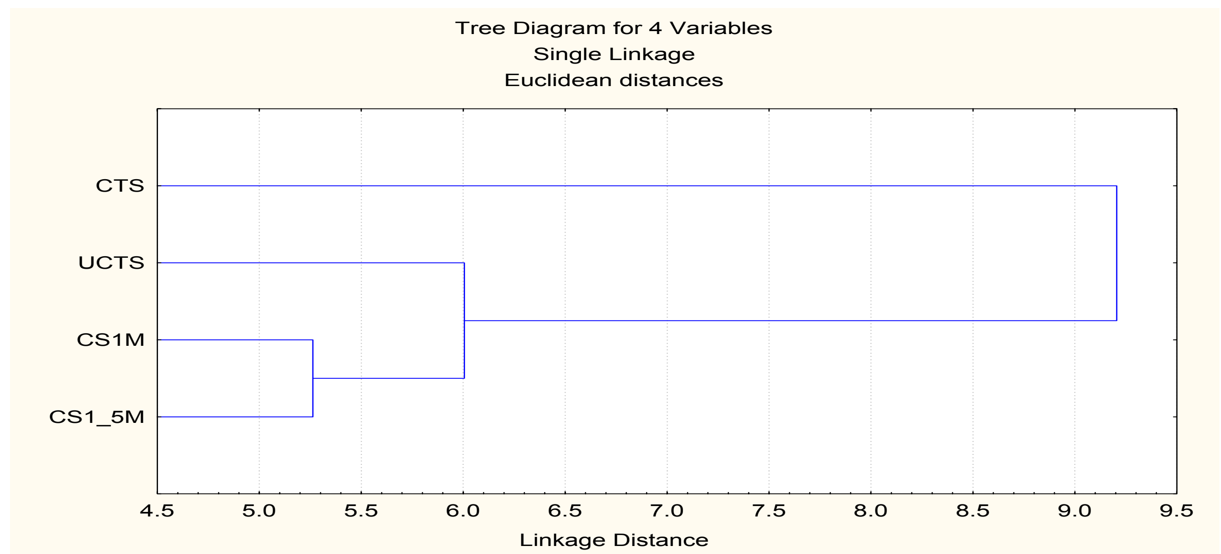
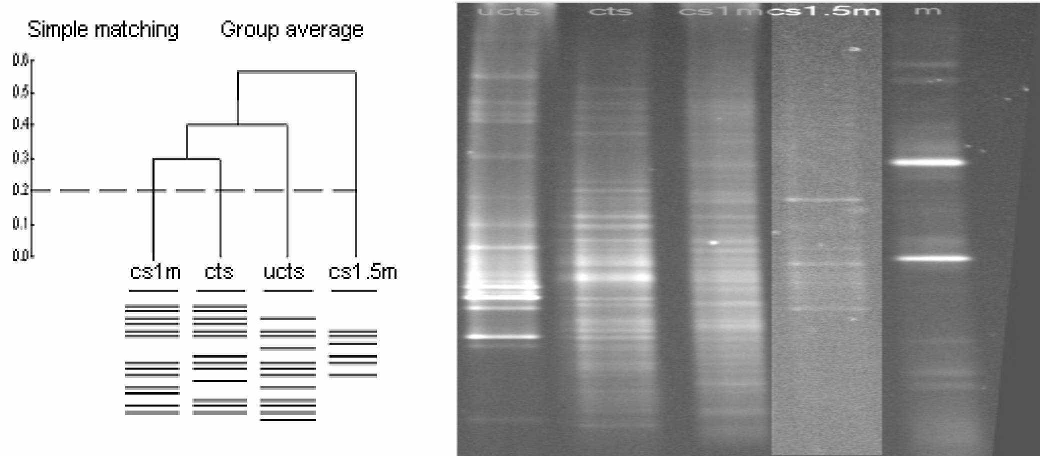


Figure 5: Cluster Analysis of the different soil layers. CTS-Contaminated topsoil, UCTS-Uncontaminated topsoil, CS1 M-Contaminated soil at 1 m, CS1.5M-Contaminated soil at 1.5 m.

PCR-DGGE

DGGE profiles of amplified 16S rDNA fragments from DNA extracted from the soil bacterial fractions revealed differences in the DNA fingerprint of the different soil layers.

The profiles of the contaminated and uncontaminated samples (5 and 6) were different (Figure 6) while the profile of the soil layer at 1.5m was difficult to resolve.



a) b)

Figure 6.(a) Cluster analysis of microbial communities at different soil layers and (b) DGGE fingerprints. UCTS - Uncontaminated topsoil, CTS - Contaminated topsoil, CS1m - Contaminated soil layer at 1 m, CS1.5m - Contaminated soil layer at 1.5 m. M - Marker. Lines beneath the numbers represent the band detected.

Table 1: Diversity Indices

*Soil Type	Shannon Index (H')	Equitability Index (J')
UCTS	2.120667	0.826787
CTS	2.52069	0.909147
CS1m	1.981935	0.731868
CS1.5m	1.84471	0.947993

*Abbreviations as in Figure 4.

Cluster analysis using a dendrogram revealed that the soil at 1 m was closely related to the contaminated topsoil than to uncontaminated topsoil. The Shannon diversity index

(H') revealed high diversity in the topsoil compared to other soil layers (table 1). The equitability (J) of all populations ranged from 0.73 to 0.95.

Discussion and Conclusion

In this study the number of TRHs and hydrocarbon-degrading bacteria decreased with soil depth. This can be attributed to nutrient and oxygen limitations to the biota of the soil subsurface. According to Zhou et al. (2002), spatial and resources factors influence microbial diversity in soil. Similar results were obtained with the hydrocarbon degrading bacteria after incubation for five days. However, there was little difference in the number of TRHs and hydrocarbon degrading bacteria in the 1-m and 1.5-m samples, possibly owing largely to similarities of nutrient levels in the soil layers.

The TPH concentration was greatest in the soil layer at 1.5 m, followed by the topsoil and the soil at 1 m. The high concentration of hydrocarbons at a depth of 1.5 m indicates the potential mobility of the pollutants to deeper soil layers. The removal of TPH reflected the number of bacteria in each soil layer. Removal efficiency was 48% in the topsoil compared with 31% and 11% in the soil at 1.5m and 1m, respectively. There was no significant difference between the TPH removal in the nutrient amended treatments and the controls (at 1 m and 1.5 m soil layers). The depth to ground water was 2 m and owing to the proximity of the 1.5 m layer to water, microbial activity could be higher at 1.5 m than at 1 m.

The respiration rate corresponded positively with TPH removal rate and reflected the difference in the number of bacteria in each soil layer and also the availability of nutrients. The nutrients were added to the contaminated soil to stimulate the biological removal of hydrocarbons (Churchill et al., 1995; Braddock et al*.,* 1997; Seklemova, 2001).

Since it is well known that different soil layers harbour different numbers of bacteria, it was expected that the soil layers would have a different pattern of substrate utilisation, especially as both PCA and cluster analysis revealed differences between the soil layers.

The relationship between the substrate utilisation patterns was further analysed using hierarchical clustering. In a dendrogram, the results of cluster analysis showed that cs1 m was more 'closely' related to cs1.5 m than to ucts and cts. This can be attributed to the similarities in nutritional (organic matter, limiting nutrients) and environmental conditions (pH, temperature) of the two soil layers. However, the closeness of the 1-m and 1.5-m samples was not evident when clustering the DGGE profiles of the 16S rDNA fragments.

The difference between ucts and cts evident in both substrate utilisation patterns and DGGE (Figures 4 and 6) can be attributed to the changes in the composition of microbial populations brought about by changes in hydrocarbon content (Atlas *et al.*, 1991; Wünsche *et al.*, 1995).

The Shannon diversity index revealed high diversity in the topsoil compared with other soil layers. This can be attributed to the relatively high amount of nutrients (organic matter and possibly limiting nutrients) in the topsoil compared with the other soil layers. The diversity indices corroborated the results of functional diversity. High removal capacity of the topsoil can be attributed to the different number of bacteria that are capable of degrading the hydrocarbons.

The DGGE profiles of amplified 16S rDNA fragments for the 1.5-m layer were difficult to resolve, however, the bands detected by the software program revealed few fragments relative to the other soil layers. The use of Archae primers instead of bacterial primers may enhance the chance of revealing the differences between the DGGE profiles of the

different soil layers (Øvreås and Torsvik, 1998). The data on both the functional and genetic diversity revealed that the two approaches of studying microbial diversity can complement each other, as the community level physiological profiles (CLPP) measures the metabolic activities of different environmental samples while the PCR-DGGE provides information about the microbial structure. The data on CLPP and PCR-DGGE suggest that in combining the functional and genetic approaches of assessing microbial diversity there is the potential to provide a clear picture about the abundance of a variety of species in an ecosystem. Further studies are required in order to understand the effect of not only other pollutants but also the influence of soil components (pore volume, level of adsorbents and other environmental factors) on the microbial diversity of different soil layers in both 'shallow' and deep aquifers. The results suggest that in hydrocarbon-contaminated soil biological removal of hydrocarbons differs with soil layer, and also that the microbial diversity (as measured by CLPP and PCR-DGGE) varies with depth. However, as the study was conducted using soil that was contaminated for a 'relatively short period', further studies are required using soil that has been contaminated for longer period as this can yield further information about the structure and function of a stable microbial community in hydrocarbon contaminated soil. It was also not clear in the current study, if the Biolog test was reflecting the real functionality of a fast changing microbial community. This requires further evaluation by comparing the 'aged' and 'non-aged' hydrocarbon contaminated sites. It will also be important to investigate the influences of different soil types, groundwater level, total organic carbon and the electron acceptors on microbial diversity of different soil layers. Further studies of microbial diversity of contaminated soil layers should also include the study on microbial diversity of uncontaminated soil layers. Information about metabolic activities of different soil layers is critical when assessing the footprints of degradation processes during monitored natural attenuation (Smets et al., 2002).

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Chapter 3

EVALUATION OF MICROBIAL COMMUNITIES COLONIZING STONE BALLASTS AT DIESEL DEPOTS

A modified version of this text has been accepted for publication as:

Mphekgo P. Maila, Thomas E. Cloete (2004) Evaluation of Microbial Communities Colonizing Stone Ballasts at Diesel Depots. Journal of Water, Air and Soil Pollution

EVALUATION OF MICROBIAL COMMUNITIES COLONIZING STONE BALLASTS AT DIESEL DEPOTS

Abstract

In this study, we evaluated the heterotrophic microbial communities colonising stone ballasts at diesel depots. The number of bacteria (both total culturable heterotrophic bacteria and hydrocarbon-degrading bacteria) was proportional to the level of hydrocarbon contamination. However, there was no significant difference ($p<0.01$) in the level of total culturable heterotrophs (TCHs) and the hydrocarbon degrading bacteria. Addition of nutrients to the ballast stimulated the biological activity and possibly the removal of hydrocarbons. However, this was only evident in the highly contaminated stone ballasts samples. The biological activity was evaluated using CO_2 production. The production of CO_2 was higher in nutrient amended treatments in which high numbers of TCHs were present. Characterisation of heterotrophic communities using Biolog revealed differences in the microbial metabolic profiles for the different sites. The results suggest that the heterotrophic microbial communities at different diesel depots are different.

Introduction

Diesel oil is a complex mixture of normal, branched and cyclic alkanes, and aromatic compounds obtained from the middle–distillate, gas-oil fraction during petroleum separation (WHO, 1996). These hydrocarbons have the potential to cause considerable damage not only to the soil but also to water intakes or groundwater reservoirs due to the mobility of some of the hydrocarbon compounds. At diesel depots, the contamination of stone ballasts which are used as support structures for railroads also pose a

significant risk to both the ground water resources and to humans as they can be exposed to both volatile and non-volatile hydrocarbons.

The heterotrophic bacteria, or more specifically the hydrocarbon degrading bacteria, can play an important role in mitigating these environmental problems as they can limit the mobility of petroleum contaminants by degrading the pollutants to avoid, for example, groundwater contamination. According to Atlas (1981), Leahy and Colwell (1990), the number of hydrocarbon degrading bacteria and their relative abundance in the bacterial communities increases significantly in the presence of readily available hydrocarbons. Hydrocarbon utilizing bacteria are ubiquitously distributed in natural environments and their proportions in the heterotrophic bacterial soil communities ranges from 0.13% to 50% (Jones *et al.*, 1970; Pinholt *et al.*, 1979).

Most knowledge into the effect of hydrocarbons on microbial diversity has been generated using soil as a medium (Urzí *et al.*, 1999; Bundy *et al.*, 2002). The information about microbial diversity of hydrocarbons polluted rocky surfaces is not well documented. There is a need to study heterotrophic diversity on rocky surfaces as this information can be useful in the cleanup of hydrocarbon contaminated stone ballasts. Stone surfaces tend to accumulate inorganic and organic substances from the surrounding environment, most of which can serve as nutrients for many microorganisms (Urzí *et al.*, 1999).

At diesel depots, the ballasts are continuously contaminated with hydrocarbons from the parked locomotives and general maintenance work. These rocky surfaces used on railroads are usually of the quartzite, fine-grained basic plutonic rock and other rock types. The loading rate of the hydrocarbons on the ballast varies depending on the traffic load at the depots and therefore the degree of contamination of the ballasts reflects the

intensity of the activities at the depot. The concentration or thickness of hydrocarbons on the ballast that encourages the attachment or colonization of the heterotrophs and the hydrocarbon degrading bacteria will vary from site to site. The information about heterotrophic diversity at contaminated rocky surfaces such as the hydrocarbon-contaminated ballasts can be useful for bioremediation purposes.

In this study we investigated the heterotrophic diversity of polluted stone ballast at different diesel depots using culture dependant methods, community level physiological profiles and respiration. The aim of the experiment was to test the hypothesis that hydrocarbons deposition on the stone ballast at different diesel depots selects for similar microbial communities dominated by hydrocarbon degrading bacteria.

Materials and Methods

Stone Ballast: The contaminated stone ballasts were collected in sterile bags from Koedoespoort (Kc), Sentrarand (SRc), Pyramid (Pc) and Springs (SPc) diesel depots, in Gauteng province, South Africa. The uncontaminated stone ballasts were collected from the Pyramid (Pu) and Sentrarand (SRu) diesel depots. The ballasts were ground using Keegor jaw crusher (Dickie and Stockler Pty, SA) under sterile conditions to reduce the size of the stones before analysis. The diameter of the ground stone ballasts used in the experiment averaged 1cm. The ballasts had size distribution when collected and the proportion of exterior contaminated stones after grinding was much higher than the interior uncontaminated stones. The exterior contaminated stones averaged 1cm in diameter while the interior uncontaminated stones averaged less than 0.5cm. The ground ballasts averaging 1cm in diameter were used in the experiment. The stone used as ballasts at diesel depots were prepared using fine-grained basic plutonic rocks. Samples were kept at 4°C until analysis. All analyses were done within 24 hours.

Enumeration of Microorganisms: 250 mℓ of 0.2% tetrasodium pyrophosphate was added to 500 mℓ Erlemeyer flask containing 25 g of the grinded stone ballasts from each diesel depots. The ballasts were grinded using Keegor jaw crusher (Dickie and Stockler Pty, SA) under sterile conditions to reduce the size of the stones before adding them into the flasks. The diameter of the grinded stone ballast averaged 1cm. The flasks were placed on a shaker (140 rpm) for 45 min. The mixtures in the flasks were allowed to settle for 5 min after mixing. Serial dilutions (with saline solution) were done using the samples before inoculating both the agar plates and the Biolog GN plates. The total culturable heterotrophs (TCHs) were enumerated by spread plate technique using nutrient agar (Biolab Diagnostics). The hydrocarbon-degrading bacteria were isolated using the mineral salt medium in which the filter-sterilised diesel 3% (v/v) was used as the sole carbon and energy source. The composition of the mineral salt medium is shown in table 1. Bacteriological agar (15g/ℓ, Biolab Diagnostics) was autoclaved (121°C, 15 min) before adding the sterile solutions (table 1). Cycloheximide (200 mg/ℓ) was added in both media to inhibit fungal growth. Both plates were incubated at 28°C and counted after 24 h and 7 d respectively. Bacterial counts were done in triplicate (three true replicates stones were ground to prepare the dilution series). ANOVA (Analysis of Variance) was used to determine the difference between the TCHs and hydrocarbon degrading bacteria.

Table 1: Composition of Mineral Salt Medium

Trace mineral solutions	Compounds	g/ℓ stock	Concentration in media g/ℓ
A	Na_2HPO_4 KH_2PO_4	141.96 136.09	10 10
B	$(NH_4)_2SO_4$	100	2.5
C	$MgSO_4$	19.7	0.4
D	$CaCl_2.2H_2O$	1.15	0.05
E	EDTA $FeSO_4.7H_2O$ $ZnSO_4.7H_2O$ $MnSO_4.H_2O$ $CuSO_4.5H_2O$ $Co(NO_3)_2.6H_2O$ $(NH_4)_6Mo_7O_{24}.4H_2O$	0.64 0.55 0.23 0.34 0.075 0.047 0.025	0.0086 0.01 0.004 0.01 0.0015 0.0008 0.0001

Carbon source utilisation pattern determination: Sample dilutions were done as described above. Dilutions yielding similar bacterial counts in the samples from SRc, Kc and SPc were used for carbon utilization pattern analysis. 100 μℓ of the each sample was added to each well (Biolog GN microplates). The optical density (OD_{600}) of the plates was measured using Bio-Tek Elx800 microreader (Bio-Tek Instruments Inc) at time 0 and after 24, 48, and 72 h of incubation at 28°C. Three Biolog plates were used per treatment. Statistical analyses were done using STATISTICA for Windows release 5.1.

Respiration rate determination: The biological activity of the contaminated ballasts was evaluated by monitoring carbon dioxide production using a Micro-Oxymax Respirometer (Columbus Instruments). 20 g of the grinded contaminated stone ballasts were added to a 250 mℓ bottle containing the sterile 150 mℓ nutrients (mineral salt medium) with no carbon and energy source. The nutrients were added to the contaminated ballast to stimulate bioremediation. The treatments in which sterile deionised water was added to the ballast, instead of nutrients, served as controls. The

CO_2 production was measured over 46 h. The composition of the different solutions used to prepare the nutrient solution is shown in table 1.

Chemical Analysis: The contaminated stone ballasts were ground as described above and Total Petroleum Hydrocarbons (TPH) was analysed using the EPA 418.1 method (US Environmental Protection Agency, 1979). 25 g of the ground ballasts were used for the analysis. After extraction of the ballasts with 1,1,2-trichloro-trifluoro-ethane, the hydrocarbon content was quantified by infrared spectroscopy. The analyses were done in triplicate.

Results

Microbiological and chemical analysis

The number of bacteria (both total culturable heterotrophs and hydrocarbon-degrading bacteria) on the polluted ballast was proportional to the level of hydrocarbon contamination (Figure 1). The number of total culturable heterotrophs (TCHs) on the polluted stone ballast was highest in the Koedoespoort ballast (Kc), followed by the Spring ballast (SPc), Sentrarand (SRc) and the Pyramid (Pc) ballast. Similar bacterial counts for TCH and hydrocarbon-degrading bacteria were counted at both Pc and SRc. No hydrocarbon-degrading bacteria and TCHs were isolated in the uncontaminated Pyramid stone ballast (Pu) and uncontaminated Sentrarand ballast (SRu). The hydrocarbon concentration at the diesel depots was highest at Koedoespoort depots (Kc) followed by SPc, SRc and the Pyramid diesel depots. The number of bacteria (both total culturable heterotrophic bacteria and hydrocarbon-degrading bacteria) was proportional to the level of hydrocarbon contamination. Kc, the diesel depot sample with the highest TPH concentration had the highest number of bacteria while Pc, the diesel

depot sample with the lowest concentration of TPH had the lowest number of culturable heterotrophic bacteria (Figure 1).

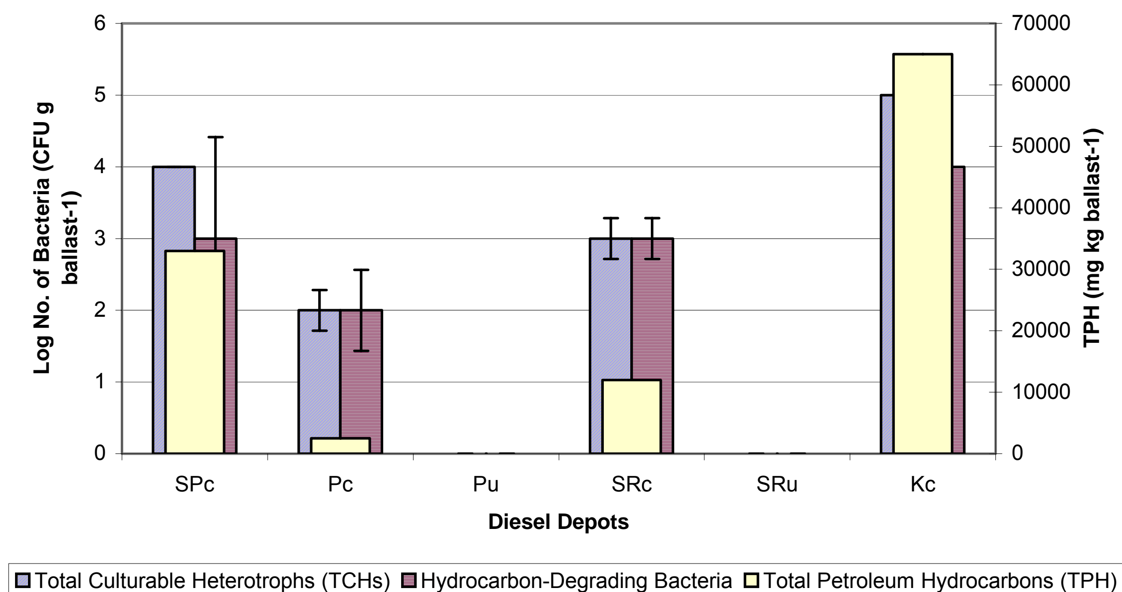

Figure 1. The number of culturable heterotrophic bacteria and the concentration of hydrocarbons at different diesel depots. Values are the means of three replicates (SD ± 1.4 or less). SPc-Springs contaminated ballast, Pc-Pyramid contaminated ballast, Pu-Pyramid uncontaminated ballast, SRc-Sentrarand contaminated ballast, SRu-Sentrarand uncontaminated ballast, Kc-Koedoespoort contaminated ballast.

Respiration rate determination

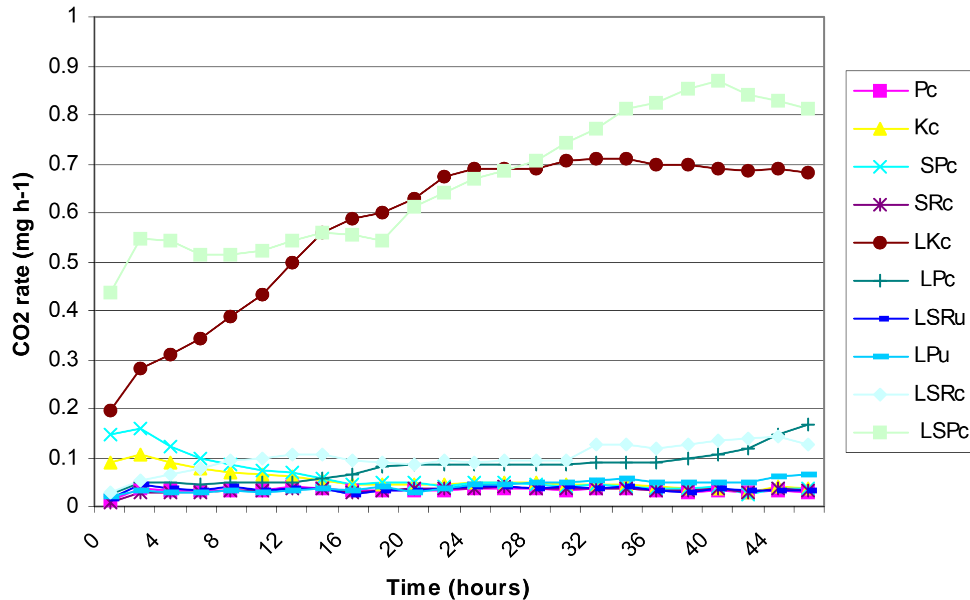

Figure 2: CO_2 production by microorganisms in different diesel depots samples. Pc- Pyramid contaminated stone ballast, Kc- Koedoespoort contaminated ballast, SPc- Springs contaminated ballast, SRc-Sentrarand contaminated ballast, LKc- nutrient amended Koedoespoort contaminated ballast, LPc- nutrient amended Pyramid contaminated ballast, LSRu- nutrient amended Sentrarand uncontaminated ballast, LPu- nutrient amended Pyramid uncontaminated ballast, LSRc- nutrient amended Sentrarand contaminated ballast, LSPc- nutrient amended Springs contaminated ballast.

Addition of nutrients stimulated the biological activity of the samples and possibly the removal of hydrocarbons. However, this was only evident in the LSPc and LKc. The biological activity as measured by CO_2 production was very low in the LSRc, LPu, LSRu, LPc and the control samples (Figure 2). The rate of CO_2 production in the diesel depots samples was higher in the nutrient amended samples in which high numbers ($\geq 10^4$ CFU/g ballast) of TCHs were present. No significant production of CO_2 was evident in

the samples with low or no culturable heterotrophs (LSRc, LPc, LPu, LSRu and the control samples).

Carbon source utilization Profiles

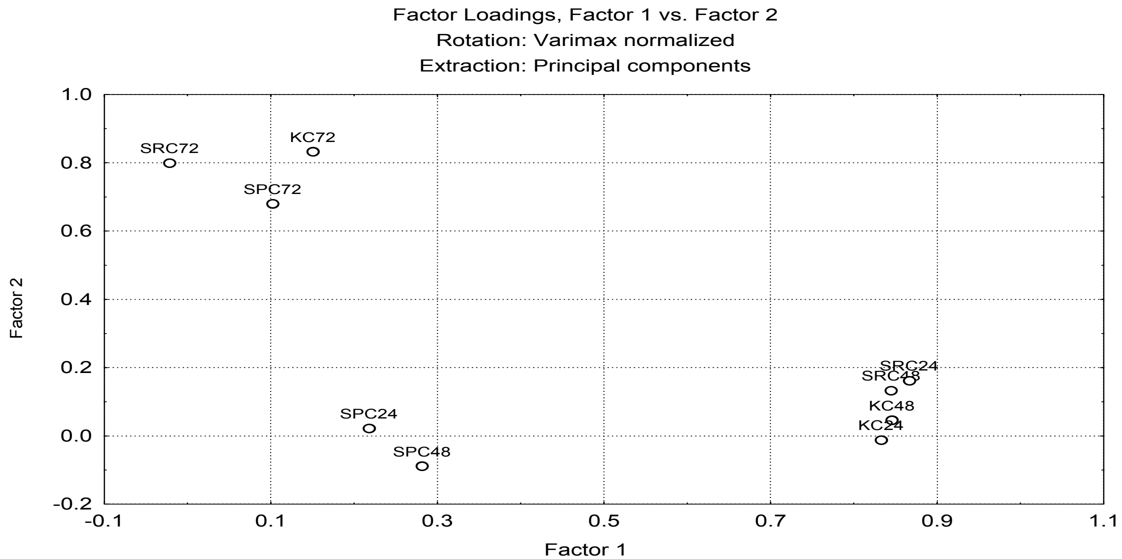

Figure 3. The relationship between microbial communities of different diesel depots as depicted by principal component analysis. Kc-Koedoespoort contaminated ballast, SPc-Springs contaminated ballast, SRc-Sentrarand contaminated ballast (readings for different incubation times are shown).

Principle Component Analysis (PCA) was performed to characterize the correlation amongst samples, taking into account the absorbance values for all 96-response wells. Two principal factors were isolated from the individual SRc, Kc, SPc (Figure 3) patterns, which explained 63% of the variation. For the three samples, factor one was related to the absorbance values for the wells, while factor 2 was related to the incubation time.

The Kc and SRc samples (at 24 h and 48 h incubation periods) had a similar pattern of carbon source utilization based on PCA (Figure 3). However, the similarities in carbon

source utilization patterns appeared to diminish with increasing incubation time (after 48 h, Fig. 3). At 72 h, Kc and SRc were less similar than at the shorter incubation times, and SPc is much less similar to Kc and SRc. The trend over time is greater divergence of carbon source utilisation patterns, more so for SPc than for Kc and SRc.

The relationship between the substrate utilization patterns was further analysed using hierarchial clustering. In a dendrogram (Figure 4), the results of cluster analysis confirmed the degree of similarities between the samples (Figure 4).

Both the dendrogram and the PCA illustrate that the substrate utilization pattern of microbial communities at different diesel depots were different.

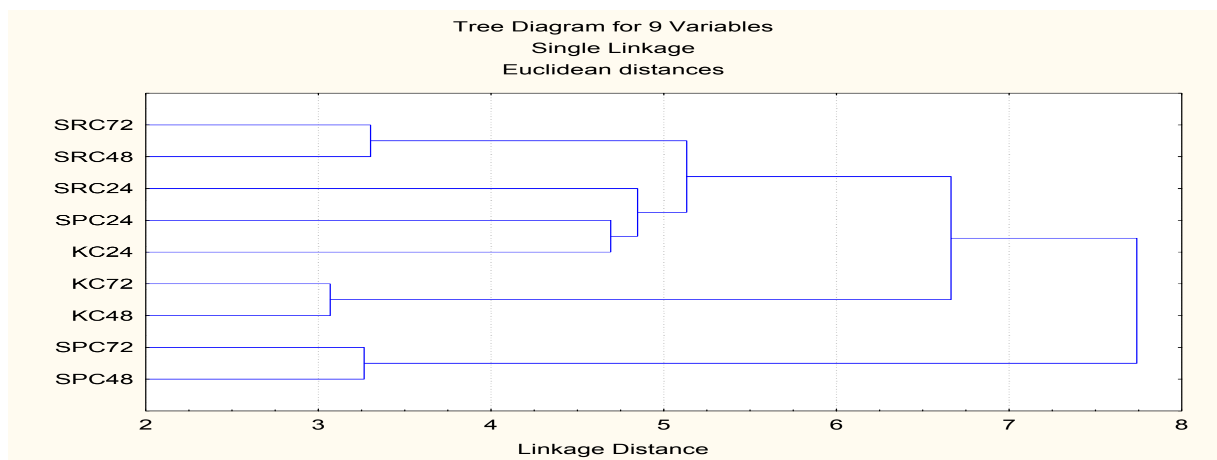

Figure 4. The relationship between microbial communities of the different polluted stone ballasts resulting from cluster analysis (readings for different incubation times are also shown, abbreviations as in Figure 3).

Discussion and Conclusion

The microbiological analysis indicated that the number of culturable bacteria on the polluted ballasts was proportional to the level of hydrocarbon concentration. Kc, the diesel depot sample with the highest TPH concentration had the highest number of culturable heterotrophic bacteria while Pc, the diesel depot sample with the lowest concentration of TPH had the lowest number of culturable heterotrophic bacteria. This can be attributed to the availability of the readily biodegradable hydrocarbons on the ballast, which can encourage the colonisation of the heterotrophs on the ballasts. According to Atlas (1981), Leahy and Colwell (1990), the number of hydrocarbon degrading bacteria and their relative abundance in the bacterial communities increases significantly in the presence of readily biodegradable hydrocarbons. The analysis of variance indicated that the number of total culturable heterotrophs (TCHs) were not significantly higher than the number of hydrocarbon-degrading bacteria. The results suggest that the number of hydrocarbon degrading bacteria forms the majority of the total culturable heterotrophic bacteria in the hydrocarbon-contaminated stone ballasts. There was no hydrocarbon degrading bacteria and the TCHs that were isolated in the uncontaminated Pyramid and uncontaminated Sentrarand ballasts. This can be attributed to the lack of the readily biodegradable organic compounds on the diesel ballasts.

The production of CO_2 by microorganisms in the diesel depots samples was higher in only two of the nutrient amended samples compared to the controls. This 'divergent' CO_2 data can be attributed to the probable heterogeneousness of the samples in terms of the real amount of hydrocarbon 'added' to the bottles. The results also suggest that the activity of LSRc and LPc was very low and the community cannot be marked as 'active'. In addition, the high CO_2 production in LKc and LSPc appears to reflect the higher

carbon source (hydrocarbon) content and larger bacterial inoculum at the beginning of the test. The addition of nutrients (mainly nitrogen and phosphorus) has been reported to stimulate the biological activity in hydrocarbon contaminated soil environments (Churchill *et al.*, 1995; Braddock *et al.*, 1997; Seklemova, 2001).

The number of carbon source of Biolog plates used by the SRc sample was four times the number of substrates used by the Springs contaminated stone ballast (SPc) after 24 hours of incubation. This suggests higher degradation capacity in the SRc sample compared to the SPc sample as both samples were adjusted to have similar cell density for Biolog plate inoculation. According to Wünsche *et al.* (1995), at lower incubation periods, Biolog patterns reflect the metabolic activities of the quantitatively dominating components of the microbial communities. However, current information reveals that carbon source utilisation profiles obtained with Biolog GN plates do not necessarily reflect the functional potential of the numerically dominant members of the microbial community used as the inoculum (Smalla *et al.*, 1998).

Influence of incubation time on the development of the substrate utilization pattern of the samples was similar to that reported elsewhere (Garland and Mills, 1991; Winding, 1994; Haack *et al.*, 1995; Kersters *et al.*, 1997). The Biolog substrate oxidation response and average well color developments often exhibit a lag phase, an exponential phase and a stationary phase. This non-linearity implies that the substrates to be most significant in discriminating microbial communities may change over the course of the experiment. A cursory glance at the results suggests that this is responsible for the dendrogram pattern.

Microbial colonization of rocky surfaces by heterotrophs is made possible by (among other things) the availability of the selective substrates that can be used to sustain the

heterotrophic community. In diesel depots in which similar human activities results in the contamination of stone ballasts by hydrocarbons, patterns of substrates utilization of the heterotrophs was expected to be similar. This was, however, not the case in this study as indicated by the substrate utilisation patterns. Both the principle component analysis (PCA) and cluster analysis indicated the degree of dissimilarity between the different diesel depots samples. Using cluster analysis, SRc and Kc were closely related compared to the SPc. However, using the PCA, the three samples (SRc, Kc and SPc) after 72 hours of incubation were lumped together, suggesting the extent of similarity in the pattern of substrate utilisation.

Microbial diversity of polluted surfaces needs to be studied further to investigate the concentration or the thickness of the hydrocarbons layer on the rock surfaces that encourages the attachment or colonization of the TCHs and the hydrocarbon-degrading bacteria. It is also not clear how the heterotrophs acquire the micronutrients from the surrounding environment or what is the structure of microbial communities under the stone ballasts. However, it is probable that the heterotrophs acquire the miconutrients from airborn pollutants. Knowledge of microbial diversity of contaminated rocky surfaces is essential as it can be applied in bioremediation of contaminated rocky surfaces as in contaminated diesel depots and contaminated rocky surfaces caused by oil spills.

Acknowledgements

We thank the Risk and Environmental managers at (Spoornet) Diesel depots for allowing and helping us to collect the samples. Special thanks also goes to Dr P Wade for assisting us with the Statistical analysis. The National Research Foundation and the CSIR supported this work.

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Chapter 4

SOIL MICROBIAL DIVERSITY: INFLUENCE OF GEOGRAPHIC LOCATION AND HYDROCARBON POLLUTANTS

A modified version of this text was sent for publication as:

Mphekgo P. Maila, P Randima, and Thomas E Cloete

(2003) Soil Microbial Communities: Influence of Geographic Location and Hydrocarbon Pollutants. Journal of Soil Biology & Biochemistry.

SOIL MICROBIAL DIVERSITY: INFLUENCE OF GEOGRAPHIC LOCATION AND HYDROCARBON POLLUTANTS

Abstract

The importance and relevance of the geographical location of the soil sample and the petroleum hydrocarbons in determining the functional or species diversity within different bacterial communities was evaluated using the community level physiological profiles. The petroleum hydrocarbons rather than the geographical location of the sample appear to be more important in determining functional or species diversity within the bacterial communities. Cluster analysis of the different community profiles revealed that the samples from different locations were as different as samples from the same location but from contaminated versus uncontaminated soils. The results of the soils from different locations artificially contaminated by different hydrocarbons also reached the same conclusion. The samples from different soils were as different as samples from the same soil contaminated by different hydrocarbons. In addition, the removal rate of the different hydrocarbons in the artificially contaminated soil was different. The results suggests that the pollutants rather than the geographical origin of the sample might be more important in determining the functional or species diversity within bacterial communities.

Introduction

The impact of petroleum hydrocarbons on soil microbial diversity has been the subject of investigation in recent years. The changes in hydrocarbon content in soils results in characteristics shifts in microbial populations and the abundance of hydrocarbon utilising bacteria (Atlas *et al.*, 1991; Wünsche *et al.*, 1995). Hydrocarbon contamination selects for a less diverse but catabolically versatile bacterial community (Atlas *et al.*, 1991;

Lindstrom *et al.*, 1999). However, information about the importance of geographical origin of the soil and the hydrocarbons in determining the functional and species diversity within bacterial communities is not well documented. There is a need to understand the importance of geographical origin of the soil and the hydrocarbons when assessing the different soil environments contaminated by hydrocarbons. The improved knowledge of the influence of the geographical origin of the soil and the hydrocarbons on microbial diversity can help to improve microbial process used in the removal of hydrocarbons from the soil.

Bundy *et al.* (2002), used community level physiological profiles (CLPP) and phospholipids fatty acid (PLFA) to study the effect of diesel on microbial communities and reported that microbial communities in different soil types do not converge after diesel contamination. However, the soil used in the study was artificially contaminated and could therefore, not adequately reflect the contaminated field sites.

Juck *et al.* (2000), found that at two oil contaminated Arctic sites investigated by DGGE and Biolog analysis, absolute diversity was decreased at one site and remained the same or increased at the other. However, the study was conducted using the cold adapted microbial communities.

In this study, we evaluated soil microbial diversity of different geographic locations contaminated by similar hydrocarbons. We also investigated the biodegradation efficiency and microbial diversity of different soils artificially contaminated by different hydrocarbons. The aim of the study was to characterise the funtional diversity of different hydrocarbon contaminated soil environments to establish the importance and relevance

of the geographic locations in relation to the stressor. The community level physiological profile was used to characterise the microbial communities.

Materials and Methods

Soil: The soil samples were taken in sterile bags from a hydrocarbon-contaminated site in Secunda (Mpumalanga Province), Coalsbrook (Free State Province), and Rosslyn (Gauteng Province), South Africa. The soils were predominantly sandy loam and had the Total Petroleum Hydrocarbon (TPH) concentration during the day of sampling of 1.2 g/kg soil, 2.5 g/kg soil and 1 g/kg soil respectively. Both the contaminated and uncontaminated soils were collected. The uncontaminated soil was also collected from the CSIR (Council for Scientific and Industrial Research) site.

Influence of different hydrocarbons on functional diversity: Crude oil (obtained from Petronet, Durban, SA), mineral oil and diesel (both from Exel Pty Ltd) were used to contaminated both the CSIR soil and the Coalsbrook uncontaminated soils. The different treatments used for the experiment are shown in table1. Each hydrocarbon was added to the soil to make the initial concentration of the artificially contaminated soil 40000 mg/kg soil. The oil and the soil were thoroughly mixed before preparing the replicates for each treatment. The replicates, containing 500g of the contaminated soil of each treatment were prepared in 10 cm pots. All the pots were incubated at room temperature in the greenhouse with normal day-night cycle. The pots were watered three times a week with 200 mℓ of water to maintain the ideal soil mositure for microbial activity. In instances where leachates were produced, the leachate was used to water the same pots. One replicate from each treatment was sacrificed once every two weeks to determine the level of hydrocarbons in the soil. The soil from each treatment (after nine weeks) was used for determining the community level physiological profiles.

Chemical Analysis: The contaminated soils were analysed using the Total Petroleum Hydrocarbons (TPH) method described in Margesin *et al.* (1999). 10 g of the polluted soil was used for the analysis. The analyses were done in triplicate.

Table 1: Treatments used during Bioremediation

Treatments	Additions/Preparations
COCOIL	Coalsbrook soil + Crude Oil
CSCOIL	CSIR soil + Crude Oil
CSD	CSIR soil + Diesel
COD	Coalsbrook soil + Diesel
CSMO	CSIR soil + Mineral Oil
COMO	Coalsbrook soil + Mineral Oil
COUN	Uncontaminated Coalsbrook soil
CSUN	Uncontaminated CSIR soil

Viable counts and Biolog assays: Microbial suspensions were prepared from soil as described by Wünsche *et al.* (1995). After appropriate dilutions in sterile saline solution, the cell suspensions were used to determine the number of culturable heterotrophs and to inoculate BIOLOG micro plates. The number of culturable heterotrophs, expressed as CFU, was determined by spreading 0.1 mℓ cell suspension on to a nutrient agar (Biolab Diagnostics, Pty Ltd, SA) medium, amended with cycloheximide (200 µg/mℓ) to suppress fungal growth. Three replicates were spread on agar plates and incubated for 24 hours at 28°C.

To obtain a substrate utilisation fingerprinting of the microbial communities, three replicates of all the soil extracts were inoculated in BIOLOG GN microtiter plates (Biolog Inc., Hayward Calif) containing 95 different sole-carbon sources and a control without a carbon source. The BIOLOG GN plates were incubated at 28°C and readings done using a Bio-Tek Elx800 (Bio-Tek Instruments Inc) micro plate reader at 600 nm after 24, 48 and 72 h. Statistical analyses were done using STATISTICA for Windows release 5.1.

Results

The Principle Component Analysis (PCA) revealed differences in the substrate utilisation patterns of both the contaminated and uncontaminated soil from each geographic location (Figure 1). However, dendrogram analysis only clustered the Rosslyn sample site based on geographic location (Figure 2). The contaminated and uncontaminated soils from the other three sites were not clustered together based on geographic location.

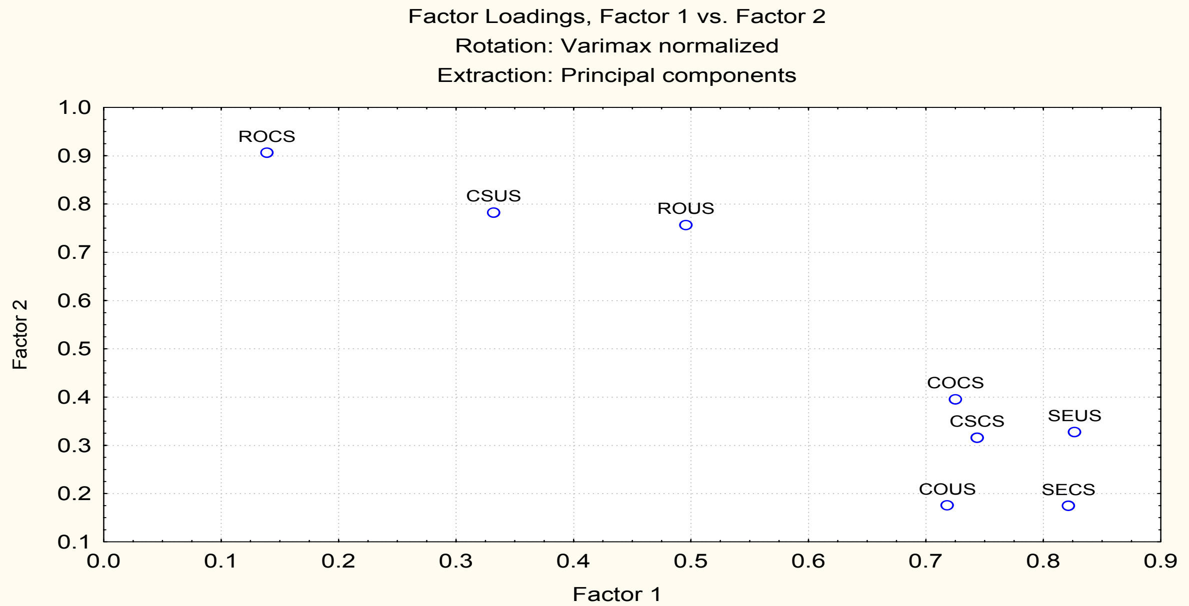

Figure 1. Principle component analysis of the different soil samples. COCS-hydrocarbon-contaminated Coalsbrook soil, COUS-Coalsbrook uncontaminated soil, CSCS-hydrocarbon-contaminated CSIR soil; CSUS-CSIR uncontaminated soil, ROCS-hydrocarbon-contaminated Rosslyn soil, ROUS-Rosslyn uncontaminated soil, SECS-ydrocarbon-contaminated Secunda soil, SEUS-Secunda uncontaminated soil.

The samples from different locations were as different as samples from the same location but from contaminated versus uncontaminated soils. Because of soil usage and heterogeneity, which can influence microbial diversity, it was expected that the geographical origin of the sample rather than the hydrocarbons was more important in determining functional or species diversity within the bacterial communities. However, the results appears not to reinforce the suggestion that geographical origin of the samples, rather than the hydrocarbons, is important in determining functional or species diversity within the mesophilic bacterial communities, as contaminated and

uncontaminated samples from the majority of the sites were not closely related (Figure 1 and 2).

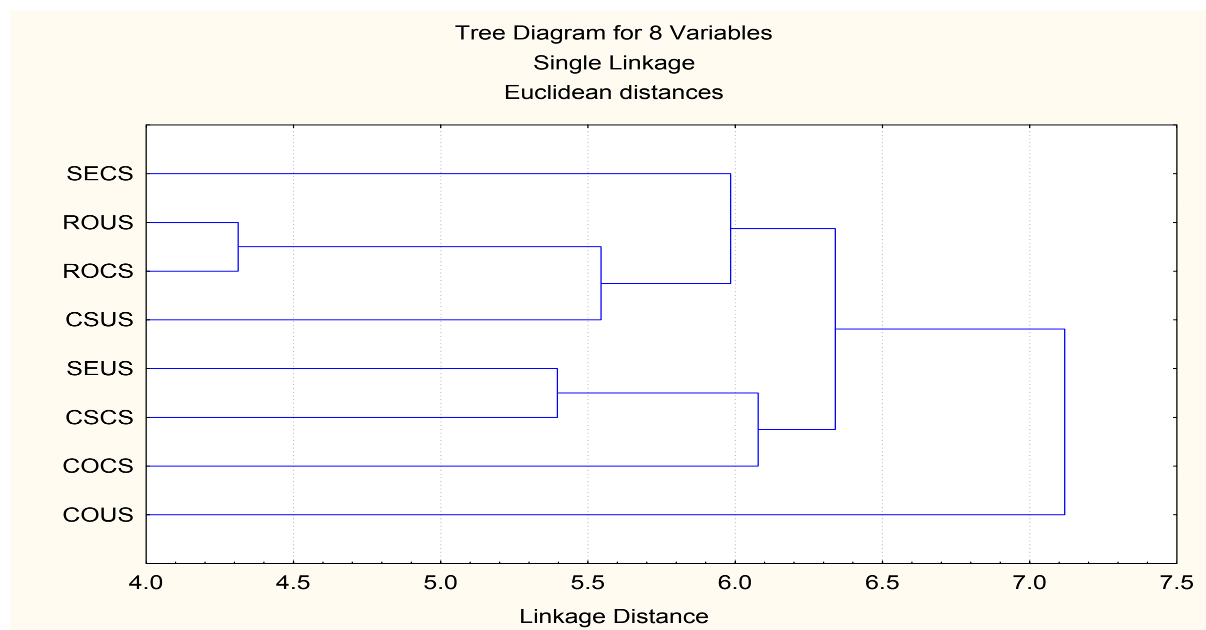

Figure 2. Cluster analysis of the different soil samples from the different geographic locations. COCS-hydrocarbon-contaminated Coalsbrook soil, COUS-Coalsbrook uncontaminated soil, CSCS-hydrocarbon-contaminated CSIR soil; CSUS-CSIR uncontaminated soil, ROCS-hydrocarbon-contaminated Rosslyn soil, ROUS-Rosslyn uncontaminated soil, SECS-hydrocarbon-contaminated Secunda soil, SEUS-Secunda uncontaminated soil.

Influence of different carbon substrates on functional diversity

The importance of geographical origin of the samples and the hydrocarbons in determining functional diversity within bacterial communities were further evaluated using two different soils artificially contaminated by different hydrocarbons. The community level physiological profiles (CLPP) of the different contaminated and uncontaminated soils were analysed after incubation using the Principle Component Analysis (PCA) and cluster analysis.

The PCA revealed differences in the substrate utilisation pattern of the contaminated and the uncontaminated soils (Figure 3). The uncontaminated soil from each of the CSIR and Coalsbrook soil (COUN and CSUN) was not closely related to any of the respective artificially hydrocarbon contaminated soils. The crude oil contaminated CSIR soil (CSCOIL) was also not closely related the crude oil contaminated Coalsbrook soil (COCOIL). However, the mineral oil contaminated CSIR (CSMO) and Coalsbrook soils (COMO) were closely related. Also related were the diesel contaminated CSIR (CSD) and Coalsbrook soil (COD, Figure 3).

The difference in the community level physiological profiles of the different hydrocarbon contaminated soils were further analysed using hierarchical clustering (Figure 4). In a dendrogram, the mineral oil contaminated CSIR soil was clustered together with the mineral oil contaminated Coalsbrook soil, while the diesel contaminated CSIR soil was also clustered with the diesel contaminated coalsbrook soil. No clustering was evident in the case of the crude oil contaminiated CSIR and coalsbrook soil.

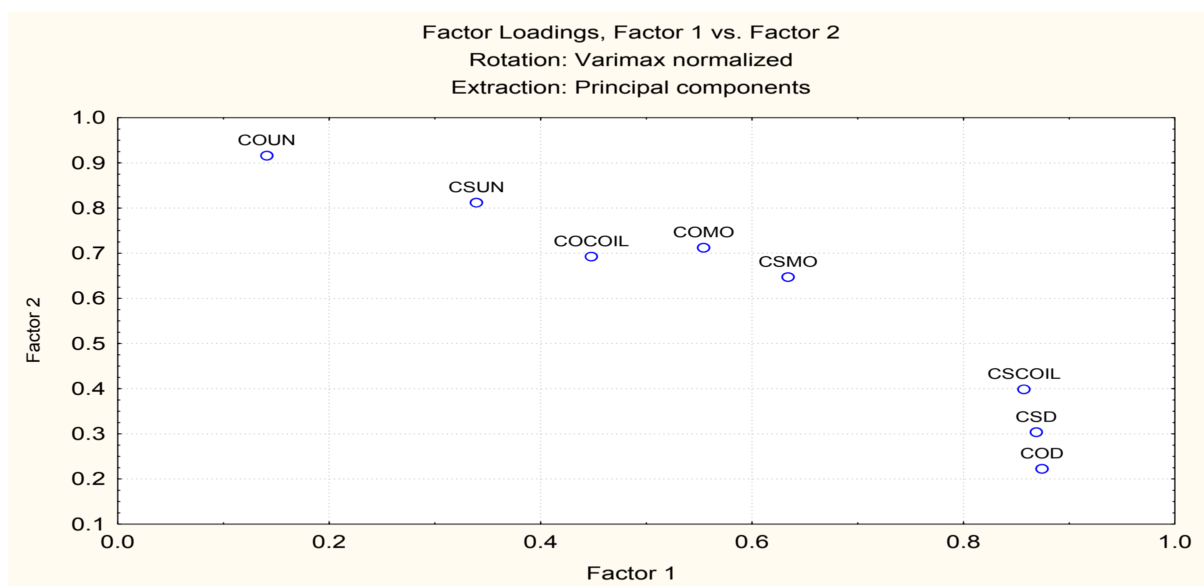

Figure 3. PCA of the different soils contaminated by different hydrocarbons. COD-diesel contaminated coalsbrook soil, COUN-uncontaminated coalsbrook soil, COCOIL-crude oil contaminated Coalsbrook soil, COMO-mineral oil contaminated Coalsbrook soil, CSMO-mineral oil contaminated CSIR soil, CSUN-uncontaminated CSIR soil, CSCOIL-crude oil contaminated CSIR soil, CSD-diesel contaminated CSIR soil.

Different soil environments may harbor different microbial diversity. However, with the availability of petroleum hydrocarbons, the functional diversity of the soil, as revealed by the substrate utilisation patterns appears to be similar. There was no clustering of either the CSIR uncontaminated soil or Coalsbrook uncontaminated soil with the contaminated soil to suggest the influence or importance of geographic location in relation to the stressor in determining functional diversity within the bacterial communities. Even though the uncontaminated CSIR and the uncontaminated Coalsbrook soil were clustered together, the linkage distances illustrated their differences. The results suggest that the

hydrocarbons rather than the geographical origin of the samples are important when determining the functional diversity within bacterial communities.

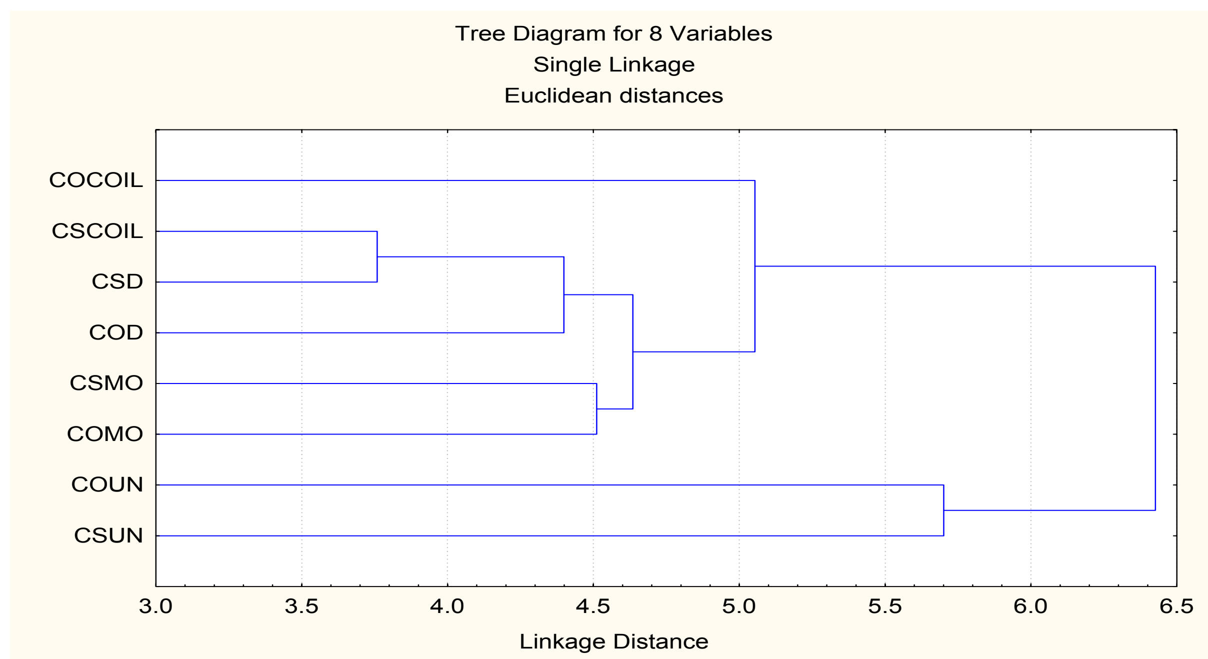

Figure 4. Cluster analysis of the soils contaminated by different hydrocarbons. COD-diesel contaminated coalsbrook soil, COUN-uncontaminated coalsbrook soil, COCOIL-crude oil contaminated Coalsbrook soil, COMO-mineral oil contaminated Coalsbrook soil, CSMO-mineral oil contaminated CSIR soil, CSUN-uncontaminated CSIR soil, CSCOIL-crude oil contaminated CSIR soil, CSD-diesel contaminated CSIR soil.

The removal of different hydrocarbons in the 'same soil' is different (Figure 5). Diesel was removed much faster than the crude oil and the mineral oil. In addition, the removal of mineral oil and crude oil was much higher in the CSIR soil than in the Coalsbrook soil. However, similar removal rate of diesel was found in both soils. The CSIR soil had a higher number of both the total culturable heterotrophs (TCHs) and the culturable hydrocarbon-utilisation bacteria than the Coalsbrook soil (results not included). The

removal of hydrocarbons from the soils was highest in the first two weeks of the ‘treatment’.

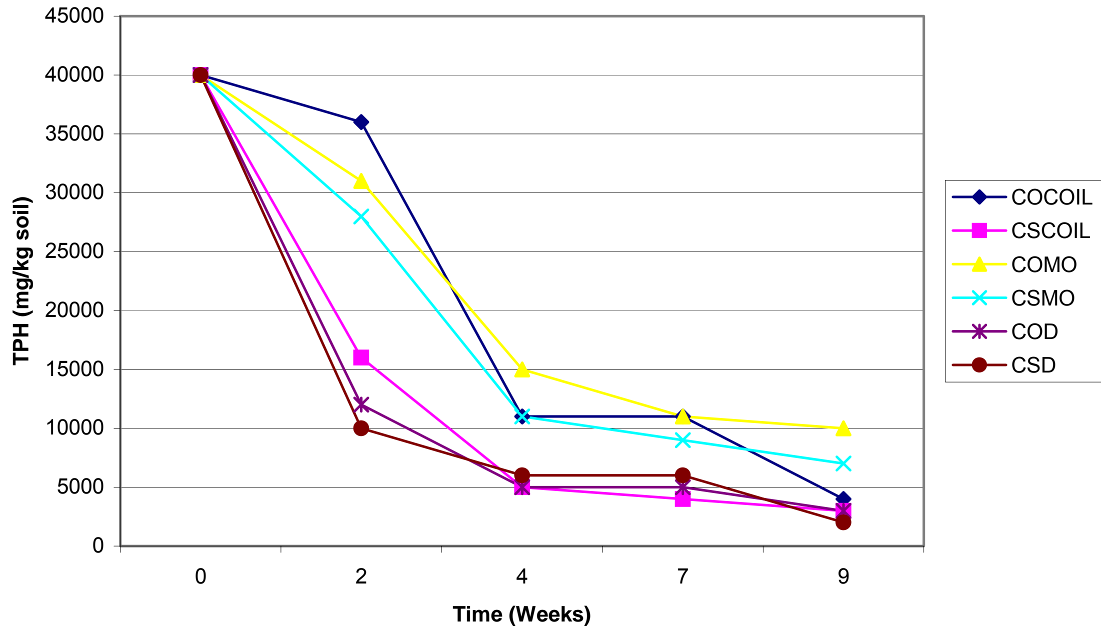

Figure 5. Biodegradation of different hydrocarbons in different soils. COD-diesel contaminated coalsbrook soil, COCOIL-crude oil contaminated Coalsbrook soil, COMO-mineral oil contaminated Coalsbrook soil, CSMO-mineral oil contaminated CSIR soil, CSCOIL-crude oil contaminated CSIR soil, CSD-diesel contaminated CSIR soil.

Discussion and Conclusion

The results indicate that different locations contaminated by different hydrocarbons have different microbial communities. The Principle Component Analysis (PCA) revealed differences in the substrate utilisation patterns of both the contaminated and uncontaminated soil from each geographic location. This is inline with similar findings that the increase in hydrocarbon content in soil results in significant changes in the microbial communities of the affected soil environments (Wünsche *et al.*, 1995; Atlas *et al.*, 1991).

The dendrogram analysis clustered only the Rosslyn sample site based on geographic location. The contaminated and uncontaminated soils from the other three sites were not clustered together. In addition, the contaminated and uncontaminated soils from each geographical location were also not closely related. This results contrast those reported by Juck *et al.* (2000), who reported clustering of samples based on the geographic origin of the samples. However, the study worked on the cold-adapted bacterial communities while the current study worked on the mesophillic bacterial communities.

Because of soil heterogeneity and usage that can influence microbial diversity, it was expected that the geographical origin of the sample rather than the hydrocarbons was more important in determining functional diversity within the bacterial communities. However, the results did not support this hypothesis as the samples from different locations were as different as samples from the same location but from contaminated versus uncontaminated soils.

The removal of hydrocarbons from the soils was highest in the first two weeks of the 'treatment'. This can be attributed to the contribution of other removal mechanisms other

than biodegradation. According to Harmsen *et al.* (1994) and Hejazi *et al.* (2003), the dominant removal mechanism of hydrocarbon during the initial phase involves the volatilisation of the low molecular weight volatile compounds. The principal removal mechanism for the non-volatile hydrocarbons appears to be biodegradation.

The removal of different hydrocarbons in the 'same soil' is different. Diesel was removed faster followed by crude oil and mineral oil. This was not surprising as the different substrates have different compositions of hydrocarbons or different aliphatic chains which can influence biodegradation (Dias and Alexander, 1971). In addition, the biodegradation of mineral oil and crude oil was much higher in the CSIR soil than in the Coalsbrook soil. However, similar biodegradation of diesel was found in both soils. This can be attributed to the significant role played by other removal mechanisms other than biodegradation. According to Morgan and Watkinson (1989), up to 40% of the hydrocarbons may evaporate in hotter climates. The differences in the biodegradation efficiency in the two different soils can be attributed to the higher number of both the total culturable heterotrophs (TCHs) and the culturable hydrocarbon-utilisation bacteria in the CSIR soil than the Coalsbrook soil.

The importance of geographical origin of the samples and the hydrocarbons in determining functional diversity in bacterial communities were further evaluated using two different soils contaminated by different hydrocarbons. As with the soil samples from the different contaminated sites, the uncontaminated soil from each of the CSIR and Coalsbrook soil was not closely related to any of their respective artificially hydrocarbon contaminated soils. However, the mineral oil-contaminated and diesel contaminated soils were closely related. In addition, the hydrocarbon-contaminated soils were clustered together. The results suggest that hydrocarbons rather than the geographical

origin of the sample are more important in determining the functional diversity within the bacterial communities.

In conclusion, the study did not support the hypothesis that the geographical origin of the sample rather than the hydrocarbons is important in determining functional or species diversity within bacterial communities. However the study opened the possibility of applying CLPP for determination of natural attenuation. Further work is required to investigate the importance of soil heterogeneity in community studies of soil environments contaminated by similar hydrocarbons using both functional and genetic diversity methods. In addition, similar studies should incorporate the physico-chemical characterisation of the various soil samples.

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Chapter 5

MULTI-SPECIES AND MONOCULTURE RHIZOREMEDIATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) FROM THE SOIL

A modified version of this text was accepted for publication as:

Mphekgo P. Maila, P Randima and Thomas E Cloete (2004) Multi-Species and Monoculture Rhizoremediation of Polycyclic Aromatic Hydrocarbons (PAHs) from the Soil. International Journal of Phytoremediation

MULTI-SPECIES AND MONOCULTURE RHIZOREMEDIATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) FROM THE SOIL

Abstract

In this study, we investigated the potential of multi-species rhizoremediation and monoculture rhizoremediation in decontaminating polycyclic aromatic hydrocarbon (PAH) contaminated soil. Plant-mediated PAH dissipation was evaluated using mono-planted soil microcosms and soil microcosms vegetated with several different grass species (*Brachiaria serrata* and *Eleusine corocana*). The dissipation of naphthalene and fluorene was higher in the 'multi-species' vegetated soil compared to the mono-planted and non-planted control soil. The concentration of naphthalene was undetectable in the multi-species vegetated treatment compared to 96% removal efficiencies in the mono-planted treatments and 63% in the non-planted control after 10 weeks of incubation. Similar removal efficiencies were obtained for fluorene. However, there was no significant difference ($p<0.05$) in the dissipation of pyrene in both the mono- and multi-species vegetated treatments. There was also no significant difference ($p<0.01$) between the dissipation of PAHs in the mono-planted treatments with different grass species. Principle Component Analysis (PCA) and Cluster analysis were used to evaluate functional diversity of the different treatments during phytoremediation of PAHs. Both PCA and Cluster analysis revealed differences in the metabolic fingerprints of the PAH contaminated and non-contaminated soils. However the differences in metabolic diversity between the multi-species vegetated and mono-planted treatments were not clearly revealed. The results suggest that multi-species rhizoremediation using tolerant plant species rather than monoculture rhizoremediation have the potential to enhance pollutant removal in moderately contaminated soils.

Introduction

Phytoremediation, the use of plants and associated microorganisms to degrade or immobilise contaminants in soil and groundwater is increasingly being considered for rehabilitating moderately contaminated soils. The technology represents a potential low-cost, effective and low maintenance alternative for waste management (Aprill and Sims, 1990). The mechanisms of phytoremediation for hazardous organic contamination in soil include, direct plant uptake, microbial degradation stimulated by plant roots, co-metabolism of contaminants in the rhizosphere and adsorption to humic or organic matter (Schnoor *et al.*, 1995; Cunningham and Ow, 1996; Chen *et al.*, 2003).

Laboratory and greenhouse experiments on plant-mediated dissipation of polycyclic aromatic hydrocarbons (PAHs) have concentrated mainly on the use of monoculture rhizoremediation of PAHs from the soil (Aprill and Sims, 1990; Lee and Banks, 1993; Walton *et al.*, 1994; Günther *et al.*, 1996; Reilly *et al.*, 1996). In these studies, the extent of organic pollutant removal in planted soil has been significantly greater than in non-planted soil. However, the information about the effectiveness of multi-species rhizoremediation of PAHs is lacking. The use of soil microcosms with mixed planted species has the potential to increase soil heterogeneity (Angers and Carnon, 1998) and microbial diversity, which can improve the microbial competence of the soil bacteria for effective pollutant removal.

In this study, the effectiveness of both monoculture rhizoremediation and multi-species rhizoremediation of PAH-contaminated soil was evaluated. In addition, the study also evaluated the functional diversity of the PAH-contaminated and non-contaminated rhizosphere and non-rhizosphere soil using community level physiological profiles.

Materials and Methods

Chemicals

All solvents (dichloromethane 99% and trichloromethane 99%) and PAH (Naphthalene 98%, fluorene 98% and pyrene 98%) were purchased from Sigma-Aldrich, SA.

Soil

The predominantly sandy loam soil used in the experiments was taken from the Pretoria Campus of the Council of Scientific and Industrial Research (CSIR), SA. The artificial contamination of the soil with PAH was done as described by Leyval and Binet (1998). The PAHs were first dissolved in trichloromethane before being mixed with 1% of the total soil to be polluted. Trichloromethane was allowed to volatilise under fumehood, and the amended soil was mixed thoroughly with the remaining 99% of the soil. After mixing, three samples of the artificially contaminated soil were collected in sealable glass containers for PAHs analysis. The concentration of the individual PAHs in the soil averaged 300mg/kg soil. Further soil sampling and storage of the rhizosphere and non-rhizosphere soil in the experiments were done as described by Wollum (1982). The rhizosphere soil (soil that adheres to the roots) was collected in sterile bags by gently shaking loose soil from the intermingled roots of the planted treatments. This rhizosphere soil was used for determining the community level physiological profiles and for PAH analysis. The non-rhizosphere soil was collected from the pots and mixed thoroughly before further analysis. Analyses were made within 24 hour of sampling. However, in cases were this was not feasible, the soil samples were stored overnight at 4°C.

Plants

The seeds of *Brachiaria serrata* (Velvet signal grass) were bought from Agricol Pty Ltd, SA and those of *Eleusine coracana* (African millet) were obtained from Plant Genetic Resources Directorate (Agriculture Department, SA). *B. serrata* is a perennial tufted

grass with shrub-like growth form. *E. coracana* is a robust annual tufted grass with slated culms and has an exceptionally dense and strong root system. The grass seeds were sown in trays (38 cm x 38 cm) containing the soil. The trays were incubated at room temperature in the greenhouse with natural day-night cycles until germination of both plant seeds occurred. No supplemental lighting was supplied. However, the average natural cycle was 9 hours of daylight. The room temperature in the greenhouse averaged 28°C during the period of incubation. The grass seedlings were used in the phytoremediation experiment (vegetated microcosms).

Vegetated and Non-vegetated microcosms

Plants mediated removal of PAH was evaluated using mono-planted and multi-planted soil microcosms. The soil was artificially contaminated with PAHs as described above. The concentration of each PAH in the soil was 300mg/kg soil. This 'time zero' concentration is the average of the individual PAHs detected. The mono-planted and multi-planted treatments were prepared as shown in table 1. Six hundred grams of each soil preparation (table 1) were placed in 10 cm pots with saucers for leachate collection. The grass seedlings were planted in different pots as shown in table 1. The density of the plants was one plant per pot for the mono-cultured treatments while two plants (as shown in table 1) were used for the multi-species treatment. The pots were placed in the green house at room temperature and natural day-night cycles. No supplemental lighting was supplied. However, the average natural cycle was 9 hours of daylight. The room temperature in the greenhouse averaged 28°C during the period of incubation. Two hundred millilitres of water was used to water the plants every two days. In instances where leachates were produced, the leachate was used to water the same pots. Each treatment had 11 pots and two pots for each treatment were sacrificed after two, six and

ten weeks of incubation to determine concentrations of PAHs in the soil. ANOVA (Analysis of Variance) was used to determine the difference between the treatments.

Chemical Analysis

Residual PAH in treatments T0 to T3 were quantified as described by Maila and Cloete (2002). PAHs were extracted from 25 g soil of each treatment using dichloromethane. PAHs were quantified after extraction with dichloromethane, using a Varian Saturn 2000 Ion Trap Gas Chromatography/Mass Spectrometer equipped with a Chrompack CP-SIL 8CB-MS (5% phenyl) Fused Silica Capillary Column (30 m* 0.25 mm* 0.25 μm). The detector was tuned according to EPA 8270C using DFTPP. Injector temperature was 230°C, oven temperature program: 30°C (6 min), 10°C/min, 300°C (7 min). Analyses were done in triplicate.

Table 1: Treatments used in the experiments

Treatments	**Additions/Preparations**
T0 (Control)	Soil + PAHs
T1 (CSEC)	Soil + PAHs + *E.corocana*
T2 (CSBS)	Soil + PAHs + *B.serrata*
T3 (CSBSEC)	Soil + PAHs + *E.corocana* + *B.serrata*
T4 (UCS)	Soil
T5 (UCSEC)	Soil + *E.corocana*
T6 (UCSBSEC)	Soil + *B.serrata* + *E.corocana*

Community Level Physiological Profiles (CLPP)

The microbial community level physiological profiles of the different treatments shown in table 1 was done using the soil samples sacrificed after 4 weeks of incubation. This

period was deemed to be sufficient to allow the acclimatisation of the plants in the contaminated and non-contaminated soils. In addition, the PAH dissipation analysis were also done (as described above) using the samples sacrificed after 4 weeks of incubation (week 4 PAH dissipation results not included). For CLPP determination, three pots per treatment were used. The rhizosphere and non-rhizosphere soils were sampled as described above and microbial suspensions were prepared from the soil for Biolog^{TM} inoculation as described by Wünsche *et al.* (1995). 10g of soil and 100 mℓ of 0.2 % tetra-sodium pyrophosphate solution were shaken in an Erlenmeyer flask for 30 min on a rotary shaker at 140 rpm. The mixture was allowed to settle for 5 min and the supernatant used for serial dilutions in a physiological saline solution.

After appropriate dilutions in sterile saline solution, the cell suspensions were used to determine the number of culturable heterotrophs and to inoculate BIOLOG^{TM} GN micro plates. The number of culturable heterotrophs (results not included), expressed as CFU, was determined by spreading 0.1 mℓ cell suspension on to a nutrient agar (Biolab Diagnostics Pty Ltd, SA) medium, amended with cycloheximide (200 μg/mℓ) to suppress fungal growth. Plate counting was done in triplicate and incubation was at 28°C for 24 hours. Dilutions giving the same number of culturable heterotrophs were used for Biolog^{TM} inoculation.

To obtain the metabolic fingerprints of the microbial communities in different treatments (table 1), three replicate (three pots per treatment) of all the soil extracts were inoculated in BIOLOG^{TM} GN microtiter plates (Biolog Inc., Hayward Calif) containing 95 different sole-carbon sources and a control without a carbon source. 100 μℓ of each soil extract was added to each well. The BIOLOG^{TM} GN plates were incubated at 28°C and readings done using a Bio-Tek Elx800 (Bio-Tek Instruments Inc) micro plate reader at 600 nm

after 24, 48 and 72 h. Statistical analyses were done using STATISTICA for Windows release 5.1.

Results

Plant mediated PAH dissipation was evaluated using mono-planted and multi-planted soil microcosms. PAH dissipation was higher in the planted soils compared to the non-planted soil (table 2-4). There was on average a 97% reduction of naphthalene in planted soils compared to 63% in the non-planted soil. The dissipation of naphthalene and fluorene was higher in the multi-planted soil compared to the mono-planted and non-planted soil (table 2 and 3). The concentration of naphthalene was undetectable in the multi-planted treatment compared to 96% reduction in the mono-planted treatments and 63% reduction in the non-planted control after 10 weeks of incubation. The standard deviations for the average values are shown in table 2. There was a 96% reduction in fluorene, from the multi-planted treatment compared to 81% reduction in the mono-planted treatment with *E. corocana* and 47% of the control treatment. However, there was no significant difference ($P<0.01$) in the dissipation of pyrene in both the mono- and multi-planted treatments (table 4). There was also no significant difference between the dissipation of PAHs in the treatment with *Brachiaria serrata* and the treatment with *Eleusine corocana* (table 2-4).

Table 2: Naphthalene concentrations (mg/kg soil) in Monocultured and Multi-planted soil treatments (Average ± Std. Dev)

Weeks of Incubation	Control (T0)	CSEC (T1)	CSBS (T2)	CSECBS (T3)
2	293 ± 0.632	224 ± 0.089	202 ± 1.032	198 ± 0.019
6	248 ± 0.059	110 ± 1.112	98 ± 0.221	66 ± 1.532
10	111 ± 0.632	12 ± 0. 321	9 ± 1.209	0

As the development of microbial communities inhabiting the root zone is influenced by plant species, it was expected that the dissipation of PAHs in treatments vegetated by different plant species would be different. However, this was not observed, as there was no significant difference ($p<0.01$) in PAH dissipation in treatments with *B. serrata* (T2) and treatment with *E. corocana* (T1).

Table 3: Fluorene concentrations (mg/kg soil) in Monocultured and Multi-planted soil treatments (Average ± Std. Dev)

Weeks of Incubation	Control (T0)	CSEC (T1)	CSBS (T2)	CSECBS (T3)
2	296 ± 0.089	241 ± 0.087	246 ± 0.009	236 ± 0.127
6	235 ± 0.635	178 ± 1.612	164 ± 0.112	110 ± 0.255
10	160 ± 1.541	56 ± 0.065	38 ± 2.872	10 ± 1.432

Table 4: Pyrene concentrations (mg/kg soil) in Monocultured and Multi-planted soil treatments (Average ± Std. Dev)

Weeks of Incubation	Control (T0)	CSEC (T1)	CSBS (T2)	CSECBS (T3)
2	298 ± 0.283	262 ± 0.064	258 ± 0.832	261 ± 0.432
6	266 ± 0.642	203 ± 1.412	218 ± 1.414	198 ± 0.432
10	255 ± 0.565	166 ± 1.632	128 ± 0.876	117 ± 1.555

In addition, as plants have the potential to increase soil heterogeneity and soil microbial diversities, it was expected that the treatment with 'multi-plants' would have a higher PAH dissipation compared to mono-planted (T1 and T2) treatments. This was observed as both naphthalene and fluorene dissipation was significantly higher ($p<0.05$) in the multi-planted treatments compared to the mono-planted treatments. However, different results were obtained for pyrene degradation.

Community Level Physiological Profiles (CLPP)

Heterotrophic microbial communities were evaluated during phytoremediation of the PAHs by determining the metabolic fingerprints of the different treatments. Both Principle Component Analysis (PCA) and Cluster analysis were used to evaluate the difference in substrate utilisation patterns of the different treatments. PCA was performed to characterize the correlation between samples, taking into account the absorbance values for all 96-response wells. Two principal factors were isolated from the individual patterns (Figure 1), which explained 63% of the variation. For the samples, factor one

was related to the absorbance values for the wells, while factor 2 was related to the incubation time.

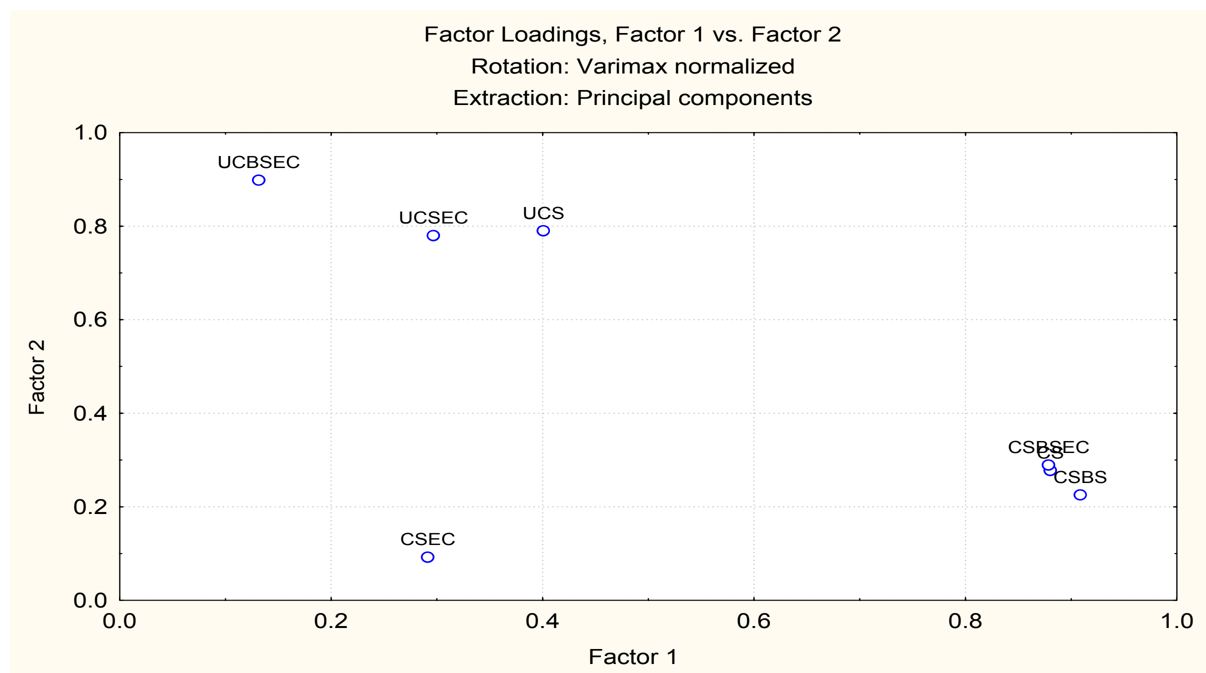

Figure 1. Metabolic diversity of PAH contaminated and non-contaminated rhizosphere and non-rhizosphere soils. UCS-uncontaminated Soil, CS-contaminated soil, CSBS-contaminated soil with *Brachiaria serrata*, UCSEC-uncontaminated soil with *Eleusine corocana*, UCBSEC-uncontaminated soil with *Brachiaria serrata* and *Eleusine corocana*, CSBSEC-contaminated soil with *Brachiaria serrata* and *Eleusine corocana*.

The metabolic fingerprints of the PAH-contaminated soils (CS, CSBS, CSBSEC, CSEC) were not closely related to the metabolic fingerprints of the non-contaminated soils, (UCS, UCSEC, UCBSEC; Figure 1). The metabolic fingerprint of the PAH-contaminated soil (CS) was more closely related to the contaminated mono-planted treatment with *Brachiaria serrata* (CSBS) and contaminated multi-planted soil with *Brachiaria serrata* and *Eleusine corocana* (CSBSEC) than to other treatments (Figure 1). The uncontaminated soils were more 'closely' related to each other than to the PAH contaminated soils.

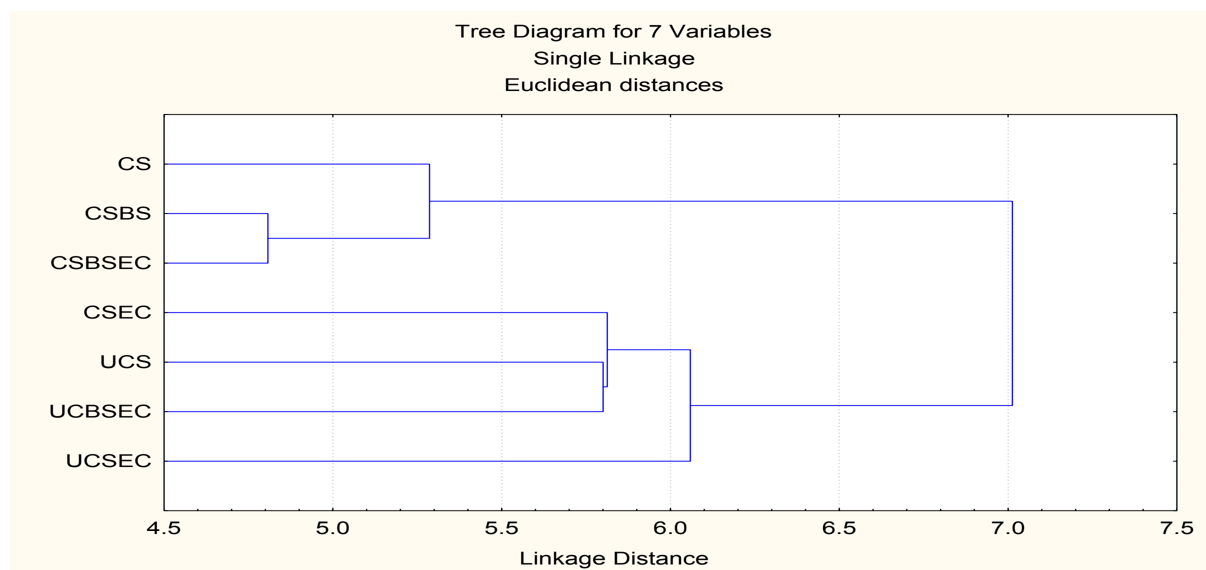

Figure 2. Cluster analysis of the PAH-contaminated and non-contaminated rhizosphere and non-rhizosphere soils. UCS-uncontaminated Soil, CS-contaminated soil, CSBS-contaminated soil with *Brachiaria serrata*, UCSEC-uncontaminated soil with *Eleusine corocana*, UCBSEC-uncontaminated soil with *Brachiaria serrata* and *Eleusine corocana*, CSBSEC-contaminated soil with *Brachiaria serrata* and *Eleusine corocana*.

The metabolic fingerprints of the different treatments were further evaluated using hierarchical clustering (Figure 2). In a dendrogram, the contaminated soils (CS, CSBS and CSBEC) were clustered together while CSEC was clustered with the uncontaminated soils. Both PCA and cluster analysis indicated that the metabolic fingerprints of PAH contaminated soils (with the exception of CSEC) were closely related as were the metabolic fingerprints of the uncontaminated soils.

As plants have the potential to increase soil heterogeneity and possibly soil diversity, it was expected that treatments with more plants will have high microbial competence, reflected by higher PAH dissipation and higher number of Biolog substrates used by the multi-planted treatments compared to mono-planted treatments. Higher PAH dissipation

was only evident with naphthalene and fluorene (table 2 and 3), while there was no significant difference in the amount of Biolog substrates (results not included) used by the multi-planted and mono-planted contaminated treatments after 72 hours of incubation. However, more Biolog substrates were used by microorganisms in the planted treatments compared to the non-planted treatments.

Discussions and Conclusion

The study investigated plant mediated PAH dissipation using mono-planted and multi-planted soil microcosms. PAH dissipation was higher in the planted soils compared to the non-planted control soil. This finding was in line with other findings, which indicated enhanced PAHs dissipation in vegetated soils compared to non-vegetated soil (Aprill and Sims, 1990; Reilly *et al.*, 1996). According to Muratova *et al.* (2003), significant PAH dissipation is attained in the vegetated soils as plants stimulate the rhizosphere micro flora, which degrade the pollutants.

The dissipation of naphthalene and fluorene was higher in the multi-planted soil compared to the mono-planted and non-planted control soil. However, there was no significant difference in the dissipation of pyrene in the mono and multi-planted treatment. This was not surprising as the rate of PAH degradation is inversely proportional to the number of rings in the PAH molecule (Cerniglia and Heitkamp, 1989; Cerniglia, 1992). In addition, the period of incubation was probably not sufficient for the complete dissipation of pyrene in both treatments or the lack of pyrene biodegraders was responsible for this difference.

There was also no significant difference ($p>0.01$) between the dissipation of PAHs in the treatment with *Brachiaria serrata* and the treatment with *Eleusine corocana*. As plant species influence the development of microbial communities inhabiting the root zone (Rovira, 1956; Rovira, 1959), it was expected that the dissipation of PAHs in treatments vegetated by different plant species will be different. This expectation emanated from the fact that the two grasses have slightly different root structure and also due to the probable difference in the root exudates, which may influence the 'competency' of the rhizosphere microbial community inhabiting the plants. However, this was not realised, as there was no significant difference ($p>0.01$) in PAH dissipation between treatments with *B. serrata* and treatment with *E. corocana*. This finding was in contrast to findings by Muratova *et al.* (2003), who reported differences in the degradation of PAH with different plant species. However, alfalfa and reed species were used in their study while different grass species were used in this study.

The data on enhanced dissipation of both naphthalene and fluorene in multi-planted treatments compared to mono-planted treatments suggests that phytoremediation can be enhanced by 'bioaugumenting' moderately contaminated soil with multi-plants instead of monocultures. However, as many organisms are known to produce toxins designed to minimise competition (Curl and Truelove, 1986), plants that are able to coexist or the so-called co-occurring plants, should be evaluated for their potential to enhance rhizoremediation of PAHs. Also longer-term studies should be considered to corroborate these results.

The metabolic fingerprints of the PAH-contaminated soils were different from the metabolic fingerprints of the non-contaminated soils. This can be attributed to the change in microbial diversity due to the presence of PAH. The availability of hydrophobic

pollutants has been reported to cause significant changes in soil microbial communities (Saxton and Atlas, 1977; Atlas *et al.*, 1991; Wünsche *et al.*, 1995). The metabolic fingerprint of the PAH-contaminated soil was more closely related to the contaminated mono-planted treatment with *Brachiaria serrata* and contaminated multi-planted soil with *Brachiaria serrata* and *Eleusine corocana* than to other treatments. This can be attributed to the 'similarities' in metabolic diversity of the contaminated treatments caused by the presence of the stressor (PAHs).

The functional diversities of the different treatments were further evaluated using hierarchical clustering. In a dendrogram, the contaminated soils (CS, CSBS and CSBEC) were clustered together while CSEC was clustered with the uncontaminated soils. These results were perplexing as it was expected that CSEC, a planted contaminated soil was going to be clustered with other contaminated soil due to presence of the stressor, which can cause significant changes in soil microbial communities (Rathbone *et al.*, 1998).

In conclusion, plant–mediated dissipation of PAHs was enhanced in the multi-planted treatments compared to the mono-planted treatments. However, enhanced PAH dissipation in the multi-planted treatments compared to the mono-planted treatments did not correspond to high functional diversity as revealed by the number of Biolog substrates used. The results suggest that multi-plant rhizoremediation should be considered when evaluating remedial approaches for moderately contaminated soils. The results further strengthen the suggestion that 'bioaugumentation' in moderately contaminated soils can be achieved by using multi-planted instead of monocultures to enhance the competence of the soil bacteria. However, further work using both functional and molecular techniques is required to understand microbial diversity in

multi-planted soil treatments, particularly as root exudates by one plant species may suppress or encourage the bacterial species 'predominant' when each plant grows in isolation.

Acknowledgement

We thank the National Research Foundation and RCN of Norway (project no 152243/730) for funding this project.

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Chapter 6

GERMINATION OF *LEPIDIUN SATIVUM* AS A METHOD OF EVALUATING THE REMOVAL OF POLYAROMATIC HYDROCARBONS (PAHS) FROM CONTAMINATED SOIL

A modified version of this text was published as:

Mphekgo P. Maila[a], Thomas E. Cloete[b] (2002) Germination of *Lepidium sativum* as a method to evaluate polycyclic aromatic hydrocarbons (PAHs) removal from contaminated soil. International Biodeterioration and Biodegradation 50: 107-113.

University of Pretoria etd – Maila, M P (2005)

GERMINATION OF *LEPIDIUM SATIVUM* AS A METHOD TO EVALUATE POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) REMOVAL FROM CONTAMINATED SOIL

Abstract

The sensitivity of *Lepidium sativum* germination to polycyclic aromatic hydrocarbons (PAHs) was investigated in soil(s) artificially and historically contaminated with mixtures of PAH. The level of germination of *L. sativum* decreased with increasing concentration of the PAH in the artificially contaminated soil, while no germination occurred in the historically polluted soil. At a concentration of 1000 and 50 ppm, the germination levels were <16% and >75%, respectively. The same germination levels, as a function of PAH concentration, were observed when a non-ionic surfactant was present in the soil(s). When used during phytoremediation of PAH, the germination level of *L. sativum* was inhibited during the first weeks, after which germination increased, possibly due to PAH dissipation from the soil. The data suggest that the germination of *L. sativum* can be used to monitor the removal of PAH pollutants from soil.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are pollutants. They occur as common constituents of petroleum, coal tar and shale oil, but are most frequently formed by incomplete combustion of fossil fuels (Pothuluri and Cerniglia, 1994). Their fate in nature is of great environmental concern due to their toxic, mutagenic and carcinogenic properties (LaFlamme and Hite, 1978; Pahlmann and Pelkonen, 1987).

Although GC-MS analysis of PAH has been successfully used to determine the concentration of PAH during the remediation of the pollutants in soil, the methodology remains expensive and requires a high degree of skill in the use of the instruments.

Previously, it was indicated that photomodified PAH had a marked inhibition of root fresh weight of the Canola plant (Ren *et al.*, 1996). The phytotoxicity of these pollutants could, therefore, possibly be used to establish a monitoring method that would indicate the presence of this compound in soil. This suggested that germination efficiency of selected plants could be used as a bioindicator of pollutants.

In this study, our objective was to select and use an appropriate plant with a short germination period and to evaluate it as a potential bioindicator of PAH pollution. In this study, we investigated the germination efficiency of *Lepidium sativum* exposed to different concentrations of PAH in both the presence and absence of a surfactant in soil. Surfactants have been shown to increase the desorption and bioavailability of PAH in soil (Aronstein *et al.*, 1991). We also evaluated the possibility of using germination of *L. sativum* as a bioindicator of PAH removal from contaminated soil.

Materials and methods

Chemicals: All solvents (dichloromethane 99% and trichloro-methane 99%), non-ionic surfactant (Triton X-100) and PAH (naphthalene 98%, pyrene 98%, coronen 98%, phenanthrene 98% and anthracene 98%) were purchased from Sigma-Aldrich, SA.

Soil: The soils used in the experiments were labeled A, B, C and D. Soil A was a predominantly sandy loam soil taken from the CSIR site (Pretoria, SA), soil B was an industrial soil taken from an industrial oil site (Secunda, South Africa), soil C was also an industrial soil (Kwazulu-Natal, SA) containing ilmenite (6%), rutile (0.4%), zircon (1%), leucoxene (0.3%), magnetite (1%) and kyanite (1%). Soil D was a white playpen sand bought from Lion Bridge SA (Pty) Ltd. Soils C and D were used in the experiment to test the germination of *L. sativum* in different soils that are not contaminated with PAH. The industrial soil (soil B) consisted of heterogeneous soil material from an oil refinery with a contamination of ≈1.2 g PAH per kg soil. The artificial contamination of soil A with PAH was done as described by Leyval and Binet (1998).

Plants: The seeds of white buffalo grass (*Panacum maximum*) were purchased from AGRICOL (Pty) Ltd, SA and those of *L. sativum* were purchased from Lion Bridge (Pty) Ltd, SA. The grass seeds were sown in trays (38 cm * 38 cm) containing soil A. The seedlings of *P. maximum* were used in the phytoremediation experiment (vegetated microcosm) while *L. sativum* seeds were used as potential bioindicator of PAH removal.

***L. sativum* bioindicator:** The seeds of *L. sativum* were exposed to different concentrations (50, 150, 300, 500 and 1000 ppm) of PAHs in soil A. For each treatment, 75 mℓ of deionised water was added to 375 g soil to bring the soil moisture to 75% field capacity (14% on wet weight). The soil was mixed and then divided into three large

polystyrene Petri dishes (150 mm * 25 mm) after which 50 seeds of *L. sativum* were sown in each Petri dish. Three replica plates were used for each treatment. The plates were placed next to the window allowing sufficient light for photosynthesis. After 3 days, the seedlings were counted. The sensitivity of *L. sativum* germination to PAH was assessed by seedling count as well as by the weight of the seedlings. Germination was defined as a visible cracking of the seed coat with a measurable root or shoot production. Five seedlings from each of the three plates were weighed and the average taken. The above experiment was repeated with the addition of Triton X-100 (a surfactant) at a concentration of 100 μg/g soil. The treatment in which PAHs were absent, as well as the treatment containing Triton X-100 with no PAH served as controls. The germination of *L. sativum* was also evaluated using different soil types. The germination procedure was repeated using the uncontaminated soil A, industrial soils (B and C) as well as soil D. The statistical processing was performed with SPSS for Windows release 7.5.2.

Table 1: Treatments used during Rhizoremediation

Treatments	Additions/preparations
T0 (Control)	Soil A + 100 μg/g Triton + grass
T1 (Treatment 1)	Soil A + 100 μg/g Triton + PAH(1000 ppm) + grass
T2 (Treatment 2)	Soil A + 100 μg/g Triton + PAH(1000 ppm)
T3 (Treatment 3)	Soil A + PAH (1000 ppm)
T4 (Treatment 4)	Diluted Soil B + 100 μg/g Triton + grass
T5 (Treatment 5)	Diluted Soil B + 100 μg/g Triton

Vegetated and non-vegetated microcosms: The use of *L. sativum* germination as a bioindicator of PAHs removal from contaminated soil was evaluated during

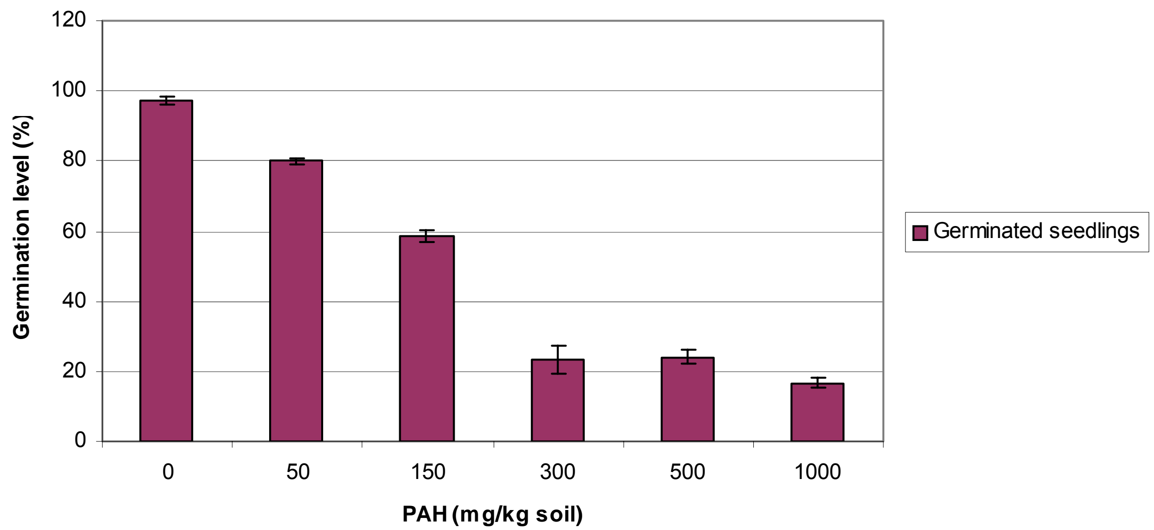
phytoremediation of PAH in different soils (table 1). Treatments 4 and 5 were prepared by mixing 350 g of soil B with 150 g of soil A to make the diluted soil B shown in the table. PAHs were added to the soil as described earlier. Five hundred grams of each soil preparation (table 1) were placed in pots with saucers for leachate collection. Two seedlings of the white buffalo grass were planted in each pot and the pots placed in the green house at ambient temperature and natural day–night cycles. Hundred milliliters of water was used to water the plants every 2 days. In instances were leachates were produced, the leachate was used to water the same pots. Each treatment had 10 pots and one pot for each treatment was sacrificed every week and the *L. sativum* germination method carried out as described previously. Three replicates plates were used for each treatment.

Chemical analysis: Residual PAH in treatments T1, T2 and T3 were quantified after extraction with dichloromethane, using a Varian Saturn 2000 Ion Trap Gas Chromatography/Mass Spectrometer equipped with a Chrompack CP-SIL 8CB-MS (5% phenyl) Fused Silica Capillary Column (30 m* 0.25 mm* 0.25 μm). The detector was tuned according to EPA 8270C using DFTPP. Injector temperature was 230°C, oven temperature program: 30°C (6 min), 10°C/min, 300°C (7 min). Chemical analysis was not carried out for T4 and T5.

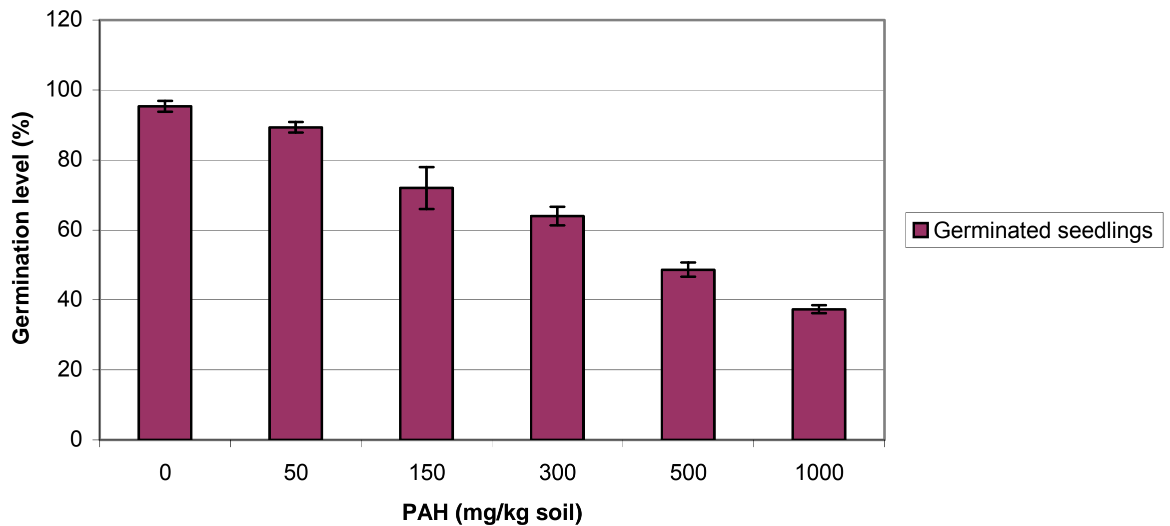
Results

L. sativum bioindicator

The ability of *L. sativum* seeds to germinate in soil contaminated with different concentrations of PAHs was investigated. Germination levels of the seeds decreased with an increase in the concentration of the PAH (Figure 1a). At higher PAH concentration (1000 ppm), the germination level of *L. sativum* was lower by a factor of



(a)



(b)

Figure 1. The level of germination of *L. sativum* in soil contaminated with different concentrations of PAH: (a) germination of *L. sativum* in the absence of the surfactant (Triton X-100) and (b) germination levels in the presence of surfactant (100 μg/g soil). The surfactant was added to make the PAH bioavailable. Error bars represent standard deviations. There was a significant difference between the treatments used (P<0.05).

three, compared to germination levels at 50 ppm. The presence of Triton X-100 in the soil did not increase the toxicity of the PAH to seed germination (Figure 1b) as the surfactant by itself had no effect on the seed germination. The level of germination also decreased with increasing concentration of PAH. The effect of PAH on the fresh weight of the seedlings was also investigated in the experiment. The seedling fresh weight of *L. sativum* decreased with an increase in the concentration of PAH (Figure 2a and b). At higher PAH concentration, the weight of the seedlings was lower by a factor of two compared to the seedlings exposed to lower PAH concentration.

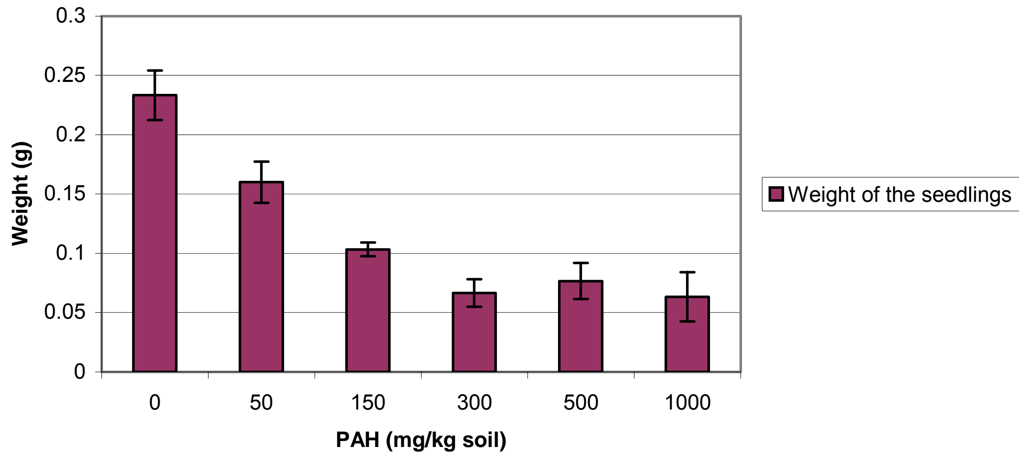
Germination of *L. sativum* in different soils shows that, where PAHs are absent, the level of germination exceeds 95% while in the case where the concentration of PAH are high (1.2 g/kg soil as in soil B), no germination of *L. sativum* occurs (Figure 3). The composition of soil C did not appear to have any inhibition on the germination of *L. sativum* seeds, as germination levels were comparable to the levels in soils with no PAH (A and D).

Germination over time in vegetated and non-vegetated soils

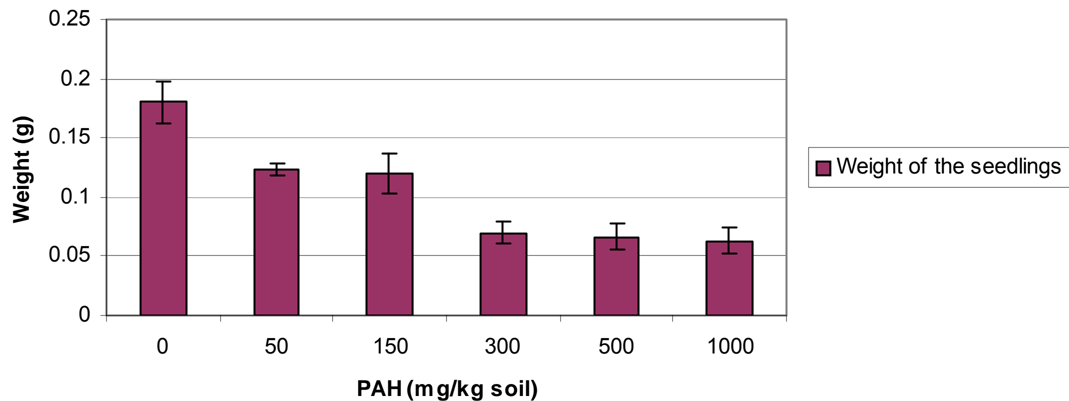
The potential of *L. sativum* germination as a bioindicator of PAH removal was investigated during phytoremediation of soil contaminated with PAH. Soil A was artificially contaminated with PAH mixtures of naphthalene, anthracene, phenanthrene, coronen and pyrene and the grass seedlings of *P. maximum* were planted in treatments T0, T1 and T4.

The level of germination in the soil of vegetated treatment (T4) was significantly ($p<0.05$) higher compared to germination levels in the non-vegetated treatment T5 (Figure 4). However, there was no significant difference in the germination level of both the vegetated treatment (T1) as well as the non-vegetated treatments (T2 and T3) containing the artificially contaminated soil A. The data suggest that *P. maximum* may

have a positive effect on the removal and or detoxification of the PAH from the soil. The extent of PAH disappearance in vegetated soil is significantly greater than in unvegetated soil (Aprill and Sims, 1990). Initial germination levels were below 15% but increased with the time of the experiment, possibly due to the reduction in the concentration of PAH in the soil (table 2).



(a)



(b) Figure 2. The fresh weight (wet) of *L. sativum* seedlings in the presence of different concentrations of PAH in soil A: (a) the seedlings fresh weight *of L. sativum* germinated in soil with no Triton X-100 and (b) seedling fresh weight of *L. sativum* in soil with Triton X-100.

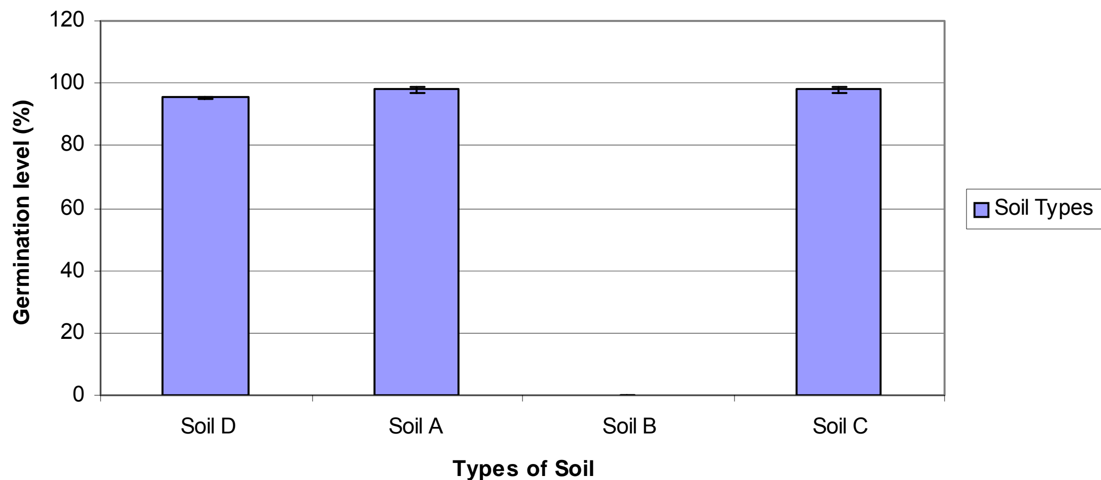


Figure 3. Germination of *L. sativum* in different soil types. Soil A (sandy loam soil not contaminated with PAH), soil B (historically PAH polluted industrial soil), soil C (soil contaminated with heavy minerals but with no PAH), soil D (white playpen sand free of PAH). Error bars represent standard deviations. There was no significant difference between the treatments in which germination occurred ($p>0.01$).

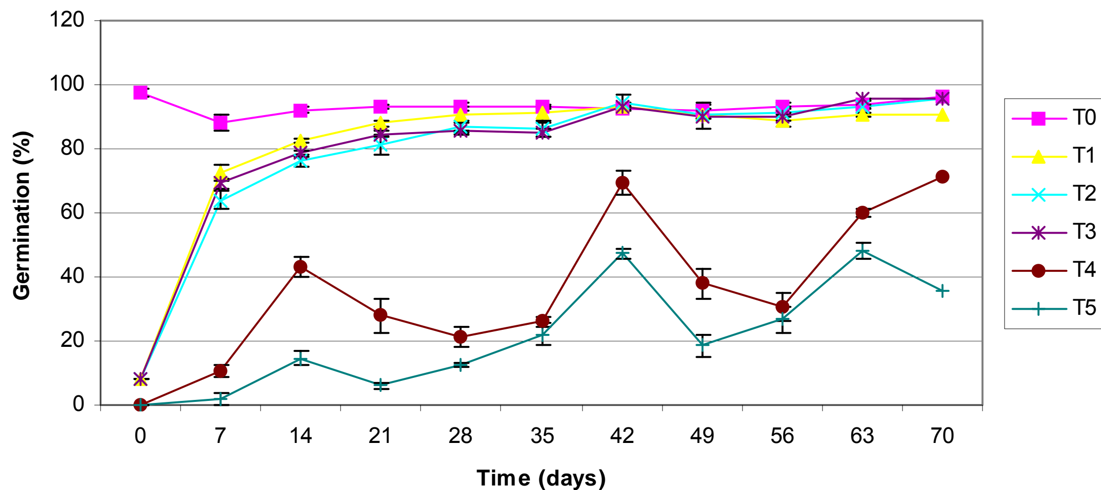


Figure 4. *L. sativum* germination during phytoremediation of contaminated industrial soil as well as soil artificially contaminated with PAH. Error bars represent standard deviations. T1 was not significantly different from T2 and T3. However, T4 was significantly different from T5 ($p<0.05$).

Table 2: PAH Concentrations (mg/kg soil) in planted and non-planted soil using EPA analytical method 8270C

Days of Incubation	Naphthalene			Fluorene			Phenathrene			Anthracene			Pyrene		
	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
7	143	106	197	433	536	572	679	757	711	737	696	703	720	824	696
42	2.46	3.81	20.4	82.7	146	183	296	510	502	571	437	512	335	402	397
70	0.82	0.52	1.7	17.1	20.3	37.4	50.4	56.5	63.4	182	135	193	184	205	211

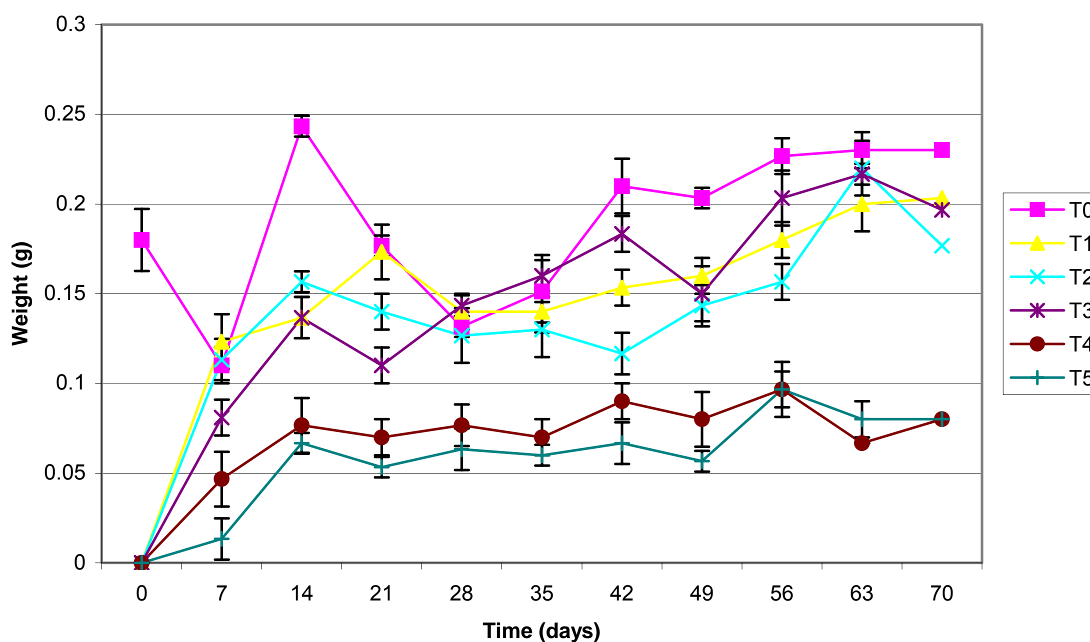


Figure 5: The average weight (wet) of *L. sativum* seedlings during phytoremediation of PAH in contaminated soil. T0-uncontaminated soil A with Triton X 100 and grass, T1-contaminated soil A with Triton X 100 and grass, T2-contaminated soil A with Triton X 100, T3-contaminated soil A, T4-diluted soil B with Triton X 100 and grass, T5-diluted soil B with Triton X 100.

The effect of PAH on the weight of the seedlings of *L. sativum* was also investigated over an experimental period of 70 days (Figure 5). As with the germination of the

seedlings, the weight of the seedlings germinated in the respective soils of the vegetated treatments gave similar results. The weight of the seedlings in the industrial soil (soil B) remained low throughout the experiment, possibly because of toxicity due to the presence of recalcitrant PAH.

Discussion

PAH phytotoxicity has been described as a physiological toxicity (Bossert and Bartha, 1985; Huang *et al*., 1996; Ren *et al*., 1996), and an indirect effect on the ability of the contaminated soil to provide water and nutrients to the plants (Reilley *et al*., 1996). In this study, *L. sativum* showed sensitivity to PAH as the level of germination decreased with an increase in the concentration of the PAH. The level of germination in soil with no surfactant (Triton X-100) was significantly lower ($p<0.05$) compared to the level of germination in soil with Triton X-100. As light dramatically enhances the toxicity of PAH (Ren *et al*., 1994), the presence of the surfactant in the soil might have interfered with the absorption of radiation by the PAH and thereby rendering the PAH less toxic. This was contrary to the findings that bioavailability/desorption of PAH in treatments with surfactant tends to be higher at certain surfactant concentration compared to treatments with no surfactant (Aronstein *et al*., 1991). According to Guha *et al.* (1998), for a given surfactant concentration, the bioavailability appears to be higher for the lower molecular PAH and there is very little difference in the bioavailability of the same compound as single solute or in different binary or ternary mixtures. The findings may be explained thus, if the PAH were of low molecular weight.

The fresh weight of *L. sativum* seedlings also decreased with an increase in the concentration of PAH in the soil. At a concentration of 300 ppm, the weight of the seedlings was lower compared to the control (0 ppm). There were no noticeable

differences in the seedling weight of *L. sativum* in both the absence and the presence of Triton X-100. PAH have been suggested to cause germinated seeds to produce fewer roots (Ren *et al*., 1996). At the early stages of plant development, root growth is due primarily to cell expansion and not cell division (Taiz and Zeiger, 1991). Cell expansion is probably being impeded, which could for example, be by inhibition of hormone action (auxin) or interference with cellular metabolism (e.g. mitochondrial function).

The data on the germination of *L. sativum* in different soils shows that where PAHs are absent, the level of germination exceeds 95% while in the case of soil B, no germination of *L. sativum* occurs. The composition of soil C did not appear to have any inhibition on the germination of *L. sativum* seeds as germination levels were comparable to the levels in soils with no PAH (soils A and D). The data suggest that *L. sativum* germination is a potential PAH bioindicator.

The data of phytoremediation showed that plants may be playing a significant role in the removal and or detoxification of PAHs in the soil. Germination level of *L. sativum* in soils of vegetated treatment (T4) was significantly higher compared to the germination level in treatment T5 while there was no significant difference between the vegetated treatment (T1) and the non-vegetated treatments containing artificially contaminated soil A. The data suggest that *P. maximum* enhanced the detoxification and or dissipation of the PAH in the soil. Vegetated treatments enhance pollutant removal in soil compared to non-vegetated treatments possibly due to the increased microbial activity as well as the deposition of root exudates in the rhizosphere (Siciliano and Germida, 1999).

The germination level of *L. sativum* seeds, increased with the reduction of the PAHs in the soil artificially contaminated with the PAHs. The level of germination in the artificially

contaminated soil was nearly the same as the germination level in the control treatment after 3 weeks of the experiment, possibly due to the dissipation of PAH in the soil.

The methodology based on the sensitivity of *L. sativum* (that has a short germination period) to PAH can be used as a monitoring tool in remediation treatments of soil contaminated with PAH. The methodology should be further developed to gain more knowledge on aspects of bioavailability of PAH in both the aged as well as the freshly spiked soil. As one can never be sure if only a specific pollutant inhibits seed germination, it is important to know if the bioindicator is also sensitivity to other pollutants (e.g. heavy metals), which are most likely to occur in the presence of the PAHs. This bioindicator is very useful to examine the bioavailability of pollutants in contaminated soil, however, extra chemical analysis are needed to complement the methodology.

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University of Pretoria etd – Maila, M P (2005)

Chapter 7

THE USE OF BIOLOGICAL ACTIVITIES TO MONITOR THE REMOVAL OF FUEL CONTAMINANTS: PERSPECTIVE FOR MONITORING HYDROCARBON CONTAMINATION

A modified version of this text was accepted for publication as:

Mphekgo P. Maila, Thomas E. Cloete (2003) The use of biological activities to monitor the removal of fuel contaminants: Perspective for monitoring hydrocarbon contamination. International Biodeterioration and Biodegradation.

THE USE OF BIOLOGICAL ACTIVITIES TO MONITOR THE REMOVAL OF FUEL CONTAMINANTS: PERSPECTIVE FOR MONITORING HYDROCARBON CONTAMINATION

Abstract

Soil biological activities are vital for the restoration of soil contaminated with hydrocarbons. Their role includes the biotransformation of petroleum compounds into harmless compounds. In this paper, the use of biological activities as potential monitoring tools or bioindicators during bioremediation of hydrocarbon-contaminated soil are reviewed. The use of biological activities as bioindicators of hydrocarbon removal in soil has been reported with variable success. This variability can be attributed partially to the spatial variability of soil properties, which undoubtedly plays a role in the exposure of organisms to contaminants. Widely used bioindicators have been enzyme activities, seed germination, earthworm survival and microorganisms or microbial bioluminescence. A mixture of some successful utilization of biological activities and several failures and inconsistencies reported, shows that at this stage there is no general guarantee for a successful utilization of biological activities as monitoring tools. Wherever possible, the use of biological activities as bioindicators of hydrocarbons removal must be used to complement existing traditional monitoring tools.

Introduction

The increasing concern about the cost of soil remediation has necessitated the need to explore not only cost effective technologies but also alternative monitoring tools. Conventional chemical analytical instruments like GC-MS usually monitor the progress of remediation of hydrocarbon-contaminated soil, which can be expensive. Due to the

cost associated with traditional monitoring tools, focus is now shifting towards using biological activities for monitoring of bioremediation of hydrocarbon-polluted soil. The use of bioindicators to evaluate hazardous chemical waste sites provides a direct, inexpensive and integrated estimate of bioavailability and contaminant toxicity (Mueller *et al.*, 1991; Wang and Freemark, 1995, Maila and Cloete, 2002). Table 1 summarises the advantages and disadvantages of bioindicators.

Many promising approaches using bioindicators as monitoring instruments have been reported (Athey *et al.*, 1989; Siciliano *et al.*, 1997; Dorn *et al.*, 1998; Marwood *et al.*, 1998; Margesin *et al.*, 1999, Maila and Cloete, 2002).

Table 1: Advantages and disadvantages of using bioindicators as monitoing instruments

Advantages	Disadvantages
• Can detect both toxicity of parent compounds and toxic metabolites; • Readily available materials are required to do the test; • The test can be performed ex or *in-situ*; • The test period in most cases is short; • Uncomplicated methodology is used to assess the extent of pollution reduction.	• Inability to distinguish toxicity resulting from parent compound and metabolites; • Bioindicator response don't always correspond with contaminant concentration; • Different tests respond differently to individual toxicants; • Sensitivity depends on the toxicant and soil (i.e. the test can be sensitive to other factors of the soil).

The list of bioindicators that have been tested as potential monitoring tool of hydrocarbon removal is shown in table 2. These approaches include the use of enzymatic activities, seed germination, earthworm survival and microorganisms or microbial bioluminescence as bioindicators. Both these biological processes have varying degrees of success as monitoring tools. Reliable bioindicators must give interpretable response curves across a range of environmental parameters (Adema and Henzen, 1989; Hund and Traunspurger, 1994), otherwise, environmental effects upon bioindicator response may confound extrapolations meant to depict the bioavailability and toxicity of contaminants in soil.

This paper reviews the types of potential bioindicators, including enzymes, seed germination, earthworm survival and microbial bioluminescence used for monitoring the remediation of soil contaminated with petroleum compounds.

Enzymes

Soil enzymes activities are attractive as indicators for monitoring various impacts on soils because of their central role in the soil environment: soil enzymes are the catalysts of important metabolic process functions including the decomposition of organic inputs and the detoxification of xenobiotics. Besides hydrocarbons, soil biological activities have been used as biological indicators of pollution with heavy metals or pesticides (Bayer *et al.*, 1982; Dick, 1997; Top *et al.*, 1999).

The degradation of hydrocarbons to simple molecules like water and carbon dioxide involves many chemical reactions in which catalytic proteins are involved. Because of their central role in hydrocarbon degradation, it is not surprising that focus is now shifting

towards using them as potential monitoring tools during bioremediation. Enzymes that have been tested for their potential to monitor hydrocarbon removal include soil lipases, dehydrogenases, catalases and ureases (table 3). However, their use has been confined to lab studies.

The lab-scale studies show great potential for the use of these proteins as bioindicators of hydrocarbon removal. Of the catalytic proteins tested, soil lipases showed great potential in monitoring bioremediation of hydrocarbon (Margesin *et al.*, 1999). The dehydrogenases, catalases and urease were found only to be useful to indicate the onset of the biodegradation process as their activities declined rapidly after the biodegradation rate has decreased (Frankenberger and Johanson, 1982; Janke *et al.*, 1992; Van der Waarde *et al.*, 1995; Margesin and Schinner, 1997).

Table 2: Different bio-indicators that were used in monitoring hydrocarbons removal

Bioindicator	**Pollutant specificity**	**Sensitivity and Range Tested**	**References**
Enzymes Soil Lipase	Diesel oil, mineral oil	Sensitive. Up to 1 mg/g soil	Margesin *et al.*, 1999; Margesin *et al.*, 2000
Soil Dehydrogenase	Crude oil and refined petroleum products	Moderately sensitive. 20-60% w/w oil/dry soil.	Frankenberger and Johanson, 1982
Urease & catalase	Mineral oil	Less sensitive. Detectable at high TPH concentration (5000 mg/kg soil)	Margesin *et al.*, 2000
Seed germination Prairie grass (Canada blue grass & Slender wheatgrass	Aromatics (Halogenated)	Sensitive. 13-133 µg/kg soil	Wang and Freemark, 1995; Siciliano *et al.*, 1997
Lepidium sativum	PAHs	Moderately sensitive 50 – 1000 mg/kg soil	Maila and Cloete, 2002
Microbial biomass	Oil contaminated soil	Moderately sensitive	Kandeler *et al.*, 1994
‘**Batteries’ of bioindicators** Microbial bioluminescence, earthworm & seed germination	Creosote, Heavy, medium and light crude oils.	Moderately sensitive. Earthworm>seed germination> bioluminescence. 25 - 17400 µg/g soil.	Wang and Freemark, 1995; Dorn *et al.*, 1998; Marwood *et al.*, 1998; Phillips *et al.*, 2000; Shakir *et al.*, 2002

In addition, the increase in soil dehydrogenase activity in hydrocarbon-contaminated soil was in proportion to the rates of oil application in that activity increased with increasing loading rates (Frankenberger and Johanson, 1982). Any influence the oils may have on soil dehydrogenase activity is dependent on the chemical composition of the oil itself. In his review Cole (1983), showed that oxygenation is a common process in pesticide and herbicide metabolism and it is an important initial mode of attack when organisms encounter what are often highly lipophilic compounds. However, not much work has been done on this particular process as a potential bioindicator of pollutant removal in soil.

Table 3: Measurements of enzymatic activities in hydrocarbon contaminated soil

Process	Enzyme	Methodology	References
Hydrocarbon mineralisation	Soil Lipase	Titration	Porkona, 1964; Schinner *et al.*, 1996; Margesin *et al.*, 1999
	Soil dehydrogenases	Spectrophotometer (Color intensity measurement)	Stevenson, 1959; Frankenberger and Johanson, 1982
	Catalases	Titration	Margesin and Schinner, 1997
	Ureases	Colorimetry	Margesin and Schinner, 1997

Most enzymatic tests are artificial and refer to the potential activity of soil enzymes. A defined amount of soil is incubated in aqueous environment with specific substrate and sometimes a buffer (Bitton and Koopman, 1992). The enzyme converts the specific substrate to another compound that can be extracted and quantified by spectroscopy for example. For lipases, tributyrin is used as a substrate, which is catalytically converted to butyric acid, which can be

extracted and quantified (table 3). The main advantage of enzymatic tests is the easy way of measuring but a disadvantage might be their indirect approach.

Not all enzymes are synthesized by a cell in the same amounts; some enzymes are present in far greater copy number than others. In bacterial cells, regulation of enzyme amount by the phenomenon called induction and repression occurs at the gene level whilst the enzyme activity is regulated through product inhibition, covalent modification and feed back inhibition (Brock and Madigan, 1991). The required amount of hydrocarbons in soil that can induce the necessary enzymes to bring about the metabolism of specific hydrocarbons is not known. However, based on the ATP required to synthesise the proteins of bacterial cells and the diffusion, limited by the volume of water surrounding cells, it is estimated that at least about 150 mg of substrate per liter of soil water should be present (Sims *et al.*, 1991).

Microorganisms

The immediate concern of the rehabilitation practitioners when assessing the strategy and outcome of bioremediation is the availability and capacity or degradative potential of the autochthonous microbial communities. The use of microorganisms as monitoring instruments of hydrocarbon-contaminated soil is not well established. However, microbial bioluminescence, microbial biomass/counts and soil respiration have been evaluated as potential monitoring tools of hydrocarbons (Delistraty, 1984; Kandeler *et al.,* 1994; Steinberg *et al.*, 1995; Van Beelen and Doelman, 1997; Phillips *et al.*, 2000).

Microbial bioluminescence involves the activities of electron transport systems, which produce substrates for the production of light. This monitoring tool has been evaluated as a potential bioindicator of a number of organic compounds (table 4).

Table 4: The use of bioluminescence to monitor/detect hydrocarbons

Organisms/System	Primary reported compound/class	Reference
Photobacterium phosphoreum	Creosote	Phillips *et al.*, 2000
Pseudomonas fluorescens	Naphthalene	King *et al.*, 1990; Heitzer *et al.*, 1994
P phosphoreum	Organics	Kaiser and Palabrica, 1991
P phosphoreum, V harveyi	Synfuel by-products	Delistraty, 1984

The use of bioluminescence is attractive since it more closely reflects toxicity than does the use of chemical analysis (Steinberg *et al.*, 1995). In addition, the application of bioengineering to produce or enhance bioluminescence properties of organisms may lead to new systems for assessing environmental toxicity. The disadvantage of using bioluminescent is the possibility of bacteria adsorbing to the soil particles and thereby being filtered out of suspension, resulting in lower luminescence than would correctly represent the level of soil toxicity (Hund and Traunspurger, 1994; Benton *et al.*, 1995; Cook and Wells 1996; Ringwood *et al.*, 1997). For further information on bioluminescence, the reader is referred to Steinberg *et al.* 1995.

It is widely assumed that the number of indigenous biodegraders increases with the reduction of hydrocarbons and that microbial population changes after hydrocarbon pollution. Wünsche *et al.* (1995), reported that changes in hydrocarbon content in soil resulted in characteristic shifts of the substrate utilization patterns by the microorganisms and that the altered pattern of substrate utilization corresponded with similar changes in abundance of hydrocarbon-utilizing bacteria and the occurrence of specific bacterial groups in the soils. Increases of hydrocarbon degrading bacteria during bioremediation have been reported elsewhere (Pearce *et al.*, 1995; Margesin *et al.*, 1999).

The concern with using microorganisms as bioindicators is that changes in bacterial numbers might be an indicative of a stimulated biodegradation process, but it does not necessarily represent an accurate measurement of the actual biodegradation process. In addition, the added biodegradative strains (in bioaugumentation process) are notoriously unreliable in their ability to compete with native microorganisms when released into the natural environment (Van Veen *et al.*, 1997). There are cases, however, where microorganisms have been used with relative success in detecting the removal of hydrocarbons in soil (Steinberg *et al.*, 1995).

Microbial processes have also been used elsewhere in monitoring pesticide removal from the soil (Top *et al.*, 1999). The most widely used microbial process to detect biotoxicity and biodegradation of contaminants is respiration (Martin *et al.*, 1978; Weissenfels *et al.*, 1992; Margesin *et al.*, 2000; and many others). This process cannot be used reliably, however, to monitor hydrocarbons removal, as it is difficult to distinguish biological hydrocarbons removal from other decomposition of soil organic compounds. Microbial respiration in soil is usually evaluated using respirometer and through titration. Phillips *et al.* (2000), evaluated six soil toxicity tests (including Microtox) to monitor bioremediation in creosote contaminated soil and found that the toxicity testing results did not always correlate with contaminant concentrations, nor were the trends indicated by each test consistent for any of the soil types used in the study.

Plants and Earthworms

Two tests that are widely used for measuring soil toxicity are the seed germination and earthworm survival assays (Green *et al.*, 1988). Other tests for water have been adapted for soil (Kwan and Dutka, 1992; Quillardet and Hofnung, 1993; Dutka *et al.*, 1995; Cook and Wells, 1996; Ringwood *et al.*, 1997). Seed germination and earthworm survival assays does also have the potential to be used as bioindicators of hydrocarbons removal in soil. The

sensitivity of earthworms to hydrocarbon-contaminated soil has been reported elsewhere (Shakir *et al.*, 2002). Earthworm survival and seed germination tests are sensitive to changes in soil toxicity during bioremediation of PAH and petroleum hydrocarbon contaminated soil (Athey *et al.*, 1989; Dorn *et al.*, 1998; Marwood *et al.*, 1998; Knoke *et al.*, 1999).

Seed germination and earthworm survival tests are useful as bioindicator response endpoints because of their simple methodology, moderate sensitivity to toxicants and their potential to be used both in situ and ex situ. The use of these tests as potential bioindicators has, however, only been confined to lab scale studies. Maila and Cloete (2002), reported that the level of germination of *Lepidium sativum* decreased with increasing concentration of the PAH in the artificially contaminated soil, while no germination occurred in the historically PAH polluted industrial soil. When used during phytoremediation of PAH, the germination level of *L. sativum* was inhibited during the first weeks, after which germination increased, possibly due to PAH dissipation from the soil.

Bioindicator response to organic pollutants varies in different plant species. Siciliano *et al.* (1997), reported a 12-fold difference in 2CBA (2-chloro benzoic acid) and 10-fold differences in Aroclor 1260 sensitivity among grass species. According to Cairns (1993), and Chapman (1995), the use of indigenous species (as bioindicators) will increase the relevance and reliability of bioindicator testing.

Dorn *et al.* (1998), evaluated the sensitivity of earthworm, microbial bioluminescence and seed germination to oil contaminated soil. Earthworms were 1.4 to 14 times more sensitive than microbial bioluminescence and 1.3 to >77 times more sensitive than seed germination to the oily soils. Overviews on the use of plants (vascular macrophytes) phytotoxicity testing and its role in environmental monitoring and assessment are available elsewhere (Wang and Freemark, 1995).

Phillips *et al.* (2000), reported that although total petroleum hydrocarbons (TPHs) in the soil was reduced following treatment, results of the earthworm and seed germination tests still showed an increase in toxicity, an indication that toxic intermediary metabolites may have formed during biodegradation.

Influence of technology and environmental conditions

The use of biological activities to evaluate hazardous chemical waste sites provides a direct, inexpensive, and integrated estimate of contaminant toxicity (Mueller *et al.*, 1991; Wang and Freemark, 1995). However, it appears that apart from the pollutants, remediation technologies do have an effect on the bioindicator response (Siciliano *et al.*, 1997; Margesin *et al.*, 1999).

Biostimulation of hydrocarbon-contaminated site had an effect on the activity of the extracellular enzyme lipase (Margesin *et al.*, 1999). The presence of inorganics (N and P) accelerated the activity of the extra-cellular enzyme lipase.

Biological treatments can have a negative effect on the bioindicator response (Belkin *et al.*, 1994; Hund and Traunspurger, 1994; Siciliano *et al.*, 1997). Siciliano *et al.* (1997), reported that the effect of soil type on bioindicator response varies in different plants. The effect of soil types on bioindicator response of Canada blue grass to 2CBA (2-chlorobenzoic acid) was significant while no significant difference was observed in the germination response of wheat grass. Differences in either one of the soil components will alter the toxicological hazard associated with a contaminated site. Implicit in this measurement of bioavailability and toxicity is the independence of bioindicator response to other organisms in the ecosystem. It is well documented that many organisms produce toxins designed to minimize competition (Curl and Truelove, 1986). It is thus important to understand the effect of the treatment technology and environmental conditions on bioindicator response.

Soil components like organic matter; pore space and pH play an important role in pollutant bioavailability in soil (DeVliegher and Verstraete, 1996). It is estimated that about one third of the initial contamination is lost through abiotic process such as volatilisaton, sorption processes and chemical transformation (Margesin *et al.*, 1999). Bioavailability, degradation and toxicity of soil contaminants are all influenced by sorption, which is influenced by time, and the physico-chemical properties of individual soils (Manilal and Alexander, 1991; Weissenfels *et al.*, 1992; Erickson *et al.*, 1993; Loehr and Webster, 1996; White and Alexander, 1996).

Effective bioindicators require a rapid and reliable methodology that characterizes the extent of contamination, minimizes worker exposure, and reduces artifacts induced by sampling the soil. Bioindicators integrate measurement of contaminant bioavailability and toxicity.

Conclusion

A number of methods exists that can be used to assess the extent of hydrocarbon contamination in soil. However, the uniqueness or heterogeneity of the soil, formation of toxic metabolites and the influence of technology contribute towards 'poor' bioindicator response of the different biological activities. Chemical and toxicity data do not always corroborate one another nor do the results of each toxicity test in a battery always agree due to the fact that each soil is unique in the response it induces and each toxicity test unique in its ability to detect different contaminant levels in different soils.

There is therefore a need to understand the influence of these factors on bioindicator response. For enzymes to be successfully used as monitoring tools during bioremediation of hydrocarbons, enzyme induction by the pollutant (hydrocarbons) as well as other soil compounds must be investigated. Not all inducers and co-repressors are substrates or end products of the enzyme involved (Brock and Madigan, 1991). Two other important aspects that must be investigated are (i) the effect of technology as in N and P addition during

biostimulation and rhizodeposition during phytoremediation and (ii) the influence of soil components that play critical roles in the bioavailability of the hydrocarbons to the biota.

The limiting concentration such as required to induce enzyme synthesis and potential product inhibition must also be evaluated. The other possibility is to monitor bioremediation processes using a 'battery' of bioindicators as attempted by Phillips *et al.* (2000). The battery must be made of different bioindicators with different sensitivities to hydrocarbon contamination. A comparison to an uncontaminated soil with identical texture must be used when determining the hydrocarbon toxicity.

In as far as microorganisms are concerned, the use of molecular techniques in characterizing both microbial communities and functional genes during soil remediation needs to be evaluated as potential monitoring tools. Substrate utilization techniques can also be used to evaluate population changes during bioremediation, however their limitations in evaluating most soil microbes offers some drawbacks.

It is not well documented that plants sensitivity to toxicants can vary substantially with environmental conditions such as organic matter, pH, ligands and toxicant interactions, and if this sensitivity can vary on a species by species basis.

The extent to which bioindicators respond to non-bioavailable poorly extractable pollutants is not well documented. The effect of non-bioavailable hydrocarbons in soil on enzyme synthesis and seed germination needs to be investigated.

In conclusion, there is still work that must be done on bioindicators before they can be used on their own to monitor hydrocarbon contamination and removal. The recommendations made by Freemark *et al.* (1990) and Freemark and Boutin (1994) are valid. There is a need to use more relevant ecological test species or activity, existing protocols must be

modified/new methods developed, tests need to be standardized and relevant test end points must be selected. At present, it is best that they be used to complement existing conventional monitoring instruments.

Acknowledgement

The author would like to thank Dr K Drønen, Bergen University (Norway) and Dr P Wade Phokus Technologies (SA) for the constructive suggestions received from them.

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Chapter 8

BIOREMEDIATION OF PETROLEUM HYDROCARBONS THROUGH LANDFARMING: ARE SIMPLICITY AND COST-EFFECTIVENESS THE ONLY ADVANTAGES?

A modified version of this text was accepted for publication as:

Mphekgo P. Maila, Thomas E. Cloete (2004) Bioremediation through Landfarming: Is simplicity and cost effectiveness the only draw cards? Reviews in Environmental Science and Biotechnology.

BIOREMEDIATION OF PETROLEUM HYDROCARBONS THROUGH LANDFARMING: ARE SIMPLICITY AND COST-EFFECTIVENESS THE ONLY ADVANTAGES?

Abstract

The biological removal of petroleum products using landfarming has been applied commercially in large scale with relative success. The technology has been widely used due to its simplicity and cost-effectiveness. However, together with these advantages, there are physical, chemical and biological aspects of the technology that can hamper the remediation process. The dominant pollutant removal mechanisms involved in landfarming are volatilisation of low molecular weight volatile compounds during the early days of contamination or treatment, biodegradation and adsorption. However, volatilisation and leaching of the petroleum products present both health and environmental challenges to the rehabilitation practitioners when designing the landfarming technology. Bioaugmentation and biostimulation are promising bioremediation approaches involving landfarming. However, due to the inherent problems related to bioaugmentation such as poor survival of augmented strains, biostimulation should be preferred in contaminated sites with indigenous pollutant-degrading bacteria. Although simplicity and cost-effectiveness are the major advantages for using landfarming, other factors generally regarded as disadvantageous to implementing the technology can be addressed. These includes requirements for large land area for treatment, availability of the pollutant degrading bacteria, effectiveness of the technology at high constituent concentration (more than 50 000 ppm), improved concentration reductions in cases requiring more than 95% of pollution reduction and the flexibility of the technology in integrating the removal of petroleum hydrocarbons with other contaminants that may occur with the petroleum products.

Introduction

The technologies that involve the biological removal of petroleum products from contaminated soil environments are today well established, and many are applied commercially on a large scale. During the 1970's, when environmental concerns associated with uncontrolled disposal became apparent, and environmental regulations were established and applied in North American and Europe (aimed at minimising the risk of air and groundwater contamination), landfarming gained popularity. This 'low tech' biological treatment method involves the controlled application and spread-out of a more-or-less defined organic bio-available waste on the soil surface, and the incorporation of the waste into the upper soil zone (Genou *et. al.*, 1994). In 1983 it was estimated that at least one-third of all United States refineries operated full-scale or pilot scale landfarmers (American Petroleum Institute, 1983). The technology has been widely used, as it is simple and cost-effective to implement compared to other treatments (American Petroleum Institute, 1983; Harmsen, 1991).

Landfarming lost its popularity in 1984 when the United States Environmental Protection Agency (US EPA) issued the land disposal restriction (LDR) as part of the hazardous and solid waste amendments (HSWA) to the resource conservation and recovery act (RCRA). The US EPA went further on 18 August 1992, by publishing a final rule, (57 FR 37194, 37252), establishing treatment standards under the land disposal restrictions program for various hazardous wastes that included petroleum products. Landfarm operators had to either operate their facilities to treat their waste below the EPA specified contaminant levels (referred to as treatment standard), or to submit a petition demonstrating that there was no migration of hazardous constituents from the injection zone (US EPA, 1984). As a result, most of the traditional landfarms in North America were closed.

Although there have been some restrictions on the application of the technology, it is still being used to treat petroleum products, with added measures for minimising or treating

volatiles and leachates (Genouw *et al.*, 1994; Harmsen *et al.*, 1994; Balba *et al.*, 1998; Picado *et al.*, 2001; Maila, 2002).

The petroleum products from the soil during landfarming are largely removed through volatilisation, biodegradation and adsorption (Morgan and Watkinson, 1989; Devliegher and Verstraete, 1996; Margesin *et al.*, 1999; Hejazi *et al.*, 2003). Lighter (more volatile) petroleum products like gasoline tend to be removed by volatilisation during landfarm aeration process and to a lesser extent, degraded by microbial respiration (EPA, 1994). The mid-range petroleum products like diesel fuel and kerosene contain lower percentage of lighter constituents than does gasoline. Biodegradation of these petroleum products is more significant than volatilisation. The more heavier or non volatile petroleum products like heating oil and lubricating oils do not volatilise during landfarm aeration, the dominant mechanisms that breaks down these petroleum products is biodegradation. Adsorption also plays an important role in the dissipation of petroleum products from the soil. According to Margesin *et al.* (1999), a third of diesel was removed from the contaminated soil by physicochemical means (adsorption and volatilisation).

The volatile organic compounds (VOCs) from the landfarm area can present air pollution problems if the treatment area is not properly covered to minimise the emissions (Hejazi *et al.*, 2003). Apart from the VOC emissions, other constraints faced by the rehabilitation practitioners considering landfarming as a treatment option include, requirements for large land area for treatment, availability of the pollutant degrading bacteria, effectiveness of the technology at high constituent concentration (more than 50 000 ppm), improved concentration reductions in cases requiring more than 95% of pollution reduction and the flexibility of the technology in integrating the removal of petroleum hydrocarbons with other contaminants that may occur with the petroleum products. Although problems associated with depth of pollution can be solved by ex-situ treatment, the polluted soil often requires a large treatment area, which can increase the risk of human exposure to the contaminants.

However, such exposure is only temporary, as contaminants will be degraded if environmental conditions are optimal (Ausma *et al.*, 2002).

Although simplicity and cost-effectiveness are the major advantages of the technology, the treatment has physical, chemical and biological 'constraints', which must be addressed. In this paper, we discuss these limitations, benefits, and possible solutions to the constraints.

Benefits and constraints of the technology

Bioremediation through landfarming is both simple and cost-effective to implement compared with other treatment technologies (Pearce and Ollerman 1998; Kelly *et al.*, 1998). On average, the costs associated with treating petroleum hydrocarbon-contaminated soil ranges from $30 to $70 per ton of contaminated soil compared with a physical treatment like soil venting which is relatively expensive ($70 to $200) per ton (Marijke and van Vlerken, 1998; Environment Canada, 2003). However, as a result of costs associated with soil excavation and transporting the contaminated soil, in situ techniques can be in general about 40 to 50% of ex-situ techniques (SCG, 2004). The technology is simple in that typical equipments, which are used for landfarming, is used widely in the farming community and is therefore 'readily' available. As most of this equipment is designed to till the soil to a depth of $\leq$ 0.5m, additional costs can be incurred during soil excavation for ex-situ treatment (Kelly *et al.* 1998). Different forms of the technology are shown in Figure 1.

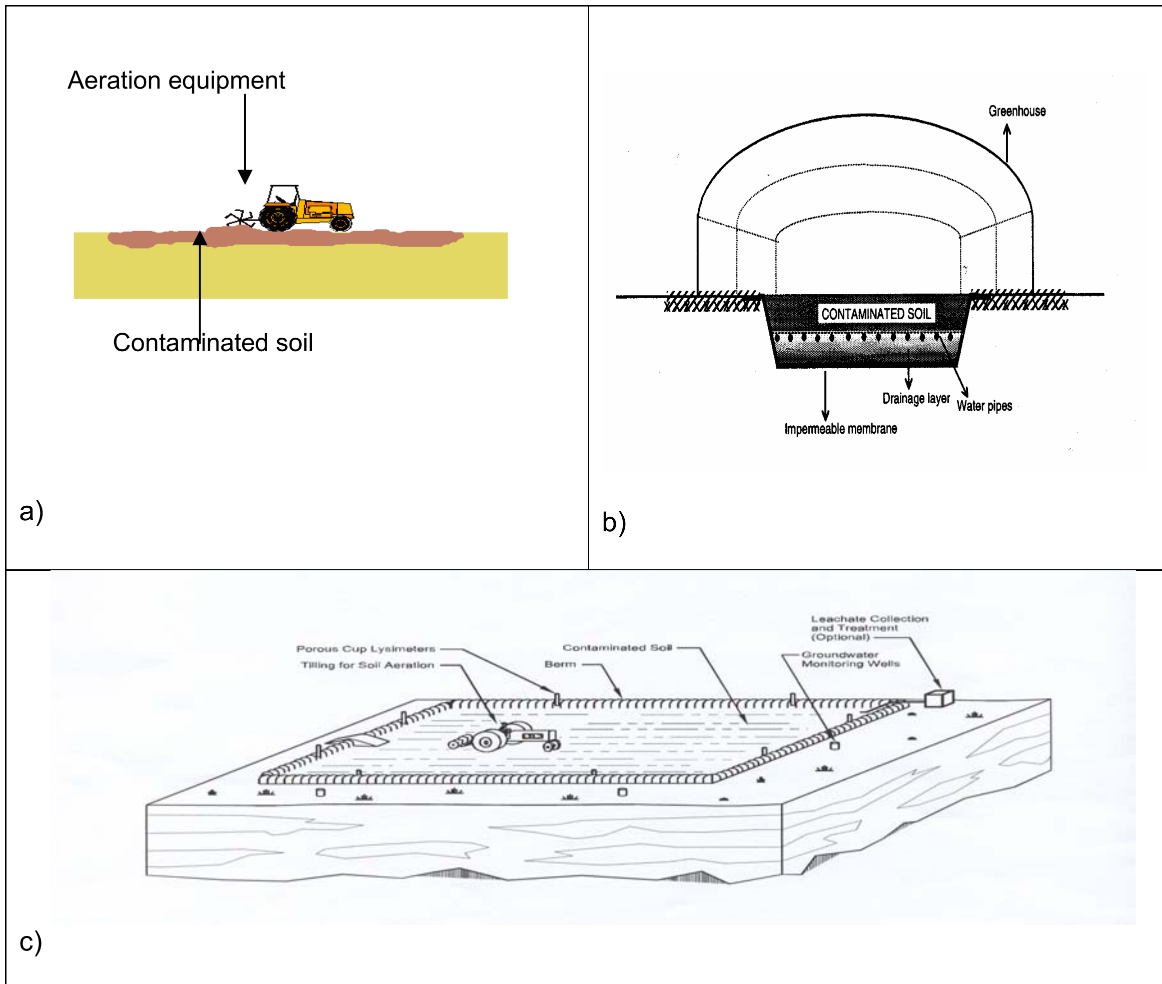


Figure 1. Different landfarm layouts a) Traditional 'landfarming' system b) 'Complex' landfarm system (Figure 1b, adapted from Picado *et al.*, 2001) c) Landfarm system without a greenhouse structure (Figure 1c, adapted from EPA, 1994).

For additional landfarm layouts or designs, the reader is referred to Doelman and Breedveld (1999) and to Battelle series (Alleman and Leeson, 1999). However, together with these advantages (table 1), there are physical, chemical and biological aspects of the technology that can hamper the remediation process. The physical aspects include the land area required for treatment, the ability and limitations of aeration equipments, mobility of pollutants in the soil, water requirements; chemical aspects include toxicity, transformation and partitioning of the petroleum products in different environmental media while biological

aspects include biostimulation or bioaugumentation for optimal biotransformation of petroleum products in the soil. The constraints of landfarming are listed in table 1.

Table 1: Benefits and constraints of Landfarming

Technology	Benefits	Constraints
Landfarming	• Very low capital input required • Technology is simple to design and implement • Large soil volumes can be treated • Both in/ex-situ can be applied • Has small environmental impact • Energy efficient	• Limited to removal of biodegradable pollutants • Large treatment area is needed • Involves risk of pollutant exposure • Substantial cost can be incurred during excavation • Limited knowledge of microbial process

Physical and Chemical aspects of Landfarming

Landfarming requires a sizeable area to treat the contaminated soil in cases where the volume of the excavated contaminated soil is large, and this can increase the risk of exposure to pollutants if ex-situ treatment is applied. The potential health hazards due to the volatilisation of lighter petroleum products from the soil during the treatment can be avoided by designing the landfarms as shown in Figure 1b. In this way exposure to harmful pollutants and dust will be minimised. However, volatilisation is only important during the loading of the greenhouse, particularly in mild climates.

The treatment of contaminated soil using landfarming can also be limited by the capacity of the aeration equipment. It is important to design landfarms in such a way that the tilling equipments are able to reach the 'subsurface' contaminated soil. The depth of the

contaminated soil varies, depending on the capacity of the tilling equipments (30 to 60 cm is commonly used, EPA, 1994). Also of importance during the treatment design is the need to incorporate an impermeable membrane with a drainage layer (as shown in Figure 1b). This membrane (high-density polyethylene membrane, ≥ 250μm thickness) prevents groundwater contamination.

Soil moisture can also impact the efficiency of removing petroleum compounds from the soil. The level of moisture in most landfarms is kept between 30 and 80% field capacity (Block *et al.*, 1992; Pope and Mathews, 1993; Malina *et al.*, 2002). The moisture level ensures the survival of the pollutant-degrading bacteria and enables dust control. However, as the size of the treatment area increases, the amount of water required to maintain the level of moisture ideal for biological activity can be enormous, especially in dry countries, and this can increase the treatment costs.

The interaction between the pollutant and micro-biota can result in the transformation of parent compounds to toxic metabolites which can lead to abortive pathways (Leisinger *et al.*, 1981; Haugland *et al.*, 1990; Lee *et al.*, 1994), while adsorbents like clay and organic matter, which are site-specific can decrease the bioavailability and therefore a lower risk for higher organisms (reduction in toxicity) and lower biodegradation efficiency as contaminants are tightly bound to the soil matrix (Volkering, 1996; Hatzinger and Alexander, 1995; Guerin and Boyd, 1992). The interaction between the pollutant and soil components is shown in Figure 2.

While the physical and chemical constraints of landfarming can hamper the efficiency of landfarming, the knowledge that has been generated during the last two decades, which addresses these limitations (Verstraete and Top, 1999; Holden and Firestone, 1997), has made it possible for the treatment of petroleum products in an environmentally safe manner.

<table>
<tr><th colspan="2">Physical</th><th>Chemical</th></tr>
<tr><td>i
Particulate pollutant
ii
liquid film
iii
surface adsorbed</td><td>iv
absorbed
v
in water phase
in pores
vi
as separate phase in pores</td><td>vii
chemically bound</td></tr>
<tr><td colspan="3">Extractability
Easily extractable poorly extractable non-extractable
(aged compounds)</td></tr>
<tr><td colspan="3">Bound pollutants</td></tr>
<tr><td colspan="3">High → low
bioavailability</td></tr>
</table>

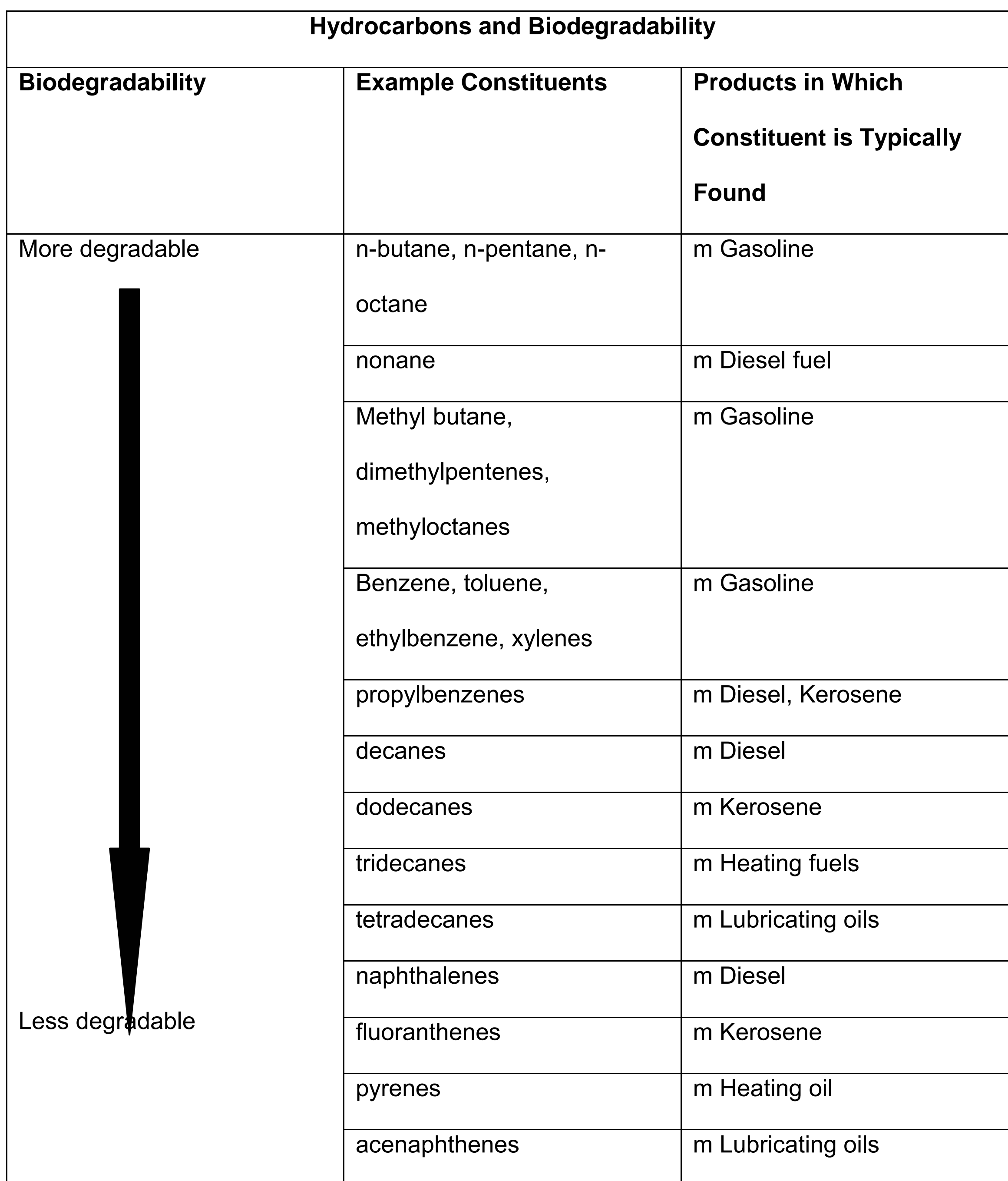
Figure 2. Different physical and chemical forms of organic pollutants in soil i: solid particles, ii: liquid film, iii: adsorbed onto soil, iv: in the water phase of soil pores, vi: as a separate phase in soil pores, vii: chemically bound to soil (adapted from Rulkens, 1992; Volkering 1996; Devliegher and Verstraete, 1996).

Bioaugmentation and Biostimulation

Bioaugmentation, the process of introducing pollutant-degrading bacteria to contaminated site, has been reported with mixed success (Van Veen *et al.*, 1997). The limitation to successful bioaugmentation in soils have been cited as being due to suppression of added strains by indigenous microbial community (poor survival of the introduced strains) and the use of readily degradable substrates, due to low concentrations and non-biodegradability of targeted pollutants (Alexander, 1994). Various efforts have been attempted to improve the success of bioaugmentation in contaminated sites (Del'Arco and de França, 1999). Strategies employed to improve bioaugmentation process for the effective removal of contaminants from the soil include the use of adapted strains or the Field Application Vector (as tested by Lajoie *et al.,* 1994). However, the most promising approach with regard to bioaugmentation has been attempted by 'seeding' the biodegradation knowledge to the indigenous microbial populations (Miethling and Karlson, 1996; El Fantroussi *et al.*, 1997; Kästner *et al.*, 1998; Top *et al.*, 1999). This involves the genetic transfer from the augmented strains to the indigenous bacteria.

With biodegradable pollutants like petroleum products (table 2), biostimulation of microbiological processes at the contaminated site is encouraged. This usually involves the modification of the site by adjusting pH, addition of limiting nutrients to achieve an ideal C:N:P ratio and improving the soil moisture. High petroleum hydrocarbon removal rates have been reported using the ratio of 100:10:1 (Genouw *et al.*, 1994). Table 3 shows some of cases in which biostimulation and bioaugmentation were attempted with relative success. The availability of petroleum hydrocarbon-degrading bacteria should be investigated during the biotreatability studies. The presence of these bacteria at contaminated site indicates that remedial approaches involving biostimulation can be used to 'encourage' the biological removal of petroleum hydrocarbons from the soil.

Table 2: The biodegradability of different hydrocarbons



Hydrocarbons and Biodegradability		
Biodegradability	**Example Constituents**	**Products in Which Constituent is Typically Found**
More degradable	n-butane, n-pentane, n-octane	m Gasoline
	nonane	m Diesel fuel
	Methyl butane, dimethylpentenes, methyloctanes	m Gasoline
	Benzene, toluene, ethylbenzene, xylenes	m Gasoline
	propylbenzenes	m Diesel, Kerosene
	decanes	m Diesel
	dodecanes	m Kerosene
	tridecanes	m Heating fuels
	tetradecanes	m Lubricating oils
	naphthalenes	m Diesel
Less degradable	fluoranthenes	m Kerosene
	pyrenes	m Heating oil
	acenaphthenes	m Lubricating oils

Biostimulation of indigenous petroleum hydrocarbon-degrading bacteria in landfarms should be encouraged ahead of bioaugmentation, as the former process relies on the degrading bacteria that have already adapted to the site's conditions.

Bioaugmentation should be implemented in contaminated sites where no indigenous petroleum hydrocarbon degrading bacteria exists, such as sites contaminated by high molecular weight polyaromatic hydrocarbons. The process of bioaugmentation should aim at 'seeding' the knowledge of degrading the pollutants to the indigenous bacteria (Fulthorpe and Wyndham, 1992; Brokamp and Schmidt, 1991; De Rore *et al.*, 1994; Top *et al.*, 1999; Top *et al.*, 1998; Verstraete and Top, 1999). As the number of microorganisms tends to increase during biostimulation, the increase in the number of degrading bacteria can be used as potential bioindicators during bioremediation (Margesin *et al.*, 1999).

Table 3: Efficiency of Full-scale Landfarming of TPH sites

Technology	Efficiency (%)	Microbial Process and Pollutants	Duration	References
Landfarming	82-90	Biostimulation (oil)	12 months	Balba *et al.*, 1998
	43	Bioaugumentation (oil)	28 days	Del'Arco and del Franca, 1999
	80-90	Biostimulation (PAHs)	3 years	Berends and Kloeg, 1986; Bossert and Bartha, 1986; Kincannon and Lin, 1985
	78	Biostimulation (PAH)	3 months	Picado *et al.*, 2001
	15	Biostimulation (heavy molecular weight PAHs)	7 months	Schenk *et al.*, 1992

Lesson learned

The objective of landfarming is to treat petroleum-contaminated sites in an environmentally safe manner by harnessing the removal efficiencies of biological, physical and chemical processes in the soil. This objective is sometimes not realised due to the constraints of the technology. In addition, no standard procedure is available for determining the allowable loading of landfarms and the time required for biodegradation of the petroleum compounds in the soil. This lack of procedure makes many landfarm designs to become a trial and error procedure with no assurances that the design will be successful in remediating the contaminated soil.

While the bio-treatability protocol recommended by Sabaté *et al.* (2004) is relevant, the urgency of the bio-treatability studies makes it difficult to gather the relevant information about optimising the processes involved in the removal of higher molecular weight petroleum compounds or the removal of poorly available part of the contaminants that are removed after the dissipation of the low molecular weight or the easily degradable petroleum compounds. There is a need to incorporate, in the biotreatability studies, investigations aimed at gathering information about the unravelling of the subsequent limiting factors during bioremediation. As this type of study may require a longer time than the 'generic' or well documented bio-treatability studies (EPA, 1994; Sabaté *et al.*, 2004), the studies can run concurrently with the full scale treatment of the contaminated site. With this approach, the information obtained from the 'urgent' bio-treatability studies, can be used to initiate the full scale treatment, while the information from the 'extended' studies about the subsequent limiting factors, used to optimise the treatment after the removal of the easily degradable petroleum compounds.

Picado *et al.* (2001) reported a 63 % reduction in total PAHs (polyaromatic hydrocarbons) concentration after the first three months of the treatment. The majority of the PAH removed during the treatment period were the 2, 3 and 4 ringed polyaromatic hydrocarbons. High

molecular weight PAHs were not removed, probably due to lack of the degrading strains, unfavourable bacterial growth conditions or due to the fact that they required a longer treatment time to dissipate, as they are difficult to degrade. Knowledge about enhancing the removal of the remaining high molecular weight hydrocarbons after the dissipation of low molecular weight hydrocarbons can help in improving the efficiency of landfarming.

Bossert *et al.* (1986) studied landfarming of 16 PAHs present in oil-contaminated sludge and reported a reduction of about 80-90% after 3 years of treatment. Low removal rates of high molecular weight petroleum compounds and the long treatment periods were experienced in some of the studies (table 3) due to the lack of process optimisation. According to Harmsen *et al.* (1994) landfarming include two steps; the first step involves an intensive treatment in which the readily available contaminants are removed. During the second step an extensive (intrinsic) treatment, the poorly available part of the contaminant is removed. In most landfarm operations, these two steps are not properly optimised by either biostimulation, in which an ideal C:N:P ratio is applied or by bioaugmentation in which the biodegraders are added to degrade petroleum compounds that are difficult to degraded by the site's indigenous biota. In addition, subsequent limiting factors (nutrients, pH, biodegraders, toxic metabolites) during landfarming are not adequately addressed, resulting in long treatment periods.

While landfarming has been able to reduce the concentration of petroleum compounds in contaminated soil (table 3), concern remains about its effectiveness in reducing the level of recalcitrant hydrocarbons and the potential toxicity of the metabolites generated during the degradation process. Also critical is the amount of time needed to reduce the concentration of petroleum compounds to levels acceptable by the regulators.

Apart from the generic approach of implementing landfarming, to treat petroleum compounds, it is important to take into account the 'added or non-additive effect' of potential

limiting factors on bioremediation. This can be achieved by a detailed bio-treatability studies which can run concurrently with the full scale treatment process, or by incorporating an improved monitoring program that include investigation of the unravelling limiting factors.

Possible solutions to the constraints

One of the earlier concern about using landfarming to treat petroleum contaminated soil has been the risk of transferring environmental pollutants from one environmental compartment (soil) to another (air or groundwater). This necessitated the need to find solutions to both the physical, chemical and biological constraints associated with landfarming. Treatment standards had to be met when applying the technology to remove petroleum compounds from the soil. The concern for further environmental contamination due to landfarming led to better treatment designs as shown in Figure 1 (b and c) from the traditional treatment approach (Figure 1a). Landfarming should be designed as shown in Figure 1b. This treatment design is able to prevent or minimise the transfer of contaminants from one environmental media to another. The design encompass a greenhouse structure that avoid or minimise dust and volatilisation of lighter petroleum compounds from the soil and also include an impermeable membrane with an impermeable layer (high density polyethylene membrane, $\geq$ 250 μm thick) which prevents ground water contamination. However, this 'physical structure' alone does not guarantee the efficient removal of petroleum compounds from the soil. The condition conducive to the proliferation of petroleum degrading bacteria in the soil has to be created for the efficient removal of petroleum compounds. This has to be evaluated during the feasibility studies. In addition, as treatment standards vary from one country to another, the success of one treatment design in one country is not a guarantee that different treatment standards will be met in another country. Landfarming principles are shown in table 4.

Table 4: The land treatment principles

Parameter	Ideal Characteristics
Soil	Well drained soil (bulky agents needed in clayey soils)
Nature of pollutants	Pollutants should be biodegradable (by existing microbiota)
Climatic conditions	Greenhouse type structure (required to minimise erosion and precipitation effects)
Microbiological	Indigenous pollutant degrading bacteria and conducive environmental conditions (pH, nutrients, moisture content etc.)

As the technology ‘relies’ on the biological process to remove petroleum compounds, the key to successful remediation is to implement removal approaches that are inline with the petroleum degrading bacteria. It is important to first conduct the feasibility studies which will yield the information about the type and metabolic activity of the indigenous microorganisms at the site, presence of possible inhibitors, biodegradability of contaminants under optimal conditions, influence of nutrients and bioavailability of pollutants in soil. This information will also help the rehabilitation practitioner to decide if biostimulation or bioaugmentation is the relevant approach for cleaning the contaminated soil. However, while this information is useful for intensive treatment of petroleum compounds, it provides very little information about the unravelling of limiting factors during bioremediation and this can have an impact on the efficiency of landfarming. Landfarming design should include a monitoring plan, which addresses the limiting factors that may occur during bioremediation, particularly as both the biological, physical and chemical processes in the soil have the potential to alter soil conditions, which may become unfavourable to petroleum degrading bacteria.

Also, the petroleum products are often present in combination with other pollutants (e.g. heavy metals as in motor washbay areas) and this creates problems, as the metals can be toxic to hydrocarbon-degrading bacteria. In this case, a bio-separation process as shown in Figure 3 is recommended. However, soil washing is recommended if the sand fraction of the contaminated soil is large, as clay matrix can be destroyed at low pH (Tichy *et al.*, 1996). With this process, metals can be removed by extraction while the petroleum hydrocarbons can be treated biologically using landfarming (Figure 3).

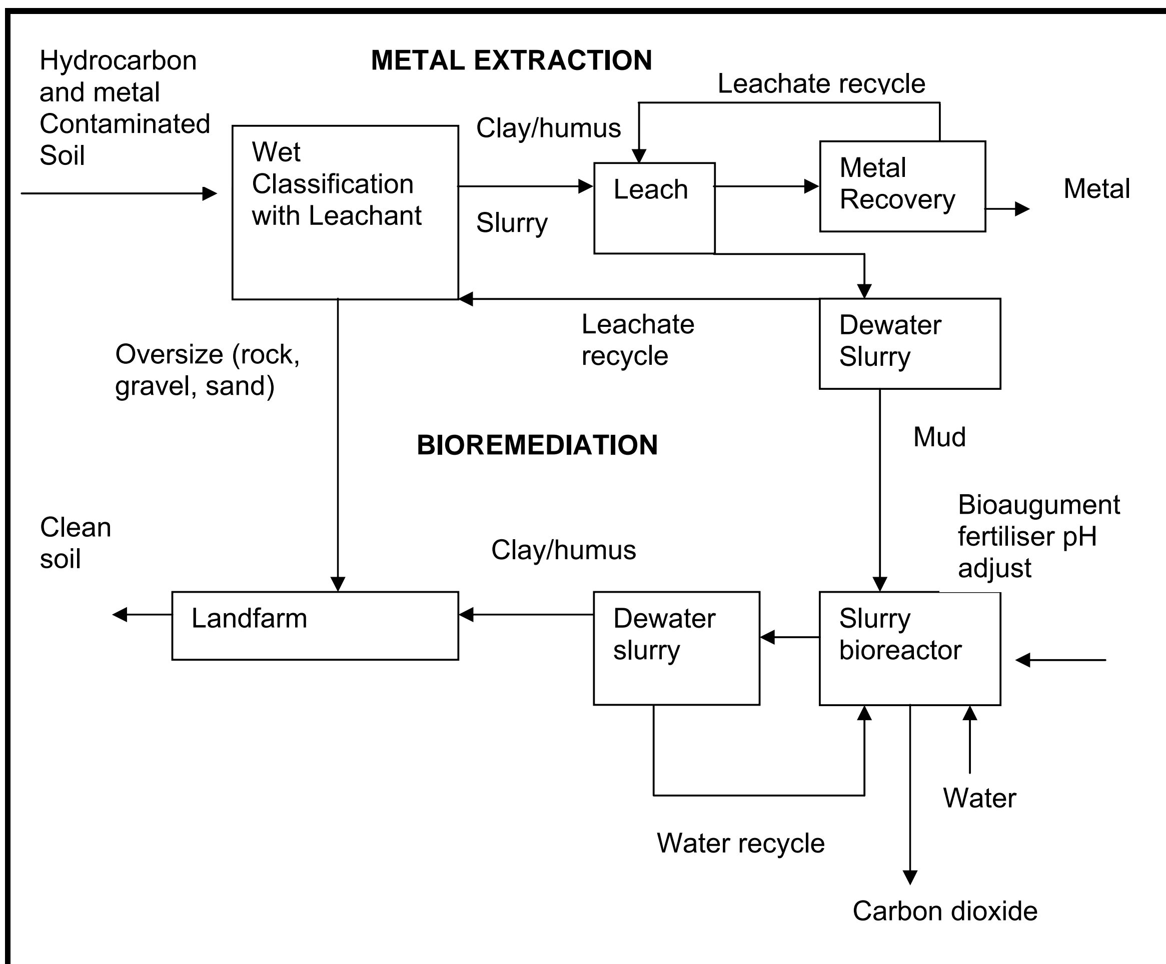


Figure 3. Metal Leaching and Bioremediation Process (adapted from US EPA, 1992).

Ecological risk management

The volatilisation of lighter petroleum products and the mobility of petroleum pollutants from landfarms constitute a threat to humans and groundwater resources. The risk to humans and groundwater can be minimised by designing landfarms as shown in Figure 1b or 1c, in which the volatiles and the downward migrating pollutants are minimised or treated.

According to Hejazi *et al.* (2003), landfarming at the site poses risk of detrimental effects through the air pathway (through the inhalation exposure route) to site workers during the initial period of landfarming. Contaminated soils are excavated and spread on a pad with a built-in system to collect any "leachate" or contaminated liquids that seep out of contaminant-soaked soil. In some cases, reduction of contaminant concentrations actually may be attributed more to volatilisation than biodegradation (Morgan and Watkinson, 1989). When the process is conducted in enclosures controlling escaping volatile contaminants, volatilisation losses are minimized.

Bioremediation through landfarming aims to remove pollutants through conversion to CO_2 and water. However, in many cases, an important fraction of pollutant and its metabolites remain untouched by the cleaning process (Devliegher and Verstraete, 1996). This amount of pollutant remaining in the soil constitutes a major concern and source of debate in relation to risk assessment. The threat posed by the pollutant residues can be minimised by adding adsorbents to form the non-bioavailable residues as suggested by DeVliegher and Verstraete (1996). Non-bioavailable pollutants can be considered as representing no direct harm to the environment. The different physical and chemical forms of organic pollutants are listed in Figure 2.

Future R&D needs

Landfarming is a cost-effective method of treating biodegradable petroleum products in the soil. However, it is important to design the treatment system in such a way that the transfer of pollutants to other environmental media is minimised or prevented. It is also important to modify the contaminated site's conditions to be 'inline' with the normal activities of the indigenous pollutant-degrading bacteria as this can improve the biological removal of petroleum products.

One of the disadvantages of landfarming is the inability of the technology to have concentration reductions of more than 95% (EPA, 1994). This pollution reduction may (in some instances) not be adequate to meet regulations or standards from specific petroleum constituencies in some countries. As this can be attributed to the unavailability of the pollutant to biota, agents (like surfactants) that improve the bioavailability of petroleum products in soil must be considered during the design phase of the technology. This should be particularly encouraged where there is a significant risk posed by the remaining residues. However, the effectiveness of this approach must be compared with the addition of adsorbents, which can make the pollutant residues, less available and therefore not harmful to higher organisms.

Landfarming may also not be effective for high constituent concentrations in the soil. As high concentration of the pollutants can be toxic to soil microorganisms, studies should be undertaken during the biotreatability studies to determine the minimum amount of soil or adsorbents (e.g. straws which can also improve soil aeration) that can be added to the soil to reduce toxicity. It is therefore important to corroborate (using other petroleum products) the findings of Del'Arco and de França (2001), who reported that the extent of oil biodegradation is inversely proportional to increasing oil contamination.

Landfarming has been used to treat volatile and biodegradable pollutants with relative success. However, the technology has not been greatly used to treat persistent organic pollutants like the high molecular weight polyaromatic hydrocarbons. There is a need to understand microbial processes and environmental conditions conducive for 'seeding' biodegradation information to the indigenous microbial communities. Remedial approaches involving bioaugumentation with the aim of increasing the removal capacity of the indigenous bacteria should therefore be evaluated at both pilot and large scale to improve the biological removal of persistent petroleum compounds using landfarming. It is also important to understand the unravelling of the subsequent limiting factors during bioremediation of both the low and high molecular weight petroleum compounds.

In conclusion, although simplicity and cost-effectiveness are the major advantages of using landfarming, the technology has 'inherent' physical, chemical and biological constraints. However, these constraints which are generally regarded as disadvantageous to implementing the technology can be addressed by applying the current wealth of knowledge on biodegradation and bioavailability of petroleum hydrocarbons, partitioning of petroleum hydrocarbons between environmental media, genetic transfer of the biodegradation knowledge to indigenous microbial communities, impact of petroleum products on soil microbial diversity and the intensive treatment of contaminated soil where space is a constraint. This wealth of knowledge on biodegradation and bioavailability of pollutants adds on to the advantages that have been well documented about landfarming. Hence, simplicity and cost effectiveness are not the only advantages associated with landfarming. Stimulated biological process and co-metabolism of recalcitrant (heavy molecular weight PAHs) are the other advantages associated with the technology. It is however, important to implement the technology in such a way that 'side effects' are minimised (i.e. there is less risk of transferring the pollution to other environmental media like the air and groundwater).

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Acknowledgement

The author would like to thank Dr K Drønen and Dr P Wade for their constructive inputs to the paper. This work was also partially funded by the National Research Foundation-Research Council of Norway grant.

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Chapter 9:

CONCLUSIONS AND PERSPECTIVES

CONCLUSIONS AND PERSPECTIVES

The methods used to study soil microbial diversity, as indicated in Chapter 1, include culture-dependent and culture-independent techniques. However, both methods have inherent limitations, which hamper efforts of understanding the genetic and functional diversity of different ecosystems. There is therefore a need to investigate the extent to which the two approaches can be used to complement each other. The literature revealed that culture-dependent methods are ideal for assessing the metabolic activities or the functional diversity of the soil while the molecular techniques and the phospholipid fatty acid are ideal for assessing the microbial community structure (Amann *et al.*, 1995; Vestal and White, 1989). Also of importance is the ability and capability of the different techniques to evaluate the effect of environmental pollutants on soil microbial communities.

In order to seek clarification on the extent to which both cultured-dependent and culture-independent methods can be used to complement each other, we used the community level physiological profiles (CLPP) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) to evaluate the influence of total petroleum hydrocarbons (TPH) on soil microbial communities. Also investigated was the influence of the hydrocarbons on both the rhizosphere and non-rhizosphere soil microbial communities.

The community-level physiological profiles (CLPP) and PCR-DGGE were used to evaluate the influence of hydrocarbons on soil microbial diversity of the different soil layers. An additional aim was to understand the distribution of hydrocarbon-degrading populations with soil depth and how the distribution patterns influence the efficiency of biodegradation. The substrate utilisation pattern of the topsoil was different from the substrate utilisation pattern of the soil layers below 1m. In addition, the substrate utilisation patterns of the contaminated and uncontaminated soil layers were different. 16S rRNA gene fragment patterns of the different soil layers were also different. While the metabolic activity of different samples as reflected by CLPP do not necessarily imply the difference in community structure of the

samples, PCR-DGGE revealed differences in 16S rRNA gene fragments of the different soils, and this complemented the results of the CLPP. The results suggest that the use of functional and genetic approaches (in combination) have a better chance of revealing a 'clearer' picture of soil microbial diversity.

The biodegradation rate of hydrocarbon was highest in the topsoil compared to other soil layers and this was supported by the high number of hydrocarbon-degrading bacteria in the topsoil compared to soil layers at and below 1 m. The results suggest that the biological removal of hydrocarbons varies in different soil layers and that microbial diversity as evaluated by CLPP and PCR-DGGE varies with depth in hydrocarbon-contaminated soil. Information about metabolic activities of different soil layers is critical when assessing the footprints of degradation processes during monitored natural attenuation (Smets *et al.*, 2002). Further studies are required to understand the effect of (not only) other pollutants, but the influence of soil components (pore volume, level of adsorbents and other environmental factors) on the microbial diversity of different soil layers in both 'shallow' and deep aquifers. It will also be important to investigate the influences of soil type, groundwater level, total organic carbon and the electron acceptors on microbial diversity of different soil layers, as these are important factors in soil remediation.

Heterotrophic bacteria play an important role in the restoration of hydrocarbon-contaminated soil. However, there is very little information about the distribution of the heterotrophic bacteria in different environments contaminated by similar contaminants. To get an understanding of the distribution of the heterotrophic bacteria in different contaminated environments, the stone ballasts at different diesel depots contaminated by hydrocarbons were used as 'models'. Hydrocarbon-utilizing bacteria colonizing the stone ballast at different diesel depots formed the majority of the total culturable heterotrophs (TCHs). The number of total culturable heterotrophs (TCHs) and the culturable hydrocarbon-utilizing bacteria were proportional to the concentration of the hydrocarbons on the ballasts. Characterisation of

heterotrophic communities using Biolog (Chapter 3) revealed differences in the microbial metabolic profiles for the different sites.

Microbial diversity of polluted surfaces needs to be studied further to investigate the concentration or the thickness of the hydrocarbons layer on the rock surfaces that encourages the attachment or colonization of the TCHs and the hydrocarbon-degrading bacteria. It is also not clear how the heterotrophs acquire the micronutrients from the surrounding environment. Knowledge of microbial diversity of contaminated rocky surfaces is essential as it can be applied in bioremediation of contaminated rocky surfaces as in contaminated diesel depots and contaminated rocky surfaces caused by oil spills.

The importance of the geographical origin of the samples and the hydrocarbons were evaluated using the contaminated and uncontaminated soils from the different soil locations. The contaminated and uncontaminated soils from the different locations were not clustered together by cluster analysis of the different community profiles (CLPP). In addition, the contaminated and uncontaminated soils from each geographic location were also not closely related. Because of different soil usage and heterogeneity, which can influence microbial diversity, it was expected that the geographical origin of the sample rather than the hydrocarbons will be more important in determining functional or species diversity within the bacterial communities. However, the results did not support this hypothesis as the samples from different locations were as different as samples from the same location but from contaminated versus uncontaminated soil. The results of the soils from different locations artificially contaminated by different hydrocarbons also reached the same conclusions. Further work is needed to investigate the importance of geographic location and hydrocarbons using molecular techniques.

Multi-planted rhizoremediation of polycyclic aromatic hydrocarbons (PAHs) was more effective compared to monoculture rhizoremediation (Chapter 5). However, selecting the

correct mixture of plant species for multi-plant rhizoremediation could create problems, particularly as organisms are known to produce toxic compounds to minimise competition (Curl and Truelove, 1986). It is therefore necessary to use plants which can coexists or the so-called co-occuring plants for multi-plant phytoremediation purposes.

The effect of PAHs on the non-rhizosphere microbial communities was found to be in line with similar findings on the impact of organic pollutants on soil microbial communities (Atlas *et al.*, 1991; Wünsche *et al.*, 1995). The metabolic diversity of the contaminated and non-contaminated soil was different. In addition, the rhizosphere microbial communites appears to be impacted in the same way as the non-rhizosphere microbial communities (Chapter 5). The Principle component analysis and cluster analysis revealed differences in the metabolic diversity of the contaminated and non-contaminated rhizosphere soil. However, the differences in the metabolic diversity of the multi-planted and monoplanted treatments were not revealed. There is a need to study further the microbial diversity in multi-planted treatments aimed at decontaminating hydrocarbon-contaminated soil using both functional and molecular approches.

The knowledge of genetic and functional diversity of the soil plays an important role during the planning and remediation of contaminated soils. However, equally important is the availability of reliable monitoring instruments to 'gauge' the progress of bioremediation (Maila and Cloete, 2002). Bioremediation as evaluated by Gas Chromatography-Mass Spectrometer (GC-MS), can be expensive. Recently, biological activities have been investigated as potential monitors of the removal of organic compounds from the soil (Margesin *et al.*, 1999; Athey *et al.*, 1989; Siciliano *et al.*, 1987; Dorn *et al.*, 1998; Marwood *et al.*, 1998; Maila and Cloete, 2002). Enzymes, earthworm survival, microbial bioluminescence and seed germination using grass species have been evaluated for their potential, but not much has been done on the use of plants with short germination period

(Margesin *et al.*, 1999; Wang and Freemark, 1995; Siciliano *et al.*, 1987; Phillips *et al.*, 2000; Shakir *et al.*, 2002).

The sensitivity of *Lepidium sativum*, a plant with short germination period, was investigated in soil(s) artificially contaminated and historically contaminated with mixtures of PAH (Chapter 6). The level of germination of *L sativum* decreased with increasing concentration of the PAH in the artificially contaminated soil while no germination occurred in the historically polluted soil. When used during phytoremediation of PAH, the germination level of *L sativum* was inhibited during the first weeks, after which germination increased, possibly due to PAH dissipation from the soil. The data suggests that the germination of *L sativum* can be used to monitor the removal of PAH pollutants from soil. The method based on the sensitivity of *L. sativum* (with a short germination period) to PAH can be used as a monitoring tool in remediation treatments of soil contaminated with PAH. The methodology should be further developed to gain more knowledge on aspects of bioavailability of PAH in both the aged as well as the freshly spiked soil. Also critical is the sensitivity of the seeds to other pollutants (e.g. heavy metals), which are most likely to occur in the presence of the PAHs.

The review article (Chapter 7) reveals that although several biological activities have the potential to monitor the removal of hydrocarbons from the soil, the methodologies have not been developed sufficiently to cater for the heterogeneity of the soil and to differentiate the toxicity by the parent compound and the metabolites. There is still work that must be done on bioindicators before they can be used on their own to monitor hydrocarbon contamination and removal. The recommendations made by Freemark *et al.* (1990) and Freemark and Boutin (1994) are valid. There is a need to use more relevant ecological test species or activity, existing protocols must be modified or new methods developed, tests need to be standardized and relevant test end points must be selected. At present, it is best that they be used to complement existing conventional monitoring instruments.

Apart from the knowledge of genetic or functional diversity of contaminated soils and monitoring instruments, the selection of the relevant treatment technology is also important for soil rehabilitation. The selection of the remediation technology should be based on: site characteristics, nature and extent of contamination, risk assessment and economic considerations. For bioremediation technologies, the pollutant of interest must be biodegradable by the existing indigenous or the added strains. As indicated in Chapter 8, bioremediation approaches involving the stimulation of the indigenous pollutant-degrading bacteria should be preferred above bioaugumentation. The latter approach should be considered when the contaminated site does not have the indigenous pollutant-degrading bacteria. Even in this case, the aim should be to 'seed' the biodegradation knowledge to the indigenous microbial populations due to poor survival of the added strains (Van Veen *et al.*, 1997; Top *et al.*, 1999).

As indicated in Chapter 8, the biological removal of hydrocarbons is cost-effective compared to other treatment technologies. Also cost-effective is the use of biological activities to monitor the removal of hydrocarbons from the soil. However, because of soil heterogeneity, bioindicator response to pollutants can be 'masked' by the availability of different adsorbents in the soil. Because of this limitation, the remediation and monitoring strategy illustrated in Figure 1 is recommended. Indeed chemical analysis is ideal for determining the levels of hydrocarbons in soil, while toxicity can be determined by using bioindicator response to not only the parent compounds, but also to toxic metabolites which are not easy to determine analytically.

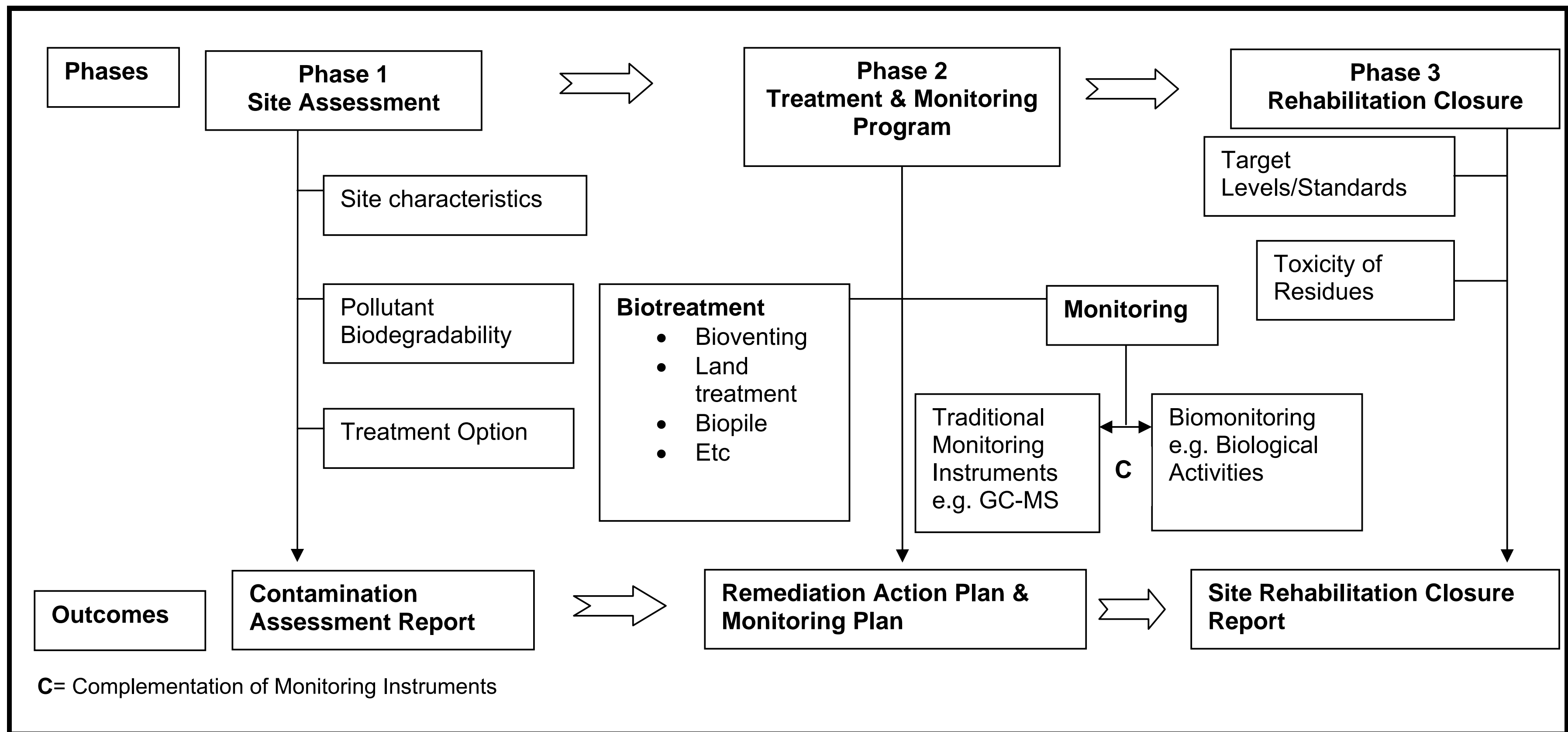

Figure 1. Strategy for bioremediation of TPH contaminated soil with an integrated monitoring program

In conclusion, the polyphasic approach is recommended when evaluating soil microbial diversity and the effect of pollutants on microbial community structure, as the approach compensates for the limitations of each existing method of evaluating microbial diversity. However, further work is needed to improve the recovery of bacteria from the soil, particularly where the interest is to evaluate the availability of the indigenous microbial populations for bioremediation. Also critical is the need to standardise or create new (biomonitoring) approaches to evaluate the removal of organic pollutants from the soil. The inherent (physical, chemical and biological) limitations of bioremediation technologies must also be investigated to improve the efficiency of bioremediation.

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