EXPLOITATION OF INDIGENOUS FUNGI IN LOW-COST *EX SITU* ATTENUATION OF OIL-CONTAMINATED SOIL

by

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PREFACE

The experimental work described in this thesis was carried out in the Department of Microbiology and Plant Pathology, University of Natal, Pietermaritzburg, from March 1994 to December 1996, under the supervision of Professor E. Senior of the Department of Microbiology and Plant Pathology.

These studies represent original work by the author and have not been submitted for degree purposes to any other university. Where use was made of the work of others, it has been acknowledged in the text.

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ABSTRACT

The central aim of this study was to determine if indigenous fungi of an oil-contaminated soil could be effectively used in a low-cost bioremediation of the soil. Since some of the contaminant had been present at the site for over two decades, the indigenous microbial species had been subjected to specific selection pressures for a protracted period, thus facilitating key enzymatic capabilities for hydrocarbon degradation. Analysis of the pertinent influential parameters of soil bioremediation indicated that an *ex situ* technique, utilising the catabolic activities of the indigenous soil fungi, was a feasible low-cost option.

Fungi were isolated from the contaminated soil through a variety of techniques. The abilities of these isolates to degrade the contaminant oil and a range of representative hydrocarbon molecules was evaluated by a systematic screening programme. Sixty-two isolates were initially examined for their growth potential on hydrocarbon-supplemented agar. A bioassay, utilising hydrocarbon-impregnated filter paper discs, was then used to examine the abilities of 17 selected isolates to catabolise three representative hydrocarbon molecules (hexadecane, phenanthrene and pristane) in different concentrations. In the same bioassay, the influence of a co-metabolite (glucose) on growth potential was also examined. Eight fungal species: *Trichophyton* sp.; *Mucor* sp.; *Penicillium* sp.; *Graphium* sp.; *Acremonium* sp.; *Chaetomium* sp.; *Chrysosporium* sp.; and an unidentified basidiomycete were then selected. Liquid batch cultures with a hydrocarbon mixture of hexadecane, phenanthrene, pristane and naphthalene facilitated quantitative analysis (HPLC) of the hydrocarbon catabolic abilities of the selected isolates.

Ex situ bioremediation was evaluated at laboratory-scale by both bioaugmentation and biostimulation in soil microcosm trials. During the course of the study, total petroleum hydrocarbon (TPH) concentration (U.S. EPA Method 418.1) was used as a simple and inexpensive parameter to monitor hydrocarbon disappearance in response to soil treatments. Soil microbial activities were estimated by use of a fluorescein diacetate hydrolysis bioassay. This was found to be a reliable and sensitive method to measure the

activity of respiring heterotrophs as compared with the unreliable data provided by plate counts.

In the bioaugmentation trial, the eight selected isolates were individually used to inoculate (30% v/v) the contaminated soil. The highest rate of biodegradation (50.5% > than the non-sterile control) was effected by an *Acremonium* species after 50 days incubation (25°C). The second highest rate of biodegradation (47% > than the non-sterile control) was achieved with a soil treatment of sterile barley/beer waste only. Comparable rates of hydrocarbon degradation were achieved in simple biostimulation trials. Thus, due to its lower cost, biostimulation was the preferred remediation strategy and was selected for further laboratory investigation. Common agricultural or industrial lignocellulosic wastes such as: wood chips; straw; manure; beer brewery waste; mushroom compost; and spent mushroom substrate were used as soil treatments, either alone or in combination. The effect of the addition of a standard agricultural fertiliser was also examined. The highest level of biodegradation (54.4% > the non-sterile control) was recorded in a microcosm supplemented (40% v/v) with chicken manure.

Finally, an *ex situ* bioremediation technique was examined in a pilot-scale field trial. Wood chips and chicken manure were co-composted with the contaminated soil in a low-cost, low-maintenance bioremediation system know as passive thermal bioventing. Extensive monitoring of the thermal environment within the biopile was made as an indirect measure of microbial activity. These data were then used to optimise the composting process. Three-dimensional graphical representations of the internal temperatures, in time and space, were constructed. From these graphs, it was determined that an inner core region of approximately 500 cm^3 provided a realistic simulation of conditions within a full-scale biopile. During this trial a TPH reduction of 68% was achieved in 130 days.

The findings of this research suggested that the utilisation of fungal catabolism is applicable to soils contaminated with a wide range of hydrocarbon contaminants. Passive thermal bioventing offers a bioremediation strategy which is highly suitable for South African conditions in terms of its low level of technological sophistication, low maintenance design

and, most importantly, its relatively low cost.

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GENERAL INTRODUCTION

This study formed part of programme initiated in 1991 to formulate a remedial strategy for some 18 000 m^3 of oil-contaminated soil at an industrial site in KwaZulu-Natal, South Africa. Contamination occurred at the site through the negligent operation of an oil recycling plant. The contaminant was a complex mixture of oils previously handled by an oil-recycling plant. The oils included: industrial/machine lubricants; transformer coolants; and motor transmission oils. Capillary gas chromatographic analysis of soil extracts revealed more than 200 different compounds and their stereoisomeric forms. These included n-alkanes, branched alkanes, alicyclics and aromatics (Lees, 1991). The contaminant concentrations ranged from 65 to 15 800 ppm over a land area of 9000 m^2. In some places, contamination had been present for >20 years, and thus was in a highly weathered state.

Wells, originally sunk for site assessment purposes, were used to monitor groundwater and off-site contamination concentrations. These operations were undertaken by an independent consulting engineer who reported to The Department of Water Affairs and Forestry (Durban, South Africa). These results indicated that migration of the hydrocarbons off the site was negligible. If this information was correct, it implied that the residual hydrocarbon product was strongly adsorbed to the soil and/or was mainly heavier insoluble fractions. In some places, free product, with a thick tar-like consistency, was visible (Plates 1a and 1b).

The contaminated soil is a sandy loam with a particulate composition of: sand, 78%; silt, 18%; and clay, 4%. The nutrient status of the soil is poor and the soil is highly acidic (see soil fertility test, Appendix A). The site is subject to perennial variation of the groundwater table level and areas of highest contamination coincided with areas where the groundwater table is perched approximately 150 mm below the surface. This had led to the development of anoxic or anaerobic conditions which are highly unfavourable for biodegradation. A full site description is provided by Lees (1997).

Preliminary investigations at the site, undertaken by Lees (1991), demonstrated significant

Plate 1a: General view of soil contamination at the project site. The leaking reservoir, containing caustic effluent, is visible in the background.

Plate 1b: Close-up of a soil sampling plot with free-product contamination indicated.

hydrocarbon-catabolic activity by indigenous soil fungi. The findings of other studies (McGugan and Ramsden, 1992; McGugan, 1993) revealed that the indigenous soil fungi were capable of utilising the contaminant as a carbon and energy source. Residual oil contamination consisted of heavy hydrocarbon fractions which are poorly soluble and, hence, are largely unavailable to bacteria. The anoxic zones formed in the subsurface, due to the perched water table, necessitate that water should be either pumped from the site or *ex situ* bioremediation should be instigated. The high sand content of the soil facilitates a relatively high oxygen diffusion rate which is ideal for an *ex situ* biopile-type of bioremediation strategy. Fungi possess a number of physiological and metabolic attributes which make them well suited for use in *ex situ* bioremediation techniques (Chapter Two).

After a critical assessment of the physical, chemical and biological conditions present at the project site, it was decided that a bioremediation programme, which specifically stimulated indigenous fungal species, was a viable solution for site remediation. The following summarise the aims and rationale of the study:

(i). To review appropriate literature on the use of bioremediation and, more specifically, the use of fungal species in *ex situ* bioremediation technologies;

(ii). To isolate fungi from the contaminated soil by a variety of techniques in order to create a representative culture collection;

(iii).To screen the abilities of the isolates to degrade the contaminant oil and a range of representative hydrocarbon molecules in a systematic experimental programme;

(iv). To critically evaluate both bioaugmentation and biostimulation *ex situ* bioremediation techniques at a laboratory-scale in soil microcosm trials;

(v). To select the more effective technique for further investigation in pilot-scale field trials; and

(vi). To determine whether optimisation of fungal catabolism is a viable, low-cost, soil remediation option, suitable for South African conditions.

CHAPTER ONE

[1] BIOREMEDIATION: A LITERATURE REVIEW

[1.1] INTRODUCTION

Globally, over two billion metric tonnes of petroleum-hydrocarbon products are produced per annum. It is estimated that 1.7-8.8 metric tonnes per annum end up polluting the world's oceans (Bartha, 1986). There are no comparable estimates of the extent of terrestrial petroleum-hydrocarbon pollution. However, due to the drastic and highly visible local effects, press coverage and public attention is disproportionately focused on incidents of marine spillage. If one considers that the greater part of petroleum is produced, refined and utilised on land, resulting routine, accidental and illegal discharges are likely to equal if not exceed the figure cited for the marine environment. Hence, there is a strong need for the development of methods for the decontamination of hydrocarbon-contaminated terrestrial environments. Previously accepted methods for the treatment/disposal of contaminated soil, such as incineration and/or burial in secure landfill, are no longer cost effective or environmentally sound practices. When the volume of contaminated soil is large, bioremediation remains the only feasible treatment option.

Over the last 25 years, bioremediation has evolved from its "novel technology" status to become a technology that is now in routine use. Numerous accounts of the successful application of bioremediation to a wide range of organic pollutant molecules are reported in the literature. In recent years, incidents such as the massive marine oil contamination from the *Exxon Valdeze* (in 1989 at Prince William Sound, Alaska) have showcased the incredible potential bioremediation has for environmental protection and land/water reclamation (Bragg *et al.*,1992). When applicable, bioremediation is now a preferred method of remediation since it uses naturally occurring microorganisms to provide a permanent solution for pollution problems as the contaminant molecules are either effectively destroyed or are transformed into less toxic molecules.

[1.2] FACTORS AFFECTING HYDROCARBON BIODEGRADATION IN NATURAL ENVIRONMENTS

Current evidence suggests that in aquatic and terrestrial environments, microorganisms are the primary agents of the biodegradation of environmentally detrimental molecules (Alexander, 1981). This chapter outlines some of the parameters that affect the biodegradation of petroleum hydrocarbons in soil matrixes. Petroleum hydrocarbon biodegradation is a complex process and depends on a number of interdependent factors. The major influential factors can be grouped into three principal categories:

(i). ***Chemistry*** of the pollutant molecule;

(ii). ***Biology*** of the pollutant-degrading microbial population; and

(iii). ***Environmental*** factors relevant to the polluted site.

Current bioremediation strategies only allow manipulation of some of these parameters to a limited degree (Blackburn and Hafker, 1993).

[1.2.1] Biological Factors

In most ecosystems, providing environmental conditions are favourable, indigenous microbial communities are capable of hydrocarbon biodegradation (Atlas, 1978a). It is suggested by some researchers (McGill, 1977; Atlas, 1978b) that all soils, except very acidic ones, contain microorganisms capable of degrading oil products. This infers that microbial seeding in most cases is not necessary and the actual problem one is faced with in bioremediation is in the supply and delivery of the correct nutrients to the microorganisms.

The ability to utilise hydrocarbons as a carbon and energy source is not restricted to a few microbial genera but is present in diverse groups of bacteria, cyanobacteria, filamentous fungi and yeasts which coexist in natural ecosystems. These organisms may act independently or in combination with each other to metabolise hydrocarbon molecules. This co-operative metabolism is especially important in the biodegradation of heavier recalcitrant fractions such as polycyclic aromatic hydrocarbons (Gibson, 1982; Cerniglia, 1984; Fedorak *et al.*, 1984). Extensive degradation of petroleum hydrocarbons is generally

only accomplished by a mixed microbial population rather than by a single species (Atlas, 1978a). While some microorganisms appear to be limited in their ability to degrade a specific group of chemicals, others have been shown to be capable of degradation of a wide range of molecules. Heterotrophic bacteria are the most important in the transformation of hazardous organic compounds and bioremediation activities are generally directed to enhancing their activity. Current studies continue to expand the list of microbial species capable of degrading petroleum hydrocarbons (Table 1.1).

Table 1.1: Genera of hydrocarbon degrading bacteria and fungi isolated from contaminated soil environments (Bossert and Bartha, 1984).

Bacteria	Fungi
Achromobacter	*Acremonium*
Acinetobacter	*Aspergillus*
Alcaligenes	*Aureobasidium*
Arthrobacter	*Beauveria*
Bacillus	*Botrytis*
Brevibacterium	*Candidia*
Chromobacterium	*Chrysosporium*
Corynebacterium	*Cladosporium*
Cytophaga	*Cochiliobolus*
Erwinia	*Cylindrocarpon*
Flavobacterium	*Debaryomyces*
Micrococcus	*Fusarium*
Mycobacterium	*Geotrichum*
Nocardia	*Gliocladium*
Proteus	*Graphium*
Pseudomonas	*Humicola*
Sarcina	*Monilia*
Serratia	*Mortierella*
Spirillum	*Paecilomyces*
Streptomyces	*Penicillium*
Vibrio	*Phoma*
Xanthomonas	*Rhodotorula*
	Saccharomyces
	Scolecobasidium
	Sporobolomyces
	Sprotrichum
	Spicaria
	Tolypocladium
	Torulopsis
	Trichoderma
	Verticillium

This list is by no means definitive but serves to exemplify the diversity of fungal and bacterial genera most frequently isolated from contaminated soil environments.

[1.2.1.a] Biological adaptation through prior exposure

Prior exposure of a microbial community to hydrocarbons, from human activity or natural sources, is important in determining how rapidly subsequent hydrocarbon inputs can be degraded. This phenomenon results in an increased hydrocarbon-oxidising potential known as adaptation. Adaptation can arise through three major mechanisms (Spain *et al.*, 1980):

(i). Induction and/or repression of specific enzymes;
(ii). Genetic changes resulting in new metabolic capabilities; and
(iii). Selective enrichment of organisms able to transform the contaminant compound(s).

Chakrabarty (1976), working with a *Pseudomonas* sp., found that the pathways for the metabolism of compounds such as naphthalene, xylene, toluene and octane were encoded on plasmids. Plasmid DNA may play a particularly important role in genetic adaptation in bacteria. This is an important attribute of bacteria that is available to researchers in the development and selection of bacterial strains for use in bioremediation.

[1.2.1b] Co-oxidation/co-metabolism

It is assumed that co-metabolism/co-oxidation plays an important role in facilitating the degradation of certain hydrocarbon molecules. A petroleum hydrocarbon mixture, with its multitude of primary substrates, provides an excellent environment in which co-oxidation can occur. Microorganisms have been found that are able to use polycyclic aromatic hydrocarbons (PAH) such as naphthalene, biphenyl, anthracene or phenanthrene as their sole carbon sources. However, there are no reports on microorganisms that are able to use polycyclic aromatic hydrocarbons consisting of four or more rings as sole carbon sources. This is not to say that they are not degraded but the presence of an alternative carbon

source may be required (Aust, 1990).

Many complex branched and cyclic hydrocarbons undoubtedly are removed from contaminated environments as a result of co-oxidation (Perry, 1979). It is now generally accepted that the higher molecular weight PAHs contained in petroleum wastes (e.g. pyrene, benzo[*a*]pyrene) are modified only through the catalytic actions of co-metabolic microorganisms (Heitkamp and Cerniglia, 1989; Keck *et al.*, 1989; McFarland *et al.*, 1992).

Co-metabolism occurs as a result of an enzyme with a broad substrate specificity coincidentally attacking a recalcitrant molecule and metabolising it (Horvath and Alexander, 1970; De Klerk and Van der Linden, 1974). The enzyme is produced by the microorganism to metabolise some other organic compound as an energy source (De Klerk and Van der Linden, 1974; McKenna, 1977). Oxygenases are often involved in co-metabolism as they can be induced and can attack a large range of substrates. Co-metabolism results in the incomplete oxidation of the substrate, thus there is often an accumulation of transformation products which may be more or less toxic than the original compound. However, other microorganisms may utilise the by-products of the co-metabolic process (De Klerk and Van der Linden, 1974; Perry, 1979) and, thus, prevent the accumulation of these molecules.

Co-metabolism may be encouraged in bioremediation operations by a technique know as analogue enrichment. Here, a more easily degradable compound (a chemical analogue to the target compound) is incorporated into the contaminated matrix. Studies made by McFarland *et al.*(1992) suggested that the maintenance of a suitable environment for co-metabolic activity is a rational approach for achieving biotransformation of high molecular weight PAH compounds in soil. Co-metabolic reactions play an important role in the degradation of molecules mediated by organisms such as the white-rot fungi and in co-composting bioremediation systems which are discussed in greater detail later (Chapter Five).

[1.2.1c] Microbial seeding

The value of seeding contaminated sites with microorganisms has yet to be substantiated as a cost-effective technique for the restoration of contaminated aquifers and soils (Riser-Roberts, 1992). Methods have to be devised to determine whether the indigenous or introduced microorganisms are more effective in a given situation. Research is also needed to determine the requirements of introduced organisms to prevent them being out-competed by the indigenous microflora. The problem of potentially pathogenic microorganisms developing in the subsurface and the long-term environmental effects of seed cultures also require further investigation to gain public and governmental legislative acceptance.

[1.2.2] Chemistry of the Pollutant Molecule

[1.2.2a] Chemical structure and toxicity of the pollutant molecule

The biodegradability of individual components in a particular hydrocarbon product will depend primarily on their chemical structures but it is also strongly influenced by the physical state and toxicity of the compounds. Susceptibility of hydrocarbons to biodegradation varies with the type and size of the components. For example, alkanes of intermediate chain length (C_{10}-C_{24}) are rapidly degraded. Very long chain alkanes, however, are resistant to biodegradation. Molecules with molecular weights exceeding 600 g mol^{-1} cease to serve as carbon sources for microorganisms (Englert *et al.*,1993). Hydrocarbons are generally ranked in the following order of decreasing susceptibility to microbial degradation: *n*-alkanes > branched alkanes > low-molecular weight aromatics > cyclic alkanes. This pattern is not, however, universal and it is reasonable to envision that specific selection pressures at a particular site will impart specific enzymatic capabilities on the indigenous microorganisms at that site. For example, Cooney *et al.* (1985) reported degradation rates of an aromatic compound (naphthalene) which were higher than those of a straight chain alkane (hexadecane).

Slow degradation rates of an organic compound may be as a result of a number of factors including: low microbial populations; an unfavourable nutrient balance; and toxicity from

contaminant overloading (Pettyjohn and Houndslow, 1983). Oils are extremely complex compounds which contain hundreds of hydrocarbon and non-hydrocarbon components. The presence of certain inhibitory substances can retard or prevent the biodegradation of otherwise labile substrates (Bartha and Atlas, 1977). When the structural features which confer toxicity to a compound are compared with those that permit degradation, differences are found among the various chemical classes (Kaufman and Plimmer, 1972). In some cases, structural features contributing to toxicity are coincident with those necessary for degradability, in other chemicals they are diametrically opposite. In all cases catabolism is mediated by substituent type, number and position.

While *n*-alkanes are most readily biodegradable, low molecular weight hydrocarbons (C_5-C_{10}) solvate and, hence, destroy the phospholipid-containing membrane structures in microorganisms (Bartha and Atlas, 1977). Liquid hydrocarbons of the *n*-alkane, *iso*-alkane, cycloalkane and aromatic type, with carbon chain lengths of under ten, all share this property to varying degrees. The *n*-alkanes, *n*-alkylaromatic and aromatic compounds of the C_5-C_6 range are biodegradable in low concentrations by some microorganisms but in most environments they are removed by volatilisation rather than biodegradation. Gaseous alkanes (C_1 to C_4) are biodegradable but are used only by a narrow range of specialised hydrocarbon degraders.

The *n*-alkanes, *n*-alkylaromatic and aromatic compounds of the C_{10} - C_{22} range are the least toxic and most readily biodegradable. The *n*-alkanes, *n*-alkylaromatic and aromatic compounds above C_{22} are hydrophobic solids at physiological temperatures. This has a dramatic effect in reducing their water solubility and, hence, their bioavailability (Bartha and Atlas, 1977). Branching of structures (e.g. asphaltics) typically reduces biodegradation rates and aromatic compounds are degraded more slowly than alkanes (Atlas, 1988). Tertiary and quaternary carbon atom branching constitute hindrances to β-oxidation. Branched alkanes and cycloalkanes of the C_{10}-C_{22} range are less biodegradable than their *n*-alkane and aromatic analogues.

Primary microbial attack of intact hydrocarbons always requires the action of oxygenases

and, thus, the presence of free oxygen is required. Most microbial enzymes attack alkanes terminally although some perform subterminal oxidations. The alcohol product is oxidised further to an aldehyde and, finally, to a fatty acid and acetyl co-enzyme A. The fatty acid is degraded further by β-oxidation with the eventual liberation of CO_2. Any degree of methylation strongly interferes with β-oxidation thereby increasing the resistance of the hydrocarbon to microbial attack (Pirnik, 1977). With these molecules, additional strategies such as α-oxidation (Cerniglia and Perry, 1973), Ω-oxidation (Pirnik, 1977) or β-alkyl group removal (Cantwell *et al.*, 1978) are required.

Cycloalkanes are highly resistant to microbial attack and often account for the most persistent components of hydrocarbon-contaminated environments (Atlas, 1981). There have been reports of oxidative and co-oxidative degradation of both substituted and unsubstituted cycloalkanes (Perry, 1979). Molecules containing up to six ring structures have been reported to be susceptible to microbial degradation (Cobet and Guard, 1973). Cycloalkanes are transformed by an oxidase system (not fully characterised) to a cyclic alcohol which is dehydrogenated to a ketone. Monoxygenases (distinctly different from the monoxygenase described above) lactonise the ring which is subsequently opened by a lactone hydrolase. The fact that the two oxygenase systems described above rarely occur in the same microorganism foils attempts to isolate monocultures which grow on cycloalkanes. However, synergistic action in microbial communities is capable of dealing with various cycloalkanes quite effectively (Bartha, 1986). Bacterial degradation of aromatic compounds normally involves the formation of a diol followed by cleavage and formation of a diacid. In contrast, fungi only incorporate one atom of molecular oxygen into the aromatic nucleus, as has also been found in mammalian hydrocarbon hydroxylase systems (Cerniglia and Gibson, 1979).

The toxicity of PAHs to microorganisms is also related, in part, to their water solubility (Sims and Overcash, 1983). While low molecular weight aromatic hydrocarbons are relatively toxic to microorganisms, they can be metabolised when present in low concentrations. Condensed PAHs are less toxic to microorganisms but are metabolised only rarely and at slow rates. Cycloalkanes are highly toxic, especially those with carbon chain

lengths below C_{10}, and are metabolised by selected microorganisms in exceptional cases. Highly condensed aromatic and cycloparaffinic systems, with four or more condensed rings, and partially oxygenated and condensed components of tar, bitumen and asphalt degrade very slowly. The biotransformation of PAHs with more than three rings generally appears only achievable with co-metabolism (Sims and Overcash, 1983). The ability of microorganisms to degrade PAHs is a function of the number of aromatic rings in the compound (Bumpus, 1989). While low molecular weight PAHs are usually readily degraded, high molecular weight PAHs, of five or more rings, resist extensive bacterial degradation in soils and sediments (Bossert and Bartha, 1984; Heitkamp and Cerniglia, 1989). The metabolic pathways for the degradation of asphaltenes are probably the least well understood and no uniform degradative pathway has been established. These are complex structures and advances in determining degradative pathways appear to be dependent on improved chemical analytical methods.

[1.2.2b] Physical state of the hydrocarbon

The physical state of a contaminant hydrocarbon will, firstly, define its behaviour in the soil matrix after a spillage. The compound's viscosity will affect both vertical migration and vadose zone formation. Infiltration prevents evaporative losses of the volatile components which are toxic to microorganisms. Particulate organic matter in the soil can reduce, by adsorption, the effective toxicity of some petroleum components but may contribute to the formation of persistent residues. The formation of hydrocarbon emulsions through the microbial production and release of biosurfactants plays an important role in the uptake and metabolism of hydrocarbons by both bacteria and fungi (Singer and Finnert, 1984). Over the last four decades a large number of synthetic surfactants have been studied as a means of increasing the surface area of hydrocarbons for microbial attack. Some of the anionic dispersant formulations developed in the late 1950's were highly toxic to indigenous fauna and flora of the marine environments in which they were applied (Smith, 1968; Colell, 1971). New varieties of synthetic dispersants have been developed which are considerably less toxic, but many of which have been shown to produce an inhibitory effect on various microbial processes (Griffiths *et al.*, 1981). The overall efficacy of the various

dispersants in various studies has been extremely variable and dependent, primarily, on the chemical formulation, its concentration and the surfactant/oil application ratio. Decreased, transitory and/or only slightly increased hydrocarbon degradation rates have been reported (Mulkins-Phillips and Stewart, 1974). As a result, commercial surfactants are not used routinely in bioremediation.

[1.2.2c] Hydrocarbon concentration

The rates of microbial uptake and mineralisation of hydrocarbons in the aqueous phase are proportional to the substrate concentration, thus conforming to Michaelis-Menten kinetics (Pfaender and Bartholomew, 1982). However, with higher molecular weight aromatic hydrocarbons, mineralisation is related to aqueous solubilities rather than total substrate concentration (Wodzinski and Bertolini, 1979; Thomas *et al.*, 1986). Microbial degradation of carbon chain alkanes exceeding C_{12}, for which the solubilities are less than 0.01 mg l^{-1}, occurs at rates exceeding those of hydrocarbon dissolution and are thus a function of the hydrocarbon surface area available for emulsification or physical attachment to the microbial cells. Concentrations of hydrocarbons above certain threshold concentrations in solid or liquid matrixes prevent biodegradation due to oxygen and nutrient limitation.

[1.2.3] Environmental Factors

[1.2.3a] Temperature

Temperature exerts a strong influence on the biodegradation of xenobiotics as it influences both the physical and chemical composition of the hydrocarbons. Low temperatures reduce the volatilisation of short carbon chain alkanes and increase the viscosity of the oil. Water solubility of hydrocarbons is also decreased at lower temperatures, thus delaying the onset of biodegradation (Atlas and Bartha, 1972).

Metabolic rates of degrading microorganisms and the overall compositions of the microbial populations are also affected by temperature (Atlas, 1981). Increases in rates of

biodegradation associated with increases in temperature are related to the "Q_{10}" effect on enzyme activity. The rates usually increase to a maximum around 40°C, after which membrane toxicity to hydrocarbons is increased. Thermophilic hydrocarbon-utilising microorganisms are able to tolerate higher temperatures (Klug and Markovetz, 1967). These types of microorganisms play an important role in co-composting bioremediation systems (Chapter 4).

[1.2.3b] Electron acceptors

(i). Oxygen

Oxygen concentration has been identified as the most critical rate-limiting variable in petroleum biodegradation (Van Wedel *et al.*, 1988). This is due to the initial stages of hydrocarbon-substrate degradation involving oxygenases for which molecular oxygen is required. The availability of oxygen in soil is influenced by a number of interdependent factors including type of soil, soil porosity and water activity. Oxygen concentration decreases with an increase in soil depth and this results in stratification of microorganisms in the soil sub-surface. Biological factors can also lead to oxygen depletion in soil as the presence of labile substrates affects microbial biomass and activity.

Anaerobic degradation of petroleum hydrocarbons by microorganisms does occur (Ward and Brock, 1978; Evans and Fuchs, 1988) although the rates are so low that its ecological significance is considered to be minor (Atlas, 1981; Bossert and Bartha, 1984). Microbial degradation of a large number of aromatic compounds, including halogenated aromatic compounds such as halobenzoates (Suflita *et al.*, 1982) and chlorophenols (Boyd and Shelton, 1984), have been shown to occur under anaerobic conditions. Numerous polyhalogenated organics have been responsive to bioremediation through the use of alternating anaerobic and aerobic conditions. Under favourable conditions, polyhalogenated compounds, such as highly chlorinated PCBs, can be reductively dehalogented under vigorous reducing conditions. Once the first group of halogens has been removed from the molecule, the treatment environment can be converted to an aerobic oxidising environment

with gaseous oxygen or hydrogen peroxide to complete biodegradation (Worne and Fortune, 1992). Alternating redox potentials are potentially suitable for the successful bioremediation of soils and groundwater contaminated with a number of polychlorinated aliphatic compounds such as TCE and PCE.

(ii). Alternative electron acceptors

Concerns over the stability of hydrogen peroxide have led researchers to evaluate soluble electron acceptors such as nitrate, sulphate and iron as alternative electron acceptors. Several studies have been made to evaluate nitrate as an alternative electron acceptor for the degradation of mono-aromatic (except benzene) and polyaromatic hydrocarbons (Brown *et al.*,1995). Nitrate is inexpensive, is easily transported in the subsurface and appears to cause fewer problems than oxygen. However, nitrate does not facilitate degradation of aliphatic compounds and there are concerns over nitrite formation and the potential for eutrophication.

[1.2.3c] Nutrients

The release of hydrocarbons into the environment represents a large influx of carbon. This often results in excessively high carbon to nitrogen ratios (C:N) and/or carbon to phosphorus ratios (C:P) which are unfavourable for microbial growth (Atlas, 1981). Thus, these nutrient imbalances have to be rectified in order to promote biodegradation of the contaminant. Measurement of soil organic carbon, organic nitrogen and organic phosphorus allows the determination of the C:N:P ratio and an evaluation of nutrient availability (Sims and Bass, 1984). If the ratio of organic C:N:P is wider than about 300:15:1, and the extractable inorganic forms of nitrogen and phosphorus do not narrow the ratio, supplemental nitrogen and/or phosphorus should be added, as commercial fertilisers (Kowalenko, 1978). After conducting research on the landfarming of waste oil, Dibble and Bartha (1979) determined that the C:N and N:P ratios of 60:1 and 800:1, respectively were optimal for biodegradation under the applicable conditions.

Great difficulty often lies in the choice and the effective delivery of the fertiliser to the contaminated matrix. As a consequence, nutrient supplementation often represents a major cost component of bioremediation projects.

[1.2.3d] Soil moisture content

Water is an essential molecule for microbial metabolic processes and hydrocarbon biodegradation can become limited by the soil moisture content. In the context of bioremediation, water also exerts a major impact by virtue of its physical presence. A primary reason for the recalcitrance of many hydrocarbons in the environment is that microbial biodegradation is only able to take place in the aqueous phase. Unfortunately, most hydrocarbons are hydrophobic and, hence, are only poorly soluble in water. Under excessive moisture levels aerobic microbial growth is significantly reduced due to the reduced gaseous exchange in the water-saturated soil pore spaces. The optimum level capable of sustaining high growth and metabolic activities of aerobic bacteria usually occurs at 50% to 70% of the soil water holding capacity (Worne and Fortune, 1992).

[1.2.3e] pH

Soil is the most complex portion of the biosphere when compared to the fairly uniform marine environment which is well buffered against acidification. Most bacteria have a limited tolerance of acidic conditions while fungi tend to be more tolerant of pH extremes. Most heterotrophic bacteria and fungi favour a pH near neutral. In acidic soils, one can expect hydrocarbon degradation to be mediated predominantly by fungi (Atlas, 1988). Jones *et al.* (1970) reported that the pH values of an acidic moorland soil, heavily contaminated with kerosene, sank to between 1 and 2 pH units, while the non-contaminated soil had a pH of 4.2-4.6. The fungal component of the contaminated soil increased by a factor of five while the bacterial component declined. The population of fungal hydrocarbon degraders increased by tenfold, while the increase in the bacterial community was small. It is accepted that fungi play an important role in hydrocarbon degradation at low pH values although the overall rate of biodegradation under low pH conditions is lower

than the rate obtained by a mixed bacterial and fungal community at neutral or slightly alkaline pH's (Atlas, 1988). Dibble and Bartha (1979) observed an optimal pH of between 5.0 and 7.8 for the mineralisation of an oily sludge. Depending on the type of contamination, soil may need the addition of lime. This also prevents possible leaching of heavy metals.

[1.3] BIOREMEDIATION TECHNOLOGIES

A wide range of bioremediation strategies is available for the clean-up of hydrocarbon-contaminated soil. Selecting the type of strategy most appropriate for a specific site is determined by a number of interdependent factors and can be guided by considering three basic principles:

(i). **Biochemistry:** The amenability of the pollutant to biological transformation to less toxic products;

(ii). **Bioavailability:** The accessibility of the contaminant to the degrading microorganisms is strongly dependent on the nature of the petroleum hydrocarbon, the soil chemistry and the soil matrix. In some cases, bioavailability may be relatively unimportant while in others it may be critical. The influence of site-specific bioavailability on bioremediation must be considered; and

(iii). **Bioactivity:** The opportunity for optimisation of biological activity includes parameters that have long been recognised as influencing the rate and extent of bioremediation (discussed in Section [1.2] above).

Other factors include: site constraints; clean-up criteria based on projected land use; and local legislation. Bioremediation can be grouped into two broad types: ***intrinsic*** and ***engineered***. Intrinsic bioremediation uses the inherent capabilities of naturally occurring microbial communities, present in the contaminated environment, to degrade the pollutant. Engineering steps are not implemented to enhance the process. Intrinsic bioremediation differs from *no-action* alternatives in that a comprehensive site evaluation in terms of sampling and analysis of the pollutant and microbial population is made. The site will also

require continuous monitoring for pollution migration. Engineered bioremediation accelerates microbial activity through engineered site-modification procedures such as the installation of wells to circulate fluids and nutrients to stimulate microbial growth. Site engineering serves to provide some control so that field sites, in essence, become *in situ* bioreactors. Other terms used to describe engineered bioremediation include *"biorestoration"* and *"enhanced bioremediation"*.

The emphasis of the following discussion is directed towards examining *ex situ* enhanced bioremediation technologies to consolidate the objective of this research project. *In situ* bioremediation is discussed only briefly.

[1.3.1] *In situ* Bioremediation

In situ bioremediation evolved from the *pump-and-treat* systems developed in the past decade for the treatment of contaminated groundwater. Pump-and-treat systems use a series of wells to raise water to the surface for biological or non-biological treatment. These methods are able to control contaminant migration and remove the contaminant mass. However, because the contaminants can become trapped in the subsurface, flushing them out may require extremely large volumes of water and long time periods. *In situ* bioremediation treats contaminants in place instead of requiring extraction and, as a result, is able to speed up the remediation process in most cases. *In situ* treatment is used largely for soils contaminated with aqueous- or non-aqueous phase liquids and light hydrocarbon molecules which are not strongly sorbed to the soil. *In situ* bioremediation is only considered effective in permeable sandy types of soils (with permeabilities $>10^{-4}$ cm s^{-1}), where soil excavation is too expensive or impractical and when the contaminant chemicals are not easily removed by vapour extraction (Hoeppel *et al.*, 1991).

There are three major types of *in situ* bioremediation practices: bioventing; soil washing; and low temperature thermal methods.

[1.3.1a] Bioventing

Bioventing is a hybrid *in situ* remedial technology which combines the physical processes of soil vapour extraction (SVE) with the degradation potential of enhanced biodegradation. Soil vapour extraction is practised routinely (Europe, U.K., U.S.A.) for the physical removal of volatile and semi-volatile contaminants (Johnson *et al.*, 1990). Bioventing is particularly promising for the remediation of soil contaminated with landfill leachate, middle distillate fuels (e.g. JP-4 jet fuel), oil refinery products and certain chemical production facility products (Bossert and Compeau, 1995). During SVE, a vacuum is used to induce a negative pressure in the vadose zone thus accelerating the volatilisation of hydrocarbon compounds. As air is pulled through the soil, the volatile organics are evacuated with the flow to the surface for aboveground treatment. The success of the technology is ultimately limited by the vapour pressure of the organic compound present. Generally, organic compounds with vapour pressures >0.1 atm. are amenable to SVE (Dupont, 1992). The key advantage of SVE (and bioventing) is the limited disruption to on-going site operations and the potential to clean soil located below the practical limits of excavation. This has the additional benefit of massive potential cost savings related to decreased excavation costs. Bioventing extends the advantages of SVE by facilitating biological degradation of residual hydrocarbons in place.

The addition of oxygen and nitrogen is accomplished by pulling air through the vadose zone wells. Reduced nitrogen is provided by injecting low concentrations of anhydrous ammonia gas into the air stream which passes through the soil. The negative pressure that develops in the soil pores greatly increases volatilisation of hydrocarbon compounds sorbed onto the soils in the vadose zone. This is also increased by a simultaneous lowering of the groundwater table. The primary purpose of soil venting in the contaminant zone is to stimulate aerobic biodegradation since middle distillate fuels are comprised mainly of low to medium volatility compounds. Volatilisation of the fractions with higher vapour pressures should also function to increase fuel surface areas and dilute the main hydrocarbon plume. The release of volatile hydrocarbons from the soil is greatly reduced if the rate of venting is equivalent to the rate of vapour phase biodegradation in the soil

profile (Miller and Hinchee, 1990). The key parameter in controlling vapour movement in the soil is the permeability to air which is a function of soil texture (soil particle size distribution), soil moisture and bulk density.

Introduction of oxygen and nitrogen into the soil in the vapour phase, rather than the dissolved phase, has some distinct advantages (Dineen *et al.*,1990):

(i). The contaminated matrix remains unsaturated and minimises the downward migration of chemicals which may occur under saturated conditions;

(ii). Vapour phase delivery provides the soil with atmospheric concentrations of molecular oxygen, compared to the low concentrations provided by hydrogen peroxide; and

(iii). Vapour phase delivery of anhydrous ammonia as a source of reduced nitrogen minimises the risk of migration of nitrates into the groundwater.

Oxygen is required at a concentration of approximately 3.1 kg kg^{-1} hydrocarbon degraded. The maximum amount of oxygen in a well aerated soil is approximately 200 000 ppm. The maximum amount of oxygen in groundwater is approximately 8 ppm. If hydrogen peroxide is used, the concentration of dissolved oxygen can be increased to 200 - 800 ppm (Ward *et al.*, 1989). Thus, delivery via the vapour phase is the most efficient oxygen delivery mechanism to soil. Reduced nitrogen is required at a concentration of 1 kg 160 kg^{-1} hydrocarbon degraded. Nitrogen is, typically, added to the soil as urea or ammonium nitrate (which dissolves in the soil water as ammonium and nitrate). Other than phosphorus, other elements are generally not limiting and are usually present in adequate concentrations. By using oxygen as an electron acceptor, the quantity of water required for the bioremediation of 1 m^3 of soil amounts to about 3000 m^3. The cost of the same amount of electron acceptor provided by oxygen from hydrogen peroxide is up to five times higher than that of nitrate (Battermann and Meier-Lohr, 1995).

The design of a successful *in situ* bioventing system depends on subsurface parameters of the contaminated soil which will be unique to the site in question (i.e. soil chemistry, soil microbiology, soil physics, soil morphology, and hydrogeology). Environmental variables affecting bioventing are still only poorly understood. Contradictory data show that many notions regarding the factors which control bioventing rates can be wrong. For example,

considerable biodegradation of hydrocarbons has been shown to occur at very low temperatures, in soils with low permeabilities and at very low nitrogen concentrations (Hoeppel *et al.*, 1991).

Despite the widespread use of SVE in the U.S.A., only a relatively small percentage of sites using this technology have reached closure (Mohr and Merz, 1995). There is a need for engineering design methods to verify experience and intuition, which are heavily relied upon in the design of SVE treatment strategies. Most importantly, site managers must decide on how closely to space SVE wells (Mohr and Merz, 1995) and how to place these wells (Conant *et al.*, 1995) to optimise the process. In addition, the treatment time has to be estimated. In an attempt to achieve these goals, researchers have modified existing air/aqueous flow models for use in user-friendly computer software. Mohr and Merz (1995) described a two-dimensional air flow model called Piecewise Linear Triangle Multi-Grid (PLTMG) for the prediction of the effective radius of the well's influence. The effective radius of influence is defined here as the maximum distance from the well where there is sufficient air flow to remove the contaminant present within the allowable time. Similarly, Conant *et al.* (1995) used Saturated-Unsaturated Flow Model with Particle Tracking (SUNFLOWPT) to predict the appropriate vertical placement of well screens. Field results of studies with SUNFLOWPT suggested that two screens which separately capture the clean and contaminated water within an aquifer could significantly reduce the volume of water requiring treatment and, hence, reduce the treatment time.

Soil venting is a potentially cost-effective technology for removing volatile hydrocarbon components from contaminated soil. The costs may be as low as U.S. \$15 ton^{-1}. If activated carbon filters are needed to trap vapours, the operating costs may double and the hydrocarbons may still not been destroyed (Hinchee *et al.*, 1987).

[1.3.1b] Soil washing

Soil washing involves injecting synthetic surfactant or solvent into the contaminated zone to promote greater release of hydrophobic contaminants to the aqueous phase and, hence, increase their bioavailability. This technology, however, has met only limited success in both laboratory and field evaluations (Nash *et al.*, 1987; Arthur *et al.*, 1989). There are, however, few cases where the addition of surfactant has significantly increased pollutant biodegradation rates. Thus, the use of surfactants in full-scale bioremediation projects is seldom justifiable (Arthur *et al.*, 1989).

[1.3.1c] Low temperature thermal methods

As these are not solely biological forms of soil remediation, only a short note will be provided here. Low temperature thermal methods involve the injection of heated gas/steam or use radio frequencies to heat in-place soils. The processes result in the release of hydrocarbons which may then be pumped and treated on the surface. These remediation methods are expensive in terms of energy and equipment and can cost in the region of U.S. \$330 m^{-3} soil (R1485, 1996). Due to the high cost, low-temperature methods are not routinely used for the remediation of hydrocarbons (Hoeppel *et al.*, 1991).

[1.3.2] *Ex Situ* Bioremediation Treatment Technologies

Ex situ bioremediation involves the excavation of the contaminated soil and placement in lined treatment cells. It is preferable that soil treatment occurs on site as liability to the owner/operator and the overall costs are reduced. An impermeable liner (clay or a geotextile) is used to protect the underlying soil and groundwater from contamination. *Ex situ* bioremediation is applicable to soils with low permeabilities and for soils contaminated with heavier viscous hydrocarbon molecules which may be strongly adsorbed. *Ex situ* solid phase techniques consist of three main types: soil treatment units; engineered biopiles; and slurry-phase reactors.

[1.3.2a] Soil treatment units

Landfarming, in its most basic form, was practised as far back as the 1930's when it was usual for refineries to dump their sludges onto sandy soils, often on the banks of rivers. Here, reductions in sludge volume over time were noted. In the 1970's, as the first wave of stringent environmental regulations came into place, these practices became less acceptable. The United States Resource Conservation and Recovery Act (RCRA) provided a tough regulatory framework and specified new standards for the construction and operation of land treatment systems. Most of the improved control methods dealt with the fate and transport of contaminants from the treatment area. Former landfarming methods encouraged leaching into the surface- or groundwater together with volatilisation as methods to reduce the contaminant volume. Current practice attempts to reduce the two methods of removal as much as possible. This treatment method is primarily applicable to organic sludges and semi-solid materials. Volatile materials are not permitted (in the U.S.A.) to be treated by this approach as a major percentage of the material volatilizes too rapidly to allow biodegradation to occur (King *et al.* 1992a).

Soil treatment or land treatment units are the simplest forms of engineered bioremediation. They consist of soil which is contained in lined treatment cells that are tilled to supply oxygen. In addition, water, nutrients and, possibly, microbial inocula are applied to the soil. Landfarming is commonly used for the treatment of petroleum products and a wide range of other liquid and solid wastes. In addition to its use as a waste disposal method, landfarming may also be used to bioremediate hydrocarbon-contaminated soils. The petroleum product is spread out over soil and is degraded by soil microorganisms. Landfarming is effective for the removal of petroleum hydrocarbons, typically reducing total petroleum hydrocarbons (TPH) concentrations to below 1000 mg kg^{-1}. Reduction in hydrocarbon concentrations to below 50 mg kg^{-1} may require extended treatment periods, more sophisticated site engineering and more frequent rototilling. Rototilling improves soil texture, increases soil permeability and soil uniformity and improves the transfer efficiency of atmospheric oxygen.

A prerequisite for landfarming treatment is a sufficient land area to spread out the soil to a depth of 15 cm to 80 cm. Depending on the availability of land at the treatment site, landfarming is one of the least expensive treatment options available. The upper 15 to 30 cm is the treatment zone or zone of incorporation (ZOI). The ZOI, in conjunction with the underlying soils where additional treatment and immobilisation of applied waste constituents occur, is referred to as the treatment zone. The volume of soil which may be treated per unit area is controlled by the aeration system used. In a typical landfarming operation, the soil is spread thinly over a liner. A layer 30-80 cm thick allows approximately 1000 m^3 of contaminated soil to be treated per hectare with a rototiller for aerating the soil. The use of specially designed equipment, such as a horizontally mounted auger, can increase this figure but may also significantly increase the total operating costs (Block *et al.*, 1990). The treatment zone may be as deep as 1.5 m. Soil conditions below this depth are generally not conducive to oxidation of the applied contaminants. The soil depth must be adequate to permit rototilling or mechanical aeration and prevent liner puncture. Underdrain piping is placed beneath the soil to intercept, and facilitate, leachate collection for recirculation or further treatment. The treatment area should be surrounded by a containment berm to enclose the contaminated soil and to prevent runoff from leaving the site. Some agencies require electronic sensing equipment such as piezometers to be installed down-gradient from the cell to demonstrate that no contaminant is migrating from the cell. Groundwater monitoring wells, initially sunk for site assessment purposes, may require periodic sampling and analysis for target compounds. Landfarming sites may often have to be covered to reduce volatile organic carbon (VOC) emissions. This will, however, depend on local air quality regulations.

A sprinkler system may be installed to increase the soil moisture. In addition, the same system may be used to deliver nutrients, surfactants and other conditioning agents to the soil. Soil moisture content should be kept at approximately 50% of field capacity. Water may be pumped from a collection sump if needed. Continual soil sampling and analysis should be conducted throughout the treatment period. The sampling protocol will depend on the depth of the soil, the shape of the cell and the sampling density requested by the local regulatory agency.

Soil nutrients, pH and buffering capacity (i.e. lime requirement), and TPH testing may be done on a monthly or quarterly basis to provide information on the remediation progress and the need for specific adjustments to keep the process within its operating range. These typical operating parameters are shown in Table 1.2. Leachate collected from the sump may also be tested for these parameters. Hydrocarbon concentration monitoring is based on a relatively inexpensive tracking parameter, such as TPH (EPA Method 418.1), even if the final closure depends on a specific compound analysis.

Table 1.2: Optimal environmental parameters for the landfarming of oily wastes (Dibble and Bartha, 1979).

Soil water holding capacity	30% to 90%
pH	7.5 to 7.8
C:N ratio	60:1
C:P ratio	800:1
Optimal temperature	20°C or above.

Dibble and Bartha (1979) found that a sludge application rate of 5% (w/w) i.e. 100 000 l ha^{-1} provided a favourable compromise between high biodegradation rates and efficient land use. Pollutant loading of the soil should also be made in frequent small applications rather than a single large application.

The following two examples demonstrate key aspects and the costs involved in landfarming. Rangal *et al.* (1988) described a landfarming operation for the disposal of a petrochemical waste. Due to the large volume of oily sludge generated (1380 m^3 $year^{-1}$), remediation was conducted on an area of 180 000 m^2 (18 ha). According to Huddleston (1979), a volume of 0.023 m^3 of oil should be applied m^{-3} soil $year^{-1}$. Thus, an area of 11470 m^2 was needed for the application of the 1380 m^3 of petrochemical sludge generated

in one year. The sludge was applied in two applications during the year. An impermeable clay layer ($K = 10^{-7}$ cm^{-1}), with a minimum depth of 50 cm and an average depth of 75 cm, lined the treatment cell. A reactive layer of free material (45 cm deep) was designed to receive the oily sludge. The reactive layer received additions of nutrients and calcareous dolomite (2.38 kg m^{-2}) to correct the soil pH to between 6.5 and 9 (Torrey, 1979). The latter helped to prevent the leaching of heavy metals.

The nutrient application rates were as follows: 0.0176 kg m^{-2} triple super phosphate; 0.0088 kg m^{-2} potassium chloride; and 0.220 kg m^{-2} urea. The nutrients were added 30 days before the sludge was applied. Urea was added again prior to the second sludge application. The costs of landfarming are generally low when compared to other treatment alternatives. An analysis of this treatment process estimated the cost to be approximately U.S. \$30 m^{-3} soil (R135, 1996). It was determined that the sludge should be distributed without exceeding a maximum limit of 10% (w/w) of oil in the reactive layer (Huddleston, 1979) which was checked by laboratory analysis. The reactive layer was ploughed every 14 days.

Lynch and Genes (1993) estimated the cost of on-site treatment and capping of a soil at a Superfund site contaminated with wood preservative to be U.S. \$59 ton^{-1}. Manure (120 tonnes) was spread over the treatment area and the loading rates were calculated to achieve a C:N ratio of 50:1. In addition to the 2% nitrogen, the manure provided organic matter which enhanced absorption of the hazardous waste contaminants. Maintaining soil moisture to near field capacity was determined to be a key operating parameter in pilot-scale studies. A centre pivot system was used to deliver water and nutrients to the soil. Benzene-extractable (BE) hydrocarbons were found to decrease by $\pm$60% over the first year of operation. Most of this decrease occurred during the first 120 days. The authors, however, gave no estimation of how much of the hydrocarbon disappearance could be attributed to volatilisation. Little decreases in BE hydrocarbons were observed in the winter and autumn months.

[1.3.2b] Engineered biopiles

Contaminated soil may be heaped into "biopiles" and oxygen is supplied to the media through an internal network of pipes with active or passive aeration. This technology is a combination of the *in situ* bioventing treatment process [1.3.1a] and the *ex situ* landfarming process previously described [1.3.2a] and, thus, some of the shortcomings associated with landfarming are addressed. Many researchers have found that there are substantial benefits to be realised over conventional landfarming systems by using vented biopiles. McGinns *et al.* (1994) found that vented biopiles operated more effectively than conventional landfarms and unvented soil piles with respect to microbial respiration and pollutant (PCP in their particular case) degradation rates. They claimed that these significant differences were due to uniform oxygen conditions produced within the biopile due to positive air injection. Forced aeration reduces the possible formation of anoxic zones in the biopile. Improved oxygen distribution in the vented biopile can lead to a more uniform degradation as opposed to only on the surface soil of landfarms. More uniform degradation leads to an increased homogeneity and greater confidence in acquiring representative samples which demonstrate statistically significant degradation. In addition to their increased biodegradation efficiency, there are major cost benefits associated with their use. The amount of land surface area required for treatment is reduced. Thus, the cost of runoff and leachate containment is reduced. Biopiles require smaller liners and smaller runoff water treatment systems, representing substantial cost savings in most situations. Plants may be grown on the soil pile since tilling is not required. These plants may eliminate dust and erosion problems as well as stimulate microbiological activity in their rhizosphere. Some biopiles, however, may be covered with polyethylene tarpaulins during operation. While these prevent wash-aways by rainfall, volatilisation of contaminant compounds is also reduced. Water and nutrients are applied to the surface of the pile and are allowed to percolate through the matrix. Although not usually required, microbial inoculum may be added. If aeration is provided by a vacuum pump, an air emission recovery system may be installed. Often local regulatory restrictions may require the capture of VOCs. During operation, the pH and moisture are maintained within ranges conducive to microbial activity.

A bioventing installation, as described by Guthrie *et al.* (1995), provides an useful illustration of large-scale *ex situ* bioventing. Bioventing was used as a pre-treatment to landfarming at a site contaminated with petrol, kerosine and fuel oils as landfarming could not be practised during the cold winter months of the year. Total petroleum hydrocarbon concentrations as high as 10 000 mg kg^{-1} and BTEX concentrations as high as 17 mg kg^{-1} were detected at the site (the target concentrations were set at 50 mg kg^{-1} for TPH and 0.25 mg kg^{-1} for BTEX). Large biopiles, consisting of three modules (4.6 m high, 33 m wide and 56 m long) were constructed. A series of slotted pipes was installed horizontally at 1.5 m and 3 m elevations. The pipes were connected via a manifold to a 5 h.p. regenerative blower. Approximately 5% -10 % of the extracted air was treated by an off-gas treatment system, which consisted of two vapour-phase carbon canisters, prior to discharge to the atmosphere. The three combined modules contained 32 700 m^3 soil. After 187 days of treatment operation, the TPH and BTEX concentrations in the two modules had decreased by 94% and 49%, respectively. This treatment lowered the contaminant hydrocarbon half life from 17 days to eight days. The soil remediation costs were approximately U.S. \$46 m^{-3} (R168, 1995). Soil satisfying clean-up criteria did not have to be reloaded into landfarming treatment cells and then unloaded for backfilling. This resulted in large savings in operation costs. It was estimated that at this site, bioventing should result in a cost saving of between U.S. \$1M and U.S. \$1.5M and a reduction in site remediation time by as much as three years. Compost biopiles are solid-phase waste treatment systems which operate at elevated temperatures due to thermophilic microbial activity. Composting, for use as a bioremediation technology, and co-composting are discussed in Chapter Five.

[1.3.2c] Slurry-phase reactors

Slurry-phase bioremediation is a process whereby contaminated soil is treated in vessel-type bioreactors which are usually located close to the contaminated site. The contaminated soil is loaded in the form of an aqueous slurry which allows for continuous mixing in the vessel. Slurry-phase treatment (SPT) has been used for a range of pollutant molecules

including: PCP (Compeau *et al.*,1990); PCBs (U.S. EPA, 1989); TNT (Montemagno and Irvine, 1989); and PAHs (Morgan *et al.*, 1991). When SPT is used, the treatment times may be dramatically reduced and much higher concentrations of contaminants may be treated than are possible with most *in situ* bioremediation applications. The improved efficiency of these systems is due to the fact that the technology allows for continual mixing which facilitates better contact between the microorganisms and the pollutant molecules. Strict control of environmental conditions within the bioreactor is also possible. Woodhull and Jurger (1994) published cost estimates associated with their pilot-scale SPT unit. All aspects of the treatment of 9100 m^3 soil were costed including labour, equipment, materials and analytical costs. A figure of U.S. \$200 - \$230 m^{-3} soil ($\pm$ R1030, 1996) was obtained. Although this figure will obviously be subject to change, the greatly improved efficiency of the process comes with a proportional increase in treatment costs and makes the technology prohibitively expensive for most applications.

[1.4] CONCLUSIONS

Petroleum hydrocarbons comprise naturally occurring materials which, by their nature, are biodegradable. Under conducive environmental conditions, most hydrocarbons are biodegraded by microorganisms already present in the environment. Hydrocarbons which persist are generally unavailable to the microbiota. It is apparent that microbial degradation of hydrocarbon pollutants is a complex process and that environmental factors have a great influence on the fates of pollutants in both soil and water. The rates of hydrocarbon degradation appear to be highly dependent on localised environmental conditions. On-going research strives to increase our ability to manipulate limiting environmental parameters, particularly for *in situ* applications, which result in improvements in current bioremediation efforts by increasing their efficacy while decreasing the treatment time. The addition of oxygen to the contaminated matrix appears to have the single most dramatic effect in increasing contaminant degradation rates. In the past decade, a variety of bioremediation technologies have been used to treat hydrocarbon-contaminated soils. Bioventing appears to be the most versatile and cost-effective method of bioremediation in both *in situ* and *ex situ* applications.

Several generalisations seem to be emerging from published reports of these applications:

(i). Many influential factors, such as the fate and behaviour of the contaminants, are site specific, therefore it may be difficult to generalise from one case to another;

(ii). The complexity of the hydrocarbon mixture, and perhaps its age, may affect the rate and extent of treatment;

(iii). There are large variations between laboratory, small- and large-scale studies; and

(iv). To determine the efficacy of bioremediation beyond mere compound removal will require well designed studies that can be statistically evaluated.

Bioremediation technologies offer cost-effective, permanent solutions to the clean-up of soil contaminated with petroleum hydrocarbons and other biodegradable organic molecules. With a better understanding of the interactions between the environment, the contaminants and the microorganisms mediating the degradation, improvements in the technology will ensue. Bioremediation is already a proven and effective option but its full potential has yet to be realised for the clean-up of contaminated environments.

CHAPTER TWO

[2] FUNGI IN BIOREMEDIATION: A LITERATURE REVIEW

[2.1] INTRODUCTION

In the preceding chapter, bioremediation as a technology was discussed in general. The aim of this chapter is to specifically examine the role of fungi in bioremedation. The relative contribution fungi make to the degradation of specific contaminants in the environment and their use in specialised bioremediation systems will be reviewed. The applicability of fungal remediation to the project site under study will also be examined.

Most studies investigating the bioremediation of hydrocarbons focus on degradation mediated by bacteria while relatively few research papers have dealt with the degradation of hydrocarbon-based products by fungi. These studies usually concentrate on strains isolated from temperate environments (Jones and Edington, 1968; Perry and Cerniglia, 1973; Davis and Westlake, 1978) or involve specific fungal species such as the white-rot fungus *Phanerochaete chrysosporium* (Aust, 1990; Morgan *et al.*, 1991). The general consensus among bioremediation engineers is that petroleum hydrocarbon degradation, in both marine and terrestrial environments, is achieved primarily as a result of bacterial activity. Hence, bioremediation systems are typically designed to enhance the bacterial component of the contaminant-degrading microbial community, while the fungal contribution is largely ignored.

The principal reason for the continuance of this belief may stem from the inadequacies of established microbiological methods to monitor changes in microbial biomass in response to the addition of chemical pollutants to a particular environment. Routine use of enrichment cultures and dilution plate techniques produce a bias in favour of bacterial species. The fungi isolated via these methods are usually species which produce large numbers of readily dispersed spores e.g. *Penicillium* and *Verticillium* spp. (Davis and Westlake, 1978; Miller and Whitney, 1981). The spores of these fungi can outnumber

those of other fungi to such an extent that when they are inoculated onto nutrient media they outgrow all other fungi (Kirk, 1969). This phenomenon was observed in the current study. However, many fungi produce few or no spores and thus their responses to such substrates are not detected. Soil fungi are often bound to the soil particles and are not released during the dilution process (Warcup, 1955; 1957). The data of Skinner (1976), using direct microscopic observation, support this contention. In addition, conventional methods often fail to distinguish between active mycelia and dormant fungal propagules (Davis and Westlake, 1978; Kirk and Kohlmeyer, 1979) and result in the isolation of fungi that do not truly reflect the distribution of species present in the soil (Launen *et al.*, 1995).

Differences occur in the behaviour of fungi as well as in their isolation. In general, fungi are slower growing than bacteria. Bacterial biomass doubling time under optimal conditions is often in hours, while fungal biomass doubling time can be up to several days. This has important implications in pollutant degradation as bacteria are able to compete more successfully than fungi for labile substrates. In addition, the slower growth rates of fungi also make laboratory studies more difficult. Bacteria are generally involved in the biodegradation of chemicals that have a high degree of water solubility and are not strongly adsorbed. Walker and Colwell (1974) compared the abilities of fungi and bacteria to degrade hydrocarbons. Bacteria and yeasts showed decreasing abilities to degrade alkanes with increasing chain length whereas filamentous fungi did not exhibit preferential degradation for particular chain lengths. The specifity of most bacteria towards a particular chemical or closely related chemical group generally limits their efficacy against a varied profile of chemical contaminants which may be targeted sequentially by a range of bacterial species. Bacteria tend to respond more rapidly to oil contamination of soil while fungi may be initially inhibited (Pinholdt *et al.*, 1979). Conversely, the activities of fungi tend to persist long after bacterial activity has declined (Jensen, 1975).

The relative contributions bacteria, yeasts and filamentous fungi make to the biodegradation of a particular petroleum hydrocarbon have been the subject of few studies. ZoBell (1946), in his review, noted that more than 100 microbial species, representing some 30 genera, had been shown to be able to utilise hydrocarbons. Atlas and Bartha

(1972) found that a larger number of hydrocarbon-catabolising bacteria (22 genera) were isolated from a marine environment compared with fungi (14 genera). In contrast, Jones and Edington (1968) found that 11 genera of fungi and six genera of bacteria were dominant in soil hydrocarbon oxidation. Cerniglia and Perry (1973) found that several fungi, eg. *Penicillium* spp. and *Cunninghamella* spp., exhibited higher levels of biodegradation than bacteria such as *Flavobacterium, Brevibacterium* and *Arthrobacter* spp. from the same environment. In a hydrocarbon (*n*-hexadecane) degradation study conducted by Song *et al.* (1986), the bacterial contribution to mineralisation was estimated at 82% while the fungal component accounted for 13%. This was in marked contrast to the findings of Anderson and Domsch (1975) who examined biodegradation of the same substrate and reported that the fungal contribution ranged from 60% to 90% and the bacterial contribution from 10% to 40%. Studies by Walker and Colwell (1974) showed that although bacteria initiated the degradation of a synthetic petroleum mixture, twice as much was degraded when bacteria, fungi and yeasts were present in the microbial population. This synergism between microorganisms could result in an increase in the rate and amount of oil degraded than would be achieved individually (Bossert and Bartha, 1984).

Many of these studies contribute little to the advancement of our understanding of hydrocarbon mineralisation in soil, other than by providing information specific to the experimental conditions. The examples above serve only to illustrate the point that the composition of the pollutant-degrading microbial populations appears to be a function of the ecosystem and local environmental conditions.

[2.2] FILAMENTOUS FUNGI IN HYDROCARBON BIODEGRADATION

Miyoshi's observation in 1895 that *Botrytis cinerea* attacked paraffin presumably initiated hydrocarbon microbiology. Later, isolation of *Cladosporium resinae* from jet fuel tanks (Parbery, 1971) was important in providing the groundwork towards an understanding of the major environmental parameters involved in hydrocarbon degradation by microorganisms. Lowery *et al.* (1968) found that catabolism most frequently targeted

hydrocarbons in the range of C_{10} to C_{16}. One species of *Aspergillus* had a substrate assimilatory range from n-hexane to n-hexadecane. Weak responses were recorded in the carbon chain length range of C_7 to C_{11}. Nyns *et al.* (1968) examined the ability of fungi to utilise hydrocarbons as a taxonomic parameter and found it to be a property of individual strains rather than a characteristic of particular species or related taxa. For example, hydrocarbon utilisation in terrigenous moulds has been considered largely a characteristic of strains from certain environments rather than of genera or species. After extensive screening of isolates, Nyns *et al.* (1968) observed that n-alkanes with less than ten carbon atoms supported the growth of very few strains of named genera. The fungi tested showed the highest growth affinity for n-alkanes with carbon chain lengths of C_{12} to C_{16}; a result similar to that of Lowery *et al.*(1968).

Oudot *et al.* (1993) investigated the biodegradation potential of crude oil by strains of fungi isolated from tropical oil-contaminated soils and sediments. They found that the most active species isolated were members of the genera *Penicillium and Aspergillus* which was in accordance with the results of other researchers (Nyns *et al.*,1968; Snellman *et al.*, 1988; Oudot *et al.*, 1987). The biodegradation potential of the fungi varied among genera and within species. The chemical composition of hydrocarbon products has been shown to be a major factor in determining which types of bacteria are able to utilise the compound as a growth substrate (Jobson *et al.*, 1972; Westlake *et al.*, 1974). This is also true for oil-degrading fungi. The *n*-alkane content is the principal, but not the sole, factor influencing biodegradability of an oil (Westlake *et al.*, 1974). Oudot *et al.* (1993) screened fungal isolates for their biodegradation potentials by incubating (28 days, 28°C) a mineral medium supplemented with 200 μl crude oil (BAL 150). The short- and medium-chain length alkanes (C_{16}-C_{26}) were removed, while long-chain alkanes over C_{28} were not attacked. Mixed cultures showed degradation levels equal to the most active of the monocultures.

Fungi possess a number of characteristics that are potentially useful for application in bioremediation. They are able to grow under environmentally stressed conditions such as low nutrient availability, low water activity and at low pH values where bacterial growth might be limited (Davis and Westlake, 1978). The filamentous growth nature of fungi

enables the organisms to chemotactically direct their growth over and between oil-covered soil particles. This optimises surface contact with the contaminant and, thus, increases the bioavailability of the compound. Mycelial organisms are able to penetrate insoluble substances such as oil tarballs, providing an attachment surface for bacteria, thus initiating a succession of other microorganisms and the enhanced degradation of the oil. A specific example was noted by Kirk (1969) who observed bacterial attachment to the mucilaginous hyphae of *Corollospora maritima*, possibly with mutual nutritional benefits. Fungi are also highly efficient in utilising available nutrients, especially nitrogen, which is most often the most limiting nutrient in contaminated soils. Effective methods of recycling nitrogen have evolved through the translocation of internal cytoplasmic contents. Some fungi have adapted to growth on a medium with a nitrogen content as low as 0.2% (w/w). White-rot fungi are unique in their ability to produce diffusible cellulolytic enzymes at carbon:nitrogen ratios of 2000:1 (Griffin, 1972).

[2.2.1] Special Applications of Fungi in Bioremediation

Fungi may be particularly useful in specific bioremediation applications, especially where the molecule has a low solubility and is, thus, only poorly available to bacteria. One such example is polycylic aromatic hydrocarbons (PAHs). These are priority environmental pollutants as a number are known mutagens and effect significant genotoxicity (Bos *et al.*, 1988; Mersch-Sundermann *et al.*, 1992) and are also powerful chemical carcinogens (Levin *et al.*, 1976; Buening *et al.*, 1978). In addition to their toxicity, PAHs are relatively persistent compounds in the environment as abiotic factors do not contribute significantly to the loss of PAHs with more than three aromatic rings (Park *et al.*, 1990). While compounds which contain more than three aromatic rings are relatively resistant to microbial degradation, the half lives of five- and six-membered ring PAHs have been estimated to be in the order of years (Shiaris, 1989; Parks *et al.*, 1990). Soil fungi have been reported to effectively degrade long-chain aliphatic hydrocarbons and aromatic hydrocarbons containing two or three rings (Leahy and Colwell, 1990). However, the range of fungi capable of oxidising PAHs with four or more rings is less well documented.

Extensive studies have been made on the zygomycete filamentous fungus *Cunninghamella elegans* and it has been demonstrated that the fungus is capable of oxidising naphthalene (Cerniglia *et al.*, 1978), fluorene (Pothuluri *et al.*, 1993), benzo[*a*]pyrene (Cerniglia and Gibson, 1980), pyrene (Cerniglia *et al.*, 1986) and methylated anthracenes (Cerniglia *et al.*, 1990). Launen *et al.*(1995) conducted a study to determine the oxidative capabilities of non-basidiomycetous soil fungi on benzo[*a*]pyrene. Soil samples for fungal isolation were collected from a refinery oil disposal site that had been in continuous use for 16 years up to the time of sampling. From all five sites examined approximately 50% of the fungal species isolated were capable of oxidising pyrene in liquid culture (seven days incubation, 24°C in 0.3% (w/v) malt extract, 0.3% (w/v) yeast extract, 0.5% (w/v) peptone, 1% (w/v) dextrose and 0.1 mg l^{-1} pyrene). The fungal species isolated included: *Mucor* spp.; *Chaetonium globosum; Trichoderma harzianum; Fusarium* spp; and *Penicillium* spp. From their results, they were able to conclude that a wide variety of non-basidiomycete fungi were capable of oxidising both pyrene and benzo[*a*]pyrene. Representative species from all fungal divisions (other than basidiomycetes) showed some degree of PAH oxidative capability. Zygomycetes were only recovered from soils which contained PAHs in concentrations <3 μg g^{-1} which suggests that they were less tolerant of PAHs. *Penicillium* spp. (48% of the isolates) were most frequently isolated during the study.

Chromatography (TLC) conducted on extractions of the liquid cultures showed that a metabolite with the same R_f value as 9-hydroxy-benzo[*a*]pyrene was the major polar product found in all of the fungal cultures examined. This indicates that the initial route of metabolism by the fungi was similar. Different species formed the same polar products although the proportion and spectrum of fungal metabolites appeared to be species dependent. Launen *et al.* (1995) concluded that the fungi isolated most probably used a cytochrome P-450-mediated biotransformation as none of the isolates were lignase-producing basidiomycetes. In addition, the benzo[*a*]pyrene oxidation product was 9-hydroxy-benzo[*a*]pyrene, which is different from the pattern observed with quinones (1.6, 3.6 and 6.12) produced by the white-rot fungus *Phanerochaete chrysosporium* (Haemmerli *et al.*, 1986).

Increasing the initial rate of ring oxidation by fungi could accelerate the mineralisation of these compounds. However, if the main degradation route is via the cytochrome P-450 pathway there is potential for the production and accumulation of potentially carcinogenic metabolites (eg 1.6- and 1.8-quinones). However, in a mixed microbial community, toxic intermediates should not accumulate to an appreciable extent. Fungal P-450 studies have been limited due to problems of isolating active enzyme and the low specific content in some species. Although P-450 has been suggested to play a role in lignin degradation (Launen *et al.,* 1995), no direct evidence of this enzyme had been reported until the findings of Masaphy *et al.* (1996). These researchers demonstrated the presence of cytochrome P-450 in the mycelium of *P. chrysosporium* and P-450-mediated B(a)P hydroxylation *in vitro*. However, these observations were made under non-lignolytic conditions thus further research is required to determine the role of P-450 in lignin and other xenobiotic degradations.

There is an increasing number of industrial applications for immobilised biocatalysts which are mostly enzymes or non-viable microbial cells. Several approaches to the use of viable fillamentous fungi have been reported: gel entrapment; fungal pellets; adsorption on particulate matter; and rotating biological contactors (Webb *et al.*, 1986; Keshavarez *et al.*, 1990; Braun and Vecht-Lifshitz, 1991). However, the growth associated characteristics of filamentous fungi are often problematic for use in immobilised systems. Recently, Pakula and Freeman (1996) successfully demonstrated the use of the castor oil-degrading filamentous fungus, *Tyromyces sambuceus* as a model system to demonstrate the operational feasibility of a new configuration of a continuous fixed film bioreactor. In this system the thickness of the fungal biofilm was controlled to a predetermined thickness by mobile stainless steel blades. The horizontal-film rotating biological contactor received a continuous substrate feed of the oil substrate (2% v/v) as an emulsion (with Tween 80 was used as a surfactant). This technology could possibly make a valid contribution to pollutant degradation in both aqueous and solid matrixes. For example, in the system described by Field *et al.* (1995; 1996) the white-rot fungus *Bjerkandera* sp. (strain BOS55) was immobilised onto fixed films to oxidise PAHs in solvent-water extracts of polluted soils (see [2.2.2d]).

Donnelly and Fletcher (1993) screened mycorrhizal fungi for their ability to metabolise PCBs. The ectomycorrhizal fungi *Radiigera atrogleba* and *Hysterangium gardneri* were able to degrade over 80% of 2,2'-dichlorobiphenyl. This appears to be the only account so far of mycorrhizae used for organo-pollutant biodegradation. The potential use of such fungi, combined with the emerging technology of phytoremediation, may offer potential solutions for particularly problematic forms of soil pollution, i.e. where both hydrocarbon and heavy metal contamination are present.

[2.2.2] White-Rot Fungi in Bioremediation

The use of white-rot fungi as a novel bioremediation technology emerged approximately ten years ago. Interest in the white-rot fungi for this application has generated a large volume of research publications which will be reviewed in this chapter. The first reports citing the use of white-rot fungi in an industrial biotechnological application was in the bleaching of kraft pulp mill effluents (Lundquist *et al.*, 1977; Eaton, 1985). These publications demonstrated the ability of *P. chrysosporium* to degrade chlorinated organics in effluent from the kraft pulp bleaching process. Pulp mill and bleach plant effluents are highly coloured, due to the presence of polymeric lignin degradation products, and contain chlorinated aromatics which are highly toxic and mutagenic (Eaton, 1985). Dye-degrading bacteria, unlike the white-rot fungi, usually exhibit strict specificity towards a single dye and are of little practical use for the treatment of mixtures of dyes which normally occur in such industrial effluents. *Phanerochaete chrysosporium* and several other white-rot fungi are able to decolourise many types of recalcitrant dyes (Table 2.1).

Table 2.1: Publications reporting the ability of *P. chrysosporium* to degrade specific organic pollutants.

Compound tested	Reference
PAHs	Bumpus (1989) Brodkorb and Legge (1992) Morgan *et al.* (1991) Vyas *et al.* (1994) Field *et al.* (1992)
PCBs	Eaton (1985)
DDT	Kohler *et al.* (1988) Bumpus and Aust (1987) Aust (1990)
Hexachlorocyclohexane	Bumpus *et al.* (1985)
Pentachlorophenol	Mileski *et al.* (1988) Lamar and Dietrich (1990)
Alkyl halide insecticides (chlordane and lindane)	Kennedy *et al.* (1990)
Dyes	Glen and Gold (1983) (Polymeric dyes) Spadaro *et al.* (1992) (Azo dyes) Cripps *et al.*(1990) (Heterocyclic dyes)
TNT (2,4,6-trinitrotoluene) RDX (hexahydro-1,2,5- trinitro-1,2,5-triazine)	Fernando *et al.* (1990)

These findings prompted further investigations which demonstrated that white-rot fungi are capable of effecting some degree of biodegradation of a wide range of xenobiotics in aqueous media and soils. A definitive report by Bumpus *et al.* (1985) was, arguably, the first to acknowledge the potential of white-rot fungi for the bioremediation of environmental pollutants. Prior to this publication, much interest in these fungi already existed since they are the only group of organisms capable of degrading lignin to the mineralisation products of carbon dioxide and water.

There are an estimated 1400 species of white-rot fungi representing all the major groups of higher basidiomycetes and xylariaceous ascomycetes (Rayner and Boddy, 1988). While most studies have used *Phanerochaete chrysosporium* as a model organism, comparable levels of pollutant degradation have also been demonstrated in other species such as *Funalia gallica, Coriolus versicolor, Poria cinerescens, Pleurotus ostreatus, Trametes versicolor, Chrysosporium lingnorum* and *Bjerkandera* spp. (Eaton, 1985). There is a great diversity among these organisms to degrade lignin (Otjen *et al.*, 1987) and this same diversity is also seen in their abilities to degrade xenobiotics (Lamar *et al.*, 1990). To date, most of the research has involved laboratory studies aimed at elucidating the functioning and efficacy of the lignin-peroxidase enzyme system. More recently, studies have begun to examine field application and determination of the fates of metabolised pollutant molecules.

[2.2.2a] The Lignin-peroxidase biodegradation system

White-rot fungi appear to be unique in their ability to degrade lignin to the end product carbon dioxide. This ability is, in part, due to the fact that they secrete hydrogen peroxide (H_2O_2) and a form of peroxidase which catalyse the free radical-dependent oxidation and depolymerisation of lignin (Gold *et al.*, 1989). Hydrogen peroxide is produced by oxidases located between the cell wall and cell membrane. The origin of the molecule is probably glucose which results from the degradation of cellulose (Koenigs, 1972). These two enzyme systems (hydrogen peroxide-producing oxidases and the lignin peroxidases) combine to generate carbon-centred free radicals in lignin, resulting in the non-specific cleavage of the lignin polymer. The resultant low molecular weight products may then undergo further modification and metabolism to carbon dioxide (Aust, 1990). The lignin degrading system is expressed upon nutrient (nitrogen, sulphur or carbon) limitation (Crawford, 1981; Kirk, 1984). Lignin peroxidases are not synthesised when the organism is grown under high nutrient/nitrogen conditions or before nitrogen is limiting. It has been demonstrated by a number of researchers (Table 2.1) that the enzyme system possessed by *P. chrysosporium* is capable of degrading many important organic pollutants including some of the more recalcitrant molecules such as high molecular weight PAHs. It appears that these catabolisms are possible due to structural similarities of the pollutants to portions

of the lignin substructure and the low specifity of the lignases. Polyaromatic hydrocarbons with ionisation potentials at or below a cut-off of approximately 7.55eV are substrates for direct one-electron oxidation by lignin peroxidase (LiP) (Hammel *et al.*, 1986). Those with ionisation potentials above this threshold are apparently acted upon by radical species formed during manganese peroxidase (MnP)-dependent lipid peroxidation reactions (Bogan, 1996a).

The biochemistry and molecular genetics controlling the lignolytic systems of *P. chrysosporium* are relatively complex. Multiple LiP isozymes are produced and the genome contains at least 10 structurally related genes encoding LiP proteins (designated *lipA* through *lipJ*). Similarly, several MnP isozymes are produced in submerged culture, and three *mnp* genes have been characterised (Bogan *et al.*, 1996b). Bogan *et al.* (1996b) observed that *mnp* mRNA levels correlated with MnP enzyme production and with the disappearance of high ionisation potential PAHs during *P. chrysosporium*-based soil remediation. Thus, extraction of mRNA and the use of reverse transcription (RT)-PCR provides a useful tool for monitoring the physiological state of the fungus to help ensure bioremediation performance.

White-rot fungi possess a number of characteristics which make them suited for the biodegradation of environmental pollutants:

(i). Since the lignin degrading system is free radical in nature, it is non-specific and should be applicable to a wide variety of substrates. Hence, white-rot fungi have the capacity to mineralise a wide variety of structurally diverse chemicals;

(ii). The enzymes do not interact with the substrate in the manner of most microbial enzymes. Instead, the enzymes are first activated with hydrogen peroxide. The substrate is actually the hydrogen peroxide which forms compound I of lignin peroxidase. Lignin peroxidase then reacts with the pollutant chemical resulting in its oxidation. Thus, the lignases are secreted from the fungal mycelium in an inactive form and must undergo oxidation in order to carry out their catalytic function. The kinetics of these reactions

allow biodegradation to take place at rates proportional to the concentration of the pollutant chemical. Therefore, the concentration of the chemical may be reduced to non-detectable levels. However, this also means that at very low chemical concentrations the biodegradation rates will be very slow;

(iii). The advantage of a mobile reactive species is that extensive degradation of organic contaminants can take place at significant distances from the fungal mycelium (McFarland *et al.*, 1992). The extracellular peroxidases result in an improved access to substances with poorly available substrates. This is a valuable attribute as many environmental pollutants are persistent because they are either hydrophobic or are bound to the soil;

(iv). The enzyme system is expressed in response to nutrient deficency which provides some advantages. First, the organism does not have to be "adapted" to the pollutant chemical to effect biodegradation and, secondly, enzyme synthesis occurs even in the absence of the pollutant chemical. Thus, enzyme synthesis will not cease as the concentration of the pollutant chemical degraded is reduced to levels below the threshold concentration required for microbial adaptation; and

(v). The organisms occur naturally throughout the environment and are easy to culture on low-cost substrates such as agricultural and forestry wastes, eg maize cobs.

[2.2.2b] Specific applications of white-rot fungi in bioremediation

The white-rot fungi have immense potential for application in *ex situ* ventilated biopiles (Holroyd *et al.*,1992). To date, most approaches to the bioremediation of PAH-contaminated sites have depended on the action of bacteria and have been limited to low molecular weight polycyclic aromatic hydrocarbons. The recalcitrant behaviour of PAHs is largely attributed to their limited bioavailability as they are strongly adsorbed on to soil organic matter (Manilal and Alexander, 1991). Field *et al.* (1992) found PAH biodegradation to be a ubiquitous phenomenon among white-rot fungi. Field *et al.* (1993) observed that both anthracene and benzo[*a*]pyrene degradation were highly correlated to

the strain's ability to decolourise the polymeric dye Poly R-478. Thus, this dye may be used to screen for suitable isolates with the ability to degrade various PAHs. Polymeric dyes have facilitated extensive automation in screening white-rot fungi. Fletcher *et al.* (1994) selected 346 cultures of basidiomycetous fungi (isolated from contaminated or chemically treated substrate) from a computer data base, which listed over 15 000 fungi, for screening against 36 different dyes. Bioassays were conducted in 96-well titre plates which were prepared and analysed for optical density with three Beckman Biomek-1000 robots. Optical density data were logged directly by a personal computer and statistically validated choices were made on species for further investigation.

Lamar and Dietrich (1990) demonstrated the depletion of PCP in contaminated soil inoculated with *P. chrysosporium* and *P. sordida*. Initial PCP concentrations of 250-400 μg g^{-1} soil decreased by 88-91% over a period of 50 days. These decreases were achieved under sub-optimal growth temperatures. *P. chrysosporium*'s optimal growth temperature, according to Burdsall and Eslyn (1974), is 40°C. Catabolism was also achieved without the addition of inorganic nutrients other than those contained in peat moss which was added at a concentration of 1.93% (w/w). The inoculum was bulked up on wood chips which were applied to plots at a concentration of 3.35% (w/w). Initially, rapid decreases in PCP were detected but these were attributed to adsorption by the wood chips. Differences between the fungal treatments and the controls became statistically significant by day 29. Pentachlorophenol concentrations in plots which received fungal inoculum were, on average, ±49% lower than the control plots. Gas chromatography analysis of soil sample extracts did not reveal the presence of extractable transformation products other than pentachloroanisole (PCA). Most of the PCP was, however, converted to non-extractable soil-bound products.

It is appropriate to compare fungal rates of PAH degradation with those exhibited by bacteria. For example, a *Mycobacterium* sp. examined by Heitkamp *et al.* (1988) oxidised pyrene during growth on a complex liquid organic medium. Initially, this organism oxidised approximately 50% of the 2.5μM pyrene added in two days. During this time, the biomass increased by 0.1 g dry weight l^{-1}. Stationary phase *P. chrysosporium*, by contrast,

mineralised 16% of 0.2μM pyrene in two weeks, at a biomass concentration of about 2 g dry weight l^{-1}. From data such as these one could conclude that bacteria offer greater potential for practical application in PAH bioremediation than white-rot fungi. However, from an ecological standpoint, lignolytic fungi probably play a significant role in the natural biodegradation of PAH contaminants. Although these organisms are slow degraders, they are highly non-specific and are ubiquitous inhabitants of woodland soils (Frankland, 1982).

[2.2.2c] Field application of *P. chrysosporium* for bioremediation

The fungal inoculum is pregrown on a mixture of lignocellulosic matter which may be pre-soaked in water and nutrient solution. The material is spread out on a liner and inoculated with solid and/or liquid grown cultures of *P. chrysosporium*. Solid phase inoculum is grown in aerated bioreactors (200 l), which contain a mixture of ligniocellulosic material, under controlled and sterile laboratory conditions. Liquid phase inoculum is prepared by culturing the fungus in sporulation broth. The resulting spore suspension is centrifuged and formulated into a stable paste for transportation. The paste is added to water on site and applied to organic material through a spray nozzle.

After the lignocellulosic material is inoculated layer by layer, it is formed into a heap and covered with plastic sheeting. The moisture level is carefully controlled during the several weeks it takes *P. chrysosporium* to establish. This primary inoculum is then used to inoculate an even larger volume of woody material to provide a sufficient bulk of material to treat the volume of contaminated soil. Soil screening and mixing with contaminated material are carried out in a one-step process with a rotating drum screen. Processed soil is then transported to the treatment area and laid out on a high density polyethylene (HDPE) liner. From soil analysis, it can be determined if nutrient additions are necessary. The soil is placed in piles of the following approximate size: 50 m x 30 m x 2 m. Networks of perforated pipes, placed inside the piles, are connected to a blower to facilitate aeration. Each treatment bed is finally covered with HDPE sheeting. Holroyd *et al.* (1992) used this technique for the clean-up of 5000 m^3 of chlorophenol contaminated soil. They found that

bioremediation of pollutants, at concentrations normally thought of as being too high for biological processing, can be attempted. This was because the major portion of growth was on the support material which offered some protection against the toxic effects of the chemical contaminants.

Pentachlorophenol has been extensively used as a biocide, especially as a wood treating agent, and is one of the U.S. EPA priority pollutants. Reported tolerances of fungi to PCP vary greatly. The maximun concentration for fungal growth varies from 12 ppm, for *Chaetomium globosum,* to 180 ppm, for *Cephaloascus fragrans* (Cserjesi and Johnson, 1972). *Phanerochaete* spp. are relatively sensitive to PCP. Growth of several species was prevented by the presence of concentrations as low as 5ppm (Ruckdeshel and Renner, 1986). In contrast, Lamar *et al.* (1990) found that the species they tested were less sensitive and were able to grow at 25 ppm PCP. In another field study (Lamar and Dietrich, 1990), *P. chrysosporium* and *P. sordidia* were able to grow and deplete PCP in a soil with initial concentrations of from 250 to 400 mg kg^{-1} soil. Fernando *et al.* (1990) found that *P. chrysosporium* was able to tolerate the concentrations of TNT which are often found at contaminated sites; i.e. 10 000 mg kg^{-1} in soil and 100 mgl^{-1} in water. At these concentrations, approximately 85% of the TNT was degraded over 90 days.

Morgan *et al.* (1991) suggested that it is necessary to supplement soil with plant-derived carbon sources. Wheat straw was found to provide the most satisfactory results. Due to their low cost, maize cobs are frequently used in field-scale experiments. Bumpus (1989) found the optimal ratio to facilitate biodegradation was four parts cobs to one part soil (w/w). Morgan *et al.* (1993) found mineralisation of two PAHs (3,4-dichloroanaline and benzo[*a*]pyrene) was reduced in treatments which contained more straw than the optimal straw:soil ratio of 1:4 (w/w). They suggested that this was probably due to insufficient contact between the hyphae and the contaminated soil when the soil was the smaller fraction of the mixture. Other workers (Huttermann *et al.*, 1988) found that a 1:10 ratio of straw/soil was suitable for the degradation of PAHs in soil inoculated with white-rot fungi. Morgan *et al.* (1991) found that 50 to 60 days after inoculation the rate of mineralisation virtually ceased, and made experiments to determine if the active mineralisation period

could be prolonged. Soil additions such as glucose, soil mixing, re-inoculation and addition of inorganic nutrients were examined. However, none of these treatments had any effect. The results led them to conclude that the fungi did not lack the ability to mineralise 3,4-dichloroaniline but were somehow prevented from doing so. This was probably due to the residual contaminant and/or its metabolic intermediates being strongly adsorbed onto soil mineral and organic matter surfaces and were, thus, unavailable for fungal attack. An alternative explanation could be that the parent molecules had been partially converted into biomass or metabolites which fungi could not mineralise.

Zeddel *et al.* (1993) examined the abilities of three different genera of white-rot fungi to biodegrade PCBs. Degradation of PCBs up to trichlorobiphenyl was comparable for all isomers. Mono- and dichlorobiphenyls, with the exception of 4,4'-dichlorobiphenyls, were mineralised. Tetrachlorobiphenyls differed from this pattern since their degradation is more dependent on the chlorine substitution pattern. *P. chrysosporium* was unable to degrade PCBs with more than two chlorines per biphenyl. They concluded that for degradation of higher chlorinated PCBs, it may be possible to perform an anaerobic pretreatment for dechlorination followed by aerobic treatment with white-rot fungi.

[2.2.2d] Potential problems associated with white-rot fungal bioremediation

Although the ability of *P. chrysosporium* to degrade a wide range of pollutant chemicals is now well established, attempts to remediate contaminated soil by direct inoculation with this organism has produced little success. An obvious explanation for this is that soil is not the natural habitat of this fungus. With the inoculum pregrown on a suitable liginin-based substrate before it is introduced into the soil, the established mycelium should be able to withstand competition from indigenous microflora and penetrate the surrounding soil. The use of a selective nutrient provides the organism with a competitive advantage. Hence, problems associated with "seeding" a site with allochthonous microorganisms, such as the inability to compete and survive, should not be experienced. However, this is not the case. Competition from other strains of fungi and bacteria has been shown to severely limit growth. Growth and survival is poor even in sterile soils. Experience gained in other

spheres of biotechnology, such as soil inoculation with fungal biocontrol agents, has proved that it is very difficult to maintain a high fungal inoculum potential in soil.

It is difficult to compare the mineralisation rates recorded by researchers as few papers provide data concerning the biomass concentration present. While most of the organo-pollutants tested with *P. chrysosporium* have revealed that most of the environmental contaminants examined undergo some degree of mineralisation, intermediate metabolites of undetermined toxicity and/or recalcitrance may accumulate.

Brodkorb and Legge (1992) suggested that *P. chrysosporium* had a synergistic effect with indigenous soil microorganisms in an phenanthrene contaminated soil. The basis of this synergism is related to the ability of *P. chrysosporium* to partly metabolise the phenanthrene to more polar products. This results in a redistribution of the catabolic products into the aqueous phase, thus increasing the bioavailability of the contaminant for biodegradation by indigenous microflora. There is, however, the risk that the metabolites may be metabolic dead-end products, have enhanced toxicity, or may have enhanced mobility in the environment. Conditions affecting the release and rates of release of these compounds are largely unknown. Strains of the genera *Bjerkandera, Phanerochaete* and *Ramaria* were shown to effectively convert anthracene to anthraquinone, which was found to be the dead-end metabolite (Field *et al.*, 1992). The accumulation of anthraquinone can not necessarily be considered an unfortunate fate of catabolism of PAHs as it is readily degraded by bacteria (Mueller *et al.*, 1989). There are distinct differences among the fungi in their abilities to metabolise anthraquinone as strains of the genus *Trametes* degrade anthracene without any accumulation of anthraquinone. Chang *et al.* (1985) concluded that *P. chrysosporium* can degrade PCP efficiently and produce no harmful intermediates during the degradation of PCP in soil and that both PCP and PCA are readily mineralised in soil. Zeddel *et al.* (1993) found that no toxic chlorinated dioxins or furans were formed during the aerobic mineralisation of PCBs (mono-, di- and trichlorinated PCBs). Haemmerli *et al.* (1986) demonstrated that during oxidation of benzo[*a*]pyrene by *P. chrysosporium,* a veratyl alcohol free-radical cation reacted with benzo[*a*]pyrene and resulted in the formation of a benzo[*a*]pyrene free radical cation. This may then undergo several

hydrolysis and/or oxidation reactions resulting in benzo[*a*]pyrene quinone formation. Benzo[*a*]pyrene quinone may form a bound contaminant residue if it reacts with soil humus and minerals. It may also be mineralised by soil microflora. The fraction of benzo[*a*]pyrene that is mineralised or bound to soil humus depends on the specific soil chemical and microbial species.

Tucker *et al.* (1995) investigated the effects of soil components and indigenous soil microorganisms on *P. chrysosporium* mineralisation of a model polymeric dye (Poly R-478) and pyrene. They found that the soil microflora inhibited mineralisation by *P. chrysosporium* and attributed this to the possible production of antifungal agents by autochthonous bacteria. Radtke *et al.* (1994) found that some soil-associated bacteria, including fluorescent psuedomonads, were antagonistic to the growth of *P. chrysosporium* on agar medium. Growth of *P. chrysosporium* was inhibited by the pseudomonads which produced phenazines. These compounds are generated by a wide range of soil inhabiting pseudomonads and actinomycetes (Chang and Blackwood, 1968; Pierson and Thomashow, 1992). These bacteria grew readily on a medium with corn cobs as their sole carbon and nitrogen sources (Radtke *et al.*, 1994). Thus, the matrix used to support the growth or used for soil inoculation of *P. chrysosporium* needs to be considered in bioremediation attempts as growth of bacteria antagonistic to *P. chrysosporium* might be stimulated. Radtke *et al.* (1994) suggested that a preliminary screening of soils for bacteria which produce phenazines might be useful in predicting possible antagonism to the applied fungus.

Tucker *et al.* (1995) found that soil composition could modify the efficacy of *P. chrysosporium* in pollutant biodegradation. Depression of pyrene mineralisation in soil microcosms was detected with clay and silt overlays but not sand. They attributed this to several factors. Modification of pH was highlighted as a causal factor, rather than the more obvious influential factors such as oxygen transfer through differing porous media.

Results from a number of studies have indicated that white-rot fungi are affected by bioavailability limitations, especially with respect to higher PAHs in aged soil pollution. In order to increase the bioavailablitiy of PAH compounds, Field *et al.* (1995) proposed the

addition of miscible co-solvents to increase PAH solubility. Miscible solvents are generally toxic to microorganisms (Ingram and Buttke, 1994) thus a compromise has to be sought between solvent toxicity and increased PAH bioavailablity. The oxidation of anthracene (used as a model PAH compound) by *Bjerkandera* sp. strain BOS55 (a white-rot fungus) in the presence of the co-solvents acetone and ethanol was examined. The solvents were found to be toxic when added in concentrations of 5% (v/v) to batch cultures at the time of inoculation. However, when the solvents were added in concentrations up to 20% (v/v) to nine-day-old cultures, lignolytic activity, as indicated by Poly R-487 dye decolourisation and anthracene oxidation, was apparent. Solvent concentrations of 20% (v/v) are toxic to fungal cells. Hence, the anthracene oxidation was attributed to extracellular peroxidases which were shown to tolerate the solvent. Solvent additions of 11%-21% (v/v) acetone or ethanol increased the rate of anthracene bioconversion to anthraquinone in liquid medium by a factor of 2-3 compared to fungal cultures which received 1%-3% (v/v) solvent. Field *et al.* (1996) also investigated the use of *Bjerkandera* sp. strain BOS55 to oxidise PAHs in solvent-water extracts of polluted soils. The study demonstrated that the fungus was capable of metabolising 80% of a 50% (v/v) PAH solution in seven days in a soil extract which containined 2% (v/v) ethanol. The main problems identified were the poor removal efficiencies of five to six ring PAH compounds at 2% (v/v) solvent and the high toxicity of the solvent at the higher concentrations (20% v/v) needed to extract the pollutants. The advantage of this technique is that solvents can be potentially recovered by distillation. The potential also exists to apply peroxidase enzymes of white-rot fungi to soil once they are immobilised on a suitable carrier. The ionisation potential of lignin peroxidase, as an oxidising agent, is substantially more powerful than peroxidase currently derived from sources such as horseradish (Wainwright, 1992). Factors such as the nature, stability and toxicity of the soil-bound transformation products under a variety of conditions must be elucidated before application of white-rot fungi to contaminated soils.

[2.3] DISCUSSION

From the literature it appears that few generalisations can be made about the efficacy of fungi in the biodegradation of petroleum hydrocarbons. Efficacy appears to be a function of localised environmental conditions. As most fungi are obligate aerobes, their usefulness is limited, depending on the soil and extent of water saturation, to a maximum soil depth of approximately 80 cm. Thus, bioremediation systems utilising fungi can, at present, only be configured as *ex situ* ventilated soil piles. These may be most useful in the bioremediation of problematic hydrocarbons such as PAHs where the filamentous growth habit of fungi allows the penetration of insoluble molecules. An additional advantage of these systems is their low cost as co-metabolic nutrients may be derived from agricultural waste products.

Researchers have focused little attention on indigenous soil fungi while most efforts have concentrated on white-rot fungi which are not natural soil inhabitants. Although many white-rot fungi possess substantial bioremediation potentials, little field success has been reported thus far. Perhaps selection criteria other than those based purely on peroxidase activity should be examined. Efforts to isolate strains from the environment should target species which exhibit higher affinities to grow in soil. This characteristic would probably have a great effect on increasing the biodegradation potential of the strain. Recombinant DNA technology may also have a role to play in consolidating these attributes into one organism. Additional problems with their use are the time, difficulty and expense of producing the required amounts of inoculum. Optimising the oxidation capabilities of an indigenous microbial community which has already adapted to the soil habitat is probably more economic and may be an equally efficient means of breaking down PAHs when compared to the introduction of foreign microorganisms.

CHAPTER THREE

[3] ISOLATION AND SCREENING OF HYDROCARBON-DEGRADING FUNGI

[3.1] INTRODUCTION

Prior to initiation of this study, the project site had been contaminated for as long as 20 years. Thus, a considerable selection pressure had been exerted on the indigenous soil microorganisms affecting key enzymes for degradation *in situ*. The purpose of this component of the study was to isolate catabolic fungi and subject them to screening procedures. From this, the types and concentrations of hydrocarbon molecules the fungi were capable of degrading were determined. Furthermore, isolates with the greatest affinities to degrade the selected "model" hydrocarbons were noted for use in soil bioaugmentation trials.

Preceding studies (McGugan, 1993; McGugan *et al.*, 1995) demonstrated that the method used for enrichment and isolation strongly influenced the type and variety of microbial species isolated from the contaminated soil. For the present study, a number of different isolation techniques and nutrient media were used to facilitate isolation of a more representative selection of fungi. The results showed that the ability to utilise the hydrocarbon hexadecane, as a sole carbon source, was ubiquitous among fungi with any significant hydrocarbon degradative ability. Therefore, hexadecane-supplemented agar plates were used as the standard medium for isolation.

[3.2] MATERIALS AND METHODS

[3.2.1] Direct Isolation With Hydrocarbon-Supplemented Agar Plates

Czapek-Dox agar plates (CD) were supplemented with hexadecane (Sigma, reagent grade) and used for direct isolation of fungal species from the contaminated soil. Approximately

one gram of soil from a homogenised soil sample, collected from multiple sampling points at the project site, was spread over the hexadecane-supplemented CD agar (HCD) plate's surface. The plates were then incubated at 25°C. The CD plates (Malloch, 1981) contained the following (g l^{-1} glass-distilled water):

K_2HPO_4, 1.0
$NaNO_3$, 3.0
$MgSO_4.7H_2O$, 0.5
KCl,0.5
$FeSO_4.7H_20$, 0.01
glucose, 5.0
Biolab agar, 20.0
antibiotics*: penicillin, 0.01; chloramphenicol, 0.5; streptomycin, 0.025
*Filter sterilised (0.2 μm, nitrocellulose, Whatman) and added after the medium had cooled to 60°C prior to pouring.

The pH of the medium was adjusted to 4.6 with concentrated HCl before autoclaving (121°C, 15 min) to preclude the growth of bacteria.

2 ml of filter sterilised (0.2 μm, nitrocellulose, Whatman) *n*-hexadecane dissolved in 20 ml hexane were added to a flask containing 100 ml of sterile molten CD. The flask was placed in an ultrasonic bath for three minutes to disperse the *n*-hexadecane. The hydrocarbon-supplemented agar was then poured as a 2 mm overlayer on the set CD plates.

[3.2.2] Direct Isolation With a Nutrient Agar Overlayer

A modification of the method described above was made by spreading one gram of soil [3.2.1] over a set CD agar plate and then over-layering with 3 mm of molten nutrient agar (Biolab).

[3.2.3] Direct Isolation With Rose Bengal Agar

Rose Bengal agar (Malloch, 1981) was used for direct isolations as described above [3.2.2.]. The Rose Bengal agar contained the following (g l^{-1} glass-distilled water):

Peptone, 5.0
Glucose, 5.0
K_2HPO_4, 1.0
$MgSO_4.H_2O$, 0.5
Rose Bengal, 0.03
Biolab agar, 20.0
Streptomycin sulphate*, 0.01

*Filter sterilised (0.2 μm, nitrocellulose, Whatman) and added after the medium had cooled to 60°C prior to pouring.

[3.2.4] Direct Isolation With Basidiomycete Selective Media

A selective medium for basidiomycetous fungi was used for the direct isolation of these species. One gram of the contaminated soil [3.2.1] was spread evenly over the surface of the plate as previously described [3.2.1].

The medium contained the following (g l^{-1} glass-distilled water):

K_2HPO_4,0.90
KH_2PO_4, 0.20
$MgSO_4.7H_2O$, 0.75
NH_4NO_3, 2.0
KCl, 0.30
Streptomycin sulphate*, 0.03
Benomyl fungicide*, 1.50

*Filter sterilised (0.2 μm, nitrocellulose, Whatman) and added after the medium had cooled to 60°C prior to pouring.

[3.2.5] Soil Dilutions

(i).Ten grams of contaminated soil [3.2.1] were placed into each of three 250 ml Erlenmeyer flasks. Distilled water (150 ml) was added to each of the flasks which were then placed on an orbital shaker (25°C, 200 rpm.) for 18 h.

(ii). 1 ml of the supernatant was then pipetted into 99 ml sterile Ringers solution.

(iii). 1 ml of this preparation was pipetted onto HCD agar plates and incubated at 25°C for 72 hours.

(iv).Monocultures were produced by hyphal tip sub-culturing onto HCD agar.

[3.2.6] Soil Sonication

A similar procedure as detailed in part [3.2.5] above was used except the three flasks were placed in an ultra-sonication bath (Whaledent Biosonic) for a period of five minutes.

[3.2.7] Batch Cultures

Duplicate batch cultures were set up in 250 ml Erlenmeyer flasks. Five grams of soil (each sample taken a different location at the site) were placed in the flasks which contained 200 ml enrichment medium. Two types of liquid media (listed below) were used:

(i) **Soil extract medium**

A soil extract was used as a low-nutrient medium and was prepared as follows:

1 kg of fertile soil (not from the experimental site) was placed in 1l of water and was sterilised by autoclaving (121°C, 15 min). The supernatant was filtered through Whatman No.1 paper and stored under refrigeration (4°C) until use. The liquid medium contained the following (l^{-1} glass-distilled water):

soil extract, 100 ml
glucose, 1.0 g
$K_2HPO_4^*$, 0.15 g
$KH_2PO_4^*$, 0.50 g
* added as buffer

The solution was adjusted to a pH 4.6 with concentrated HCl and filter sterilised (0.2 μm, nitrocellulose, Whatman) antibiotics, as described for the CD agar [3.2.1], were added.

(ii) **Complex basal salts**

The procedure described above was repeated with a mineral salts medium (Appendix B) instead of the soil extract medium. The flasks were closed with cotton wool bungs and aluminium foil prior to sterilisation (121°C, 15 min). The medium was sparged with a small aquarium pump. Air entering the flasks was first passed through a copper sulphate solution (1M) and then through a glass fibre air filter. The vessels were incubated (25°C) until fungal growth was visible (approximately 4 days). Mycelial fragments were aseptically sub-cultured onto HCD plates until monocultures were obtained.

[3.2.8] Results and Discussion

The different techniques employed for the enrichment and isolation procedures provided a diversity of fungal species for screening for hydrocarbon catabolic activity.

The use of a nutrient overlayer on plates [3.2.2] improved contact with fungal propagules in the soil and, hence, increased their growth potential. Ultrasonication [3.2.6] resulted in cellular damage to vegetative mycelium so this method was used to isolate fungal species from spores released from the surface of the soil particles during sonication. It was noted that the most extensive growth in the batch cultures [3.2.7] resulted from a soil sample obtained from a sampling point above an effluent reservoir located on the site. Two possible reasons for this exist. Firstly, the concentration of oil contamination at this sampling point was significantly lower than at the others and, secondly, the soil was free of any additional contaminants which possibly arose through the operation of a resin processing plant presently on site. In contrast, no isolates were obtained from the two sampling points directly below the effluent reservoir. These areas were presumably contaminated with caustic floor-washings which had leaked from the reservoir. The pH (Annon., 1983) values of these soil samples were extremely low and ranged from 2.3 to 3.7. These observations were important for the selection of a location from where soil could be excavated for use in microcosms (Chapter Four). Initially, 62 fungal species were isolated and subcultured on HCD agar until monocultures were obtained. The monocultures were then maintained on HCD agar or PDA with reduced glucose concentrations. A preceding study (McGugan *et al.*, 1995), revealed that indigenous fungi undergo a rapid attenuation in their abilities to biodegrade complex organic molecules when cultured on media which contain high concentrations of labile carbon.

[3.3] PRELIMINARY STRAIN IDENTIFICATION

[3.3.1] Materials and Methods

Isolates were cultured (14 days/25°C) on standard PDA (Malloch, 1981) and their gross morphologies were examined. Visibly similar fungi were subjected to further examination by light microscopy after incubation (room temperature) under "black light" for 24 hours to induce sporulation.

[3.3.2] Results and Discussion

Examination of reproductive structures (conidia, sporangia, etc.) of the fungi by light microscopy revealed that some isolates were duplicated. These isolates, however, were not discarded as it was expected there would be a large degree of variation in the catabolic capabilities of fungal strains within the same genus. This preliminary step in identification allowed fungi belonging to the same genera to be grouped together. In the later stages of the screening process, isolates with low hydrocarbon catabolic potentials, within the same genera, were eliminated.

[3.4] PRELIMINARY STRAIN SELECTION

[3.4.1] Materials and Methods

All 62 isolates were subjected to a simple preliminary testing procedure to determine which possessed high affinities for the oil substrate. Species which showed little or no affinity to grow on CD agar plates supplemented with oil, soxhlet-extracted (Appendix C) from soil obtained from the project site, were discarded.

Oil-agar plates were inoculated with 1 cm^2 block of mycelium cut from 5-day-old PDA cultures. The oil-agar plates were prepared as follows:

Soxhlet-extracted oil (2 ml) was added to 100 ml molten CD agar in a 150 ml Erlenmeyer flask. The flask was placed in a sonicator bath for three minutes to agitate the oil into discrete droplets. This oil-agar was applied as a 2-3 mm overlayer to standard solid CD agar plates. The oil agar solidified before the oil droplets reconstituted. Fungal growth on the plates was monitored every five days.

[3.4.2] Results and Discussion

Initially, measurements were made to quantify the rates of radial mycelial growth by taking measurements of the diameters of the expanding circular colonies (two measurements were taken at right angles to each other). However, after 10 days incubation it was no longer possible to do this due to the irregular growth patterns of most of the colonies. Many colonies sporulated heavily and gave rise to numerous daughter colonies. Selection of the fungal strains was then made by subjective visual evaluation. Strains were selected on the basis of their growth characteristics rather than their growth rates. Fungal strains which produced compact, dense, circular colonies were selected rather than fungal species which produced thin, sparse and spreading mycelium. The latter growth habit is usually indicative of low substrate affinity. These cultures also tended to sporulate rapidly on the oil-agar. This is a survival mechanism and is also indicative of a low substrate affinity.

From this procedure, 17 fungal strains were selected for additional screening and were arbitrarily assigned a two-lettered identification code.

[3.5] FINAL STRAIN SELECTION

[3.5.1] Materials and Methods

A plate bioassay method, described by Alleman *et al.* (1993), was used to evaluate the abilities of the 17 fungal isolates (selected in [3.4]) to grow on three key hydrocarbon molecules.

Blocks of mycelium (1 cm^2) were transferred onto assay plates and incubated (25 °C) for four days before being subjected to testing with hydrocarbon-impregnated discs.

The bioassay agar plates contained the following (g l^{-1} glass-distilled water):

Sodium citrate,
K_2SO_4, 5.0
NH_4NO_3, 2.0
$CaCl_2$, 0.068
$MgSO_4$, 0.2
thiamine, 0.001
glucose, 1.0 or 10.0

Hydrocarbon assay discs were prepared by impregnating sterile cellulose filter discs (15 mm, Whatman No.1) with hydrocarbons dissolved in sterile (0.2 μm, cellulose-acetate, Sartorius) solvent (hexane or acetone) at selected concentrations. The solvent was allowed to volatilise before placing the discs on the assay plates. Three hydrocarbons representing three structural groups were selected as substrates:

(i). straight chain alkane: *n*-hexadecane, $C_{16}H_{34}$ (Sigma);
(ii). branched alkane: pristane (2,6,10,14-tetramethylpentadecane), $C_{19}H_{40}$ (Aldrich); and
(iii). aromatic hydrocarbon: phenanthrene, $C_{14}H_{10}$ (Sigma).

The hydrocarbon substrates were prepared by individually dissolving *n*-hexadecane and pristane in hexane and phenanthrene in acetone. The filter discs were impregnated with 0.1 ml of the appropriate dilution of the stock solution to achieve hydrocarbon concentrations of 10, 50, 75 and 100 mg l^{-1}.

[3.5.2] Results and Discussion

The responses of the selected isolates to the different hydrocarbon concentrations and the presence/absence of an alternative carbon source in the solid media are summarised in Tables 3.1 to 3.5. Only the results for the highest and lowest hydrocarbon concentrations are shown (viz. 100 and 10 mg l^{-1}). To increase the consistency of the visual evaluations, a simple method was used to rate each isolates growth potential on the substrates. Growth of the isolate's was rated as follows:

(-) : The hydrocarbon was toxic and no growth occurred. This was indicated by inhibition zones (>5 mm) around the impregnated disc.

(+) : The hydrocarbon was less toxic but the fungal mycelium only grew up to the disc but not onto the impregnated disc.

(++): The fungus experienced limited toxicity to the hydrocarbon but mycelial growth was sparse.

(+++): The hydrocarbon was not toxic to the fungus and growth was prolific over the disc surface.

Biodegradability of hydrocarbon molecules decreases with increases in structural complexity (Perry, 1984). Hence, biodegradability of the three hydrocarbon molecules tested decreased as follows: hexadecane > pristane > phenanthrene. Thus, growth of each isolate on each compound was ranked accordingly. For each positive "+" growth reaction on hexadecane a score of one was given. For each positive "+" growth reaction on pristane a score of two was given. For each positive "+" growth reaction on phenanthrene a score of three was given. If no growth occurred, a score of zero was given. The cumulative score for each isolate is displayed in the last column of each table. Hence a "+" result for phenanthrene was weighted more heavily than a "+" on hexadecane. The average score for each trial was calculated and the isolates which gave an above-average score were noted.

Table 3.1: Growth performance of fungal isolates on discs impregnated with selected hydrocarbons (100 mg l^{-1}) on agar supplemented with 1 g glucose l^{-1}.

Culture	Hexadecane	Pristane	Phenanthrene	Rating mean=2.8
E1	++	+	-	4
D1	-	-	-	0
F2	+	+	-	3
FT	+	+	-	3
B1	+	-	-	1
F1	+	-	-	1
G1	++	-	-	2
C2	++	-	-	2
DT	-	+	-	2
W1	++	+	-	4
WG	++	++	-	6
FX	++	++	-	6
BL	+	+	-	3
PX	++	++	-	6
L1	-	-	-	0
QR	-	-	-	0
SF	++	+	-	4

Table 3.2: Growth performance of fungal isolates on discs impregnated with selected hydrocarbons (100 mg l^{-1}) on agar supplemented with 10 g glucose l^{-1}.

Culture	Hexadecane	Pristane	Phenanthrene	Rating mean=5.2
E1	+++	++	-	7
D1	-	+	++	8
F2	+	+	-	3
FT	++	+	+	7
B1	+	+	+	6
F1	+	-	-	1
G1	++	+	-	4
C2	+++	+	-	5
DT	+	+	-	3
W1	++	+	-	4
WG	+++	++	-	7
FX	++	++	+	9
BL	++	++	+	9
PX	++	++	+	9
L1	+	-	-	1
QR	+	-	-	1
SF	++	++	-	6

Table 3.3: Growth performance of fungal isolates on discs impregnated with selected hydrocarbons (10 mg l^{-1}) on agar supplemented with 1 g glucose l^{-1}.

Culture	Hexadecane	Pristane	Phenanthrene	Rating mean=7.7
E1	+++	+++	+	12
D1	+	+	+	6
F2	++	+	-	4
FT	++	+	+	8
B1	+	+	-	3
F1	+++	+	-	7
G1	+++	+	+	8
C2	+++	+	++	11
DT	++	+	-	4
W1	++	+	+	7
WG	+++	++	-	7
FX	++	++	+	9
BL	++	+	+	7
PX	++	++	+	9
L1	++	++	-	6
QR	++	++	+	9
SF	++	+	+	8

Table 3.4: Growth performance of fungal isolates on discs impregnated with selected hydrocarbons (10 mg l^{-1}) on agar supplemented with 10 g glucose l^{-1}.

Culture	Hexadecane	Pristane	Phenanthrene	Rating mean=9.1
E1	+++	+++	+++	18
D1	++	++	++	12
F2	++	+	-	4
FT	++	++	+	9
B1	++	+	+	7
F1	+++	++	-	7
G1	+++	++	+	10
C2	+++	+	+++	14
DT	+++	++	-	7
W1	+++	++	+	10
WG	+++	++	-	7
FX	++	++	+	9
BL	+++	+	+	8
PX	++	++	+	9
L1	+++	++	-	7
QR	+++	++	+	10
SF	++	+	+	7

Plates 2a and 2b illustrate the rating scale used in Tables 3.1 to 3.5.

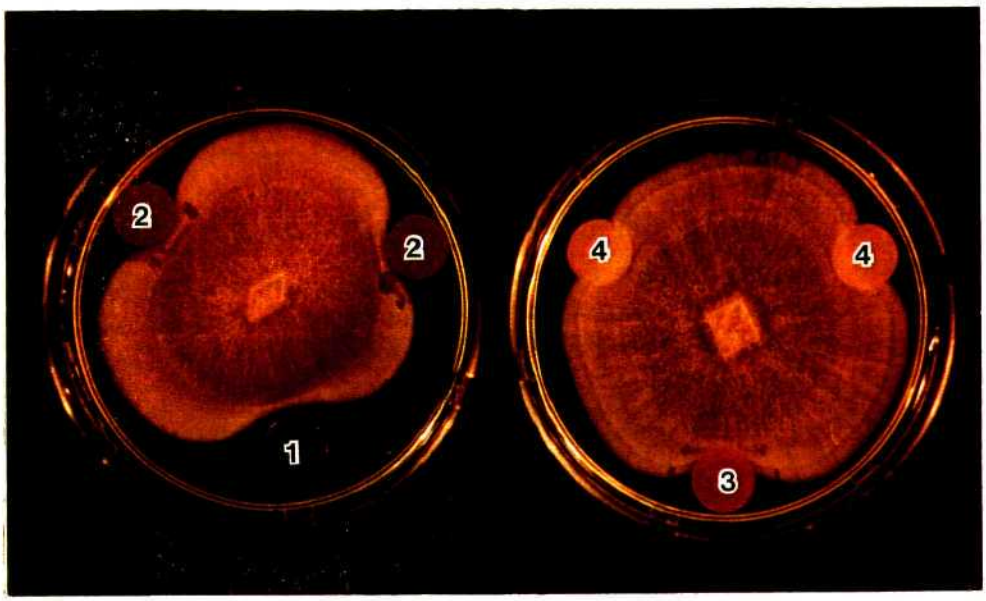

Plate 2a: Filter disc bioassay rating system. Markers indicate: "-"(1) ;"+" (2); " ++"(3); and "+++" (4) .

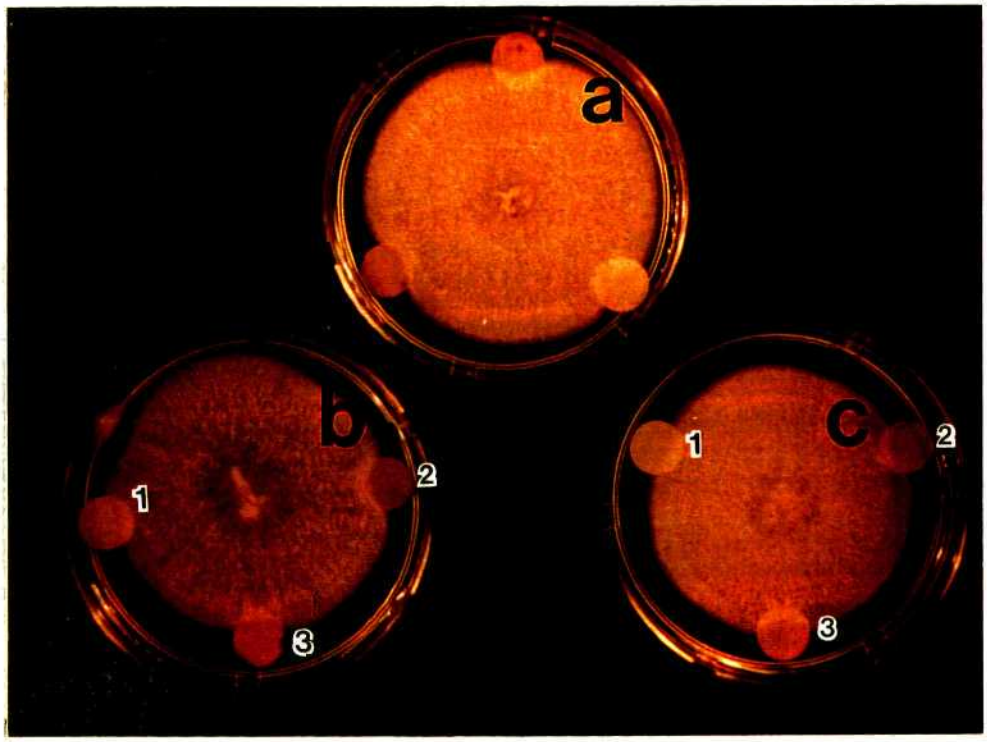

Plate 2b: Examples of the filter disc bioassay with isolate E1 (*Trichophyton* sp.). a: Hydrocarbon concentration 100 mg with 10 g l^{-1} glucose; b: Hydrocarbon concentration 10 mg with 1 g l^{-1} glucose with ratings: (1) hexadecane (+++); (2) pristane (+++); and (3) phenanthrene (+); c: hydrocarbon concentration 10 mg with 10 g l^{-1} glucose with ratings: (1) hexadecane (+++); (2) pristane (+++); and (3) phenanthrene (+++).

In Table 3.5, it can be seen that isolates E1, FX and PX performed consistently well with all four treatments. Isolates WG, SF, FT, BL and D1 performed well with the higher hydrocarbon concentration. Other isolates such as W1, QR, C2 and G1 exhibited above average performances but only with the lower hydrocarbon concentration. It can also be noted that the addition of a labile utilisable co-substrate, glucose, had a positive growth effect on some isolates, particularly in the presence of phenanthrene (Tables 3.1 and 3.2). No isolates were able to grow on a phenanthrene concentration of 100 mg l^{-1} until the concentration of the co-metabolic substrate (glucose) was increased to 10 g l^{-1}. Thereafter, five isolates gave positive growth reactions, indicating an increased tolerance to the molecule in the presence of the co-metabolite.

This method is simpler than other methods previously described (Lamar *et al.*, 1990) as it eliminates the need for hydrocarbons to be dissolved in hot agar. Due to the hydrophobic nature of most hydrocarbons, it is often a difficult task to achieve an even distribution of the hydrocarbon in the agar before an immiscible emulsion forms. Conventional procedures to screen fungi for resistance to toxic chemicals can require a large number of flasks and can be extremely time consuming (Ruckdeshel and Renner, 1986). In contrast, the method used allows one to test an organism for its ability to either grow on three different hydrocarbon molecules simultaneously or one molecule at three different concentrations. In addition, other parameters which affect biodegradation rates, such as pH and temperature and the presence of a co-metabolic substrate, may also be examined.

It is believed that quantitative assays, as made by Alleman *et al.* (1993), are not possible with this method. It is expected that significant concentrations of the hydrocarbons diffuse from the assay discs into the medium. Together with reducing the concentration of the hydrocarbons to which the isolates are exposed, extraction of residual hydrocarbons from the discs does not provide a sufficiently accurate estimate of the fungal-mediated hydrocarbon disappearance. To address this problem, a batch culture technique, which allowed quantitative gas chromatographic analysis (GC) of the residual hydrocarbons, was devised.

Table 3.5: Summary of results listing isolates which achieved "above average" ratings in hydrocarbon (HC) degradation plate-bioassays.

Table 3.1	Table 3.2	Table 3.3	Table 3.4
100 mgl^{-1} HC 1 gl^{-1} Glucose	100 mgl^{-1} HC 10 gl^{-1} Glucose	10 mgl^{-1} HC 1 gl^{-1} Glucose	10 mgl^{-1} HC 10 gl^{-1} Glucose
E1	**E1**	**E1**	**E1**
F2			
FT	**FT**		
DT			
WG	**WG**	**WG**	
FX	**FX**	**FX**	**FX**
BL	BL		
PX	**PX**	**PX**	**PX**
SF	**SF**	**SF**	
	B1		
		G1	**G1**
	C2	**C2**	**C2**
		QR	**QR**
W1		**W1**	**W1**
	D1	**D1**	**D1**
	BL		

[3.6] QUANTITATIVE ANALYSIS OF RESIDUAL HYDROCARBONS FROM BATCH CULTURES

[3.6.1] Materials and Methods

The ability of fungal isolates to degrade selected hydrocarbon molecules was tested with surface-suspended batch cultures. This experiment was designed in such a way that each fungus was exposed to the hydrocarbon molecules and, after a period of incubation, the residual hydrocarbons were extracted and subjected to GC analysis. Relative comparisons were then made to determine the extent of degradation each isolate had effected on the specific hydrocarbon molecule. Fungal mycelium was inoculated onto the surfaces of glass beads which were then immersed in a mineral salts/hydrocarbon solution. The glass beads were used to support the fungal mycelium as it developed (Plates 3a and 3b). Eight fungal isolates which exhibited the highest resistance to the hydrocarbons in the toxicity bioassay [3.5.1] were selected for batch culture. These cultures were: E1; FX; PX; WG; SF; C2: D1; and W1.

The fungal-selective basal salts solution contained the following (g l^{-1} glass-distilled water):

$NaNO_2$, 2.0
KCl,0.50
$MgSO_4.H_2O$, 0.05
KH_2PO_4, 1.0
$FeSO_4.7H_2O$, 0.01
D-glucose, 10.0
penicillin*, 0.01 mg
chloramphenicol*, 0.50 mg
streptomycin sulphate*, 0.25 mg

Hydrochloric acid (1N) was used to adjust the pH of the medium to 5 before autoclaving (121°C, 15 min). * Dissolved in sterile distilled water which contained 20% (v/v) methanol,

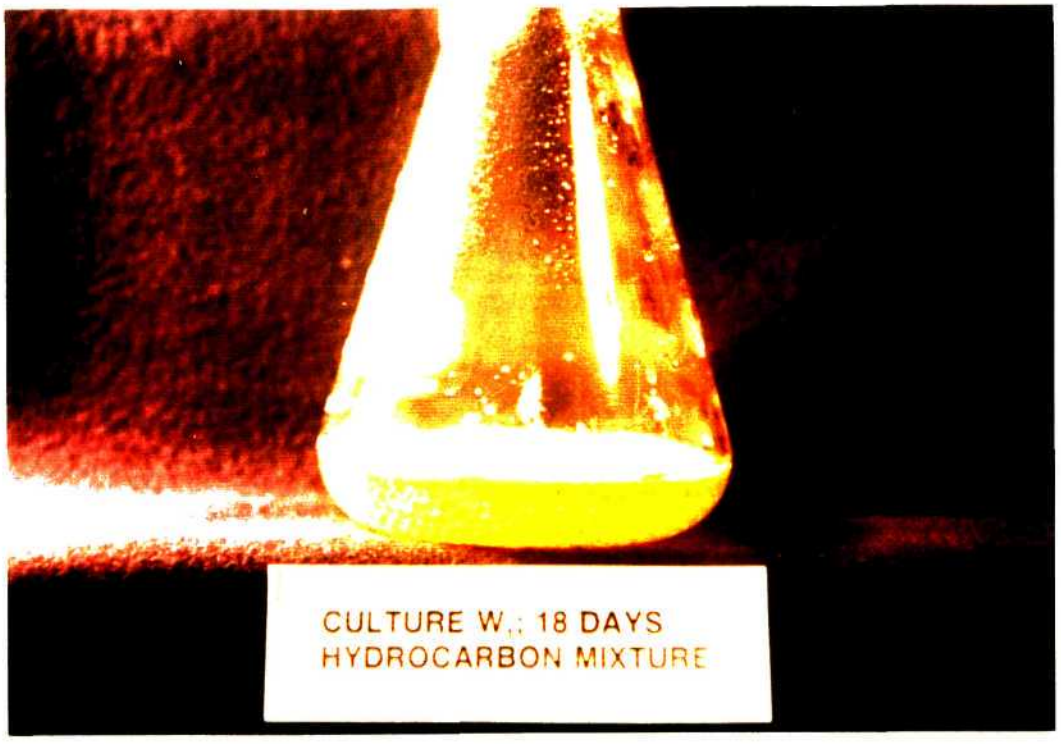

Plate 3a: Batch culture flask with complex mineral salts medium and a layer of glass beads. Note the white fungal mycelium supported on the surface of the glass beads.

Plate 3b: Close-up of batch culture flask in Plate 3a above.

diluted to 10 ml with the same solution prior to filter sterilisation (0.2 μm, nitrocellulose, Whatman) into the basal salts solution at room temperature.

25 ml of sterile basal salts solution were pipetted into ten sterile 250 ml Erlenmeyer flasks which contained a 2 cm layer of glass beads (5 mm diameter).

A hydrocarbon stock solution was made by placing 1.0 g each of *n*-hexadecane, pristane, phenanthrene and naphthalene in a sterile (121°C, 15 min) volumetric flask and then diluting to 100 ml with solvent (acetone and hexane 50% v/v).

1.0 ml of the hydrocarbon solution (10 mg of each hydrocarbon) was added to each flask. The flasks were then placed in an ultrasonic water bath (Whaledent Biosonic) for five minutes (room temperature). During this time, the flasks were agitated periodically to facilitate an even distribution of the hydrocarbons within the liquid medium and over the glass beads.

The flasks were aseptically inoculated with 1 cm^2 of fungal mycelium from seven-day-old cultures grown on PDA. The inoculated flasks were then closed with cotton wool bungs and Parafilm™. The flasks were incubated (29°C) in darkness for 30 days (Plates 3a and 3b). Each fungal treatment was made in triplicate. Three sterile controls were used to quantify abiotic losses from the flasks during the incubation period. After incubation, the residual hydrocarbons were extracted and analysed by GC (Appendix D).

[3.6.2] Results and Discussion

To quantify the effects of fungal metabolism on the hydrocarbons, mean peak area comparisons were made with standards (10 mg l^{-1}). The peak area data are summarised in Table 3.6. The four hydrocarbon compounds examined were eluted from the GC column after the following times: naphthalene, 9.9 min; phenanthrene, 15.3 min; hexadecane, 16.7 min; and pristane, 18.7 min (see 6.2.3 for GC conditions).

Table 3.6: Peak areas and percentage hydrocarbon attenuation from hydrocarbon-supplemented batch cultures.

Sample	Hydrocarbon	Peak Area ± standard deviation (volt.min^{-1})	Percentage reduction	Corrected value (percent)*
Standard	NAP	1669.63 ±160.37	-	-
	PHEN	1826.82 ±112.08	-	-
	HEX	2507.98 ±247.10	-	-
	PRIS	2093.82 ±130.70	-	-
Control	NAPT	126.98 ±4.17	92.39	-
	PHEN	1242.48 ±155	21.04	-
	HEX	1503. 45±284.26	16.92	-
	PRIS	1762.68 ±364.43	11.03	-
E1	NAPT	750.05 ±92.05	55.05	NEG
	PHEN	725.70 ±94.91	60.28	39.24
	HEX	34.46 ±9.28	99.69	81.71
	PRIS	673.20 ±80.15	66.46	57.81
FX	NAPT	877.88 ±184.51	47.42	NEG
	PHEN	861.44 ±195.47	52.84	31.81
	HEX	7.54 ±3.47	99.69	82.79
	PRIS	702.22 ±38.82	66.46	55.43
WG	NAPT	1496.68 ±182.18	10.36	NEG
	PHEN	1430.47 ± 97.51	21.69	0.66
	HEX	9.79 ±6.68	99.61	82.69
	PRIS	1304.5 ±112.18	37.69	26.66
D1	NAPT	1939.35 ±208.16	NEG	NEG
	PHEN	517.11 ±70.09	71.69	50.66
	HEX	486.54 ±8.56	80.21	63.68
	PRIS	2070.15 ±178.39	1.13	NEG

Table 3.6 continued:

Sample	Hydrocarbon	Peak Area ± standard deviation (volt. min^{-1})	Percentage reduction	Corrected value (percent)*
PX	NAPT	1636.26 ±269.77	1.99	NEG
	PHEN	1396.21 ±161.13	23.57	2.54
	HEX	342.58 ±11.27	89.34	69.42
	PRIS	1589.16 ±122.77	24.10	13.07
SF	NAPT	1337.28 ±189.73	19.90	NEG
	PHEN	892.87 ± 105.68	51.12	30.09
	HEX	15.16 ±5.73	99.39	82.47
	PRIS	1369.94 ±158.93	34.57	23.54
C2	NAPT	1021.78 ±126.79	38.80	NEG
	PHEN	876.04 ±106.79	52.04	31.01
	HEX	44.81 ±4.75	98.21	81.01
	PRIS	1063.00 ±142.84	49.23	38.21
W1	NAPT	1267.27 ±253.9	24.09	NEG
	PHEN	1360.66 ±198.61	25.51	4.48
	HEX	179.27 ±9.36	92.85	75.93
	PRIS	662.05 ±104.43	68.38	57.35

* Percentage reduction in hydrocarbons corrected for physico-chemical losses.

NAPT: Naphthalene

PHEN: Phenanthrene

HEX: *n*-Hexadecane

PRIS: Pristane

NEG: negligible degradation

SE: standard error of means

All strains tested exhibited high levels of hexadecane degradation. This was expected as the isolates were cultured on hexadecane-supplemented medium throughout the course of the study. The degradation levels were all above 75% and in one case (Isolate FX) was as high as 82.79%. With the exception of D1, all cultures were able to effect pristine degradation levels of more than 10%. The highest level of pristane degradation recorded was 57.81% (Isolate E1). Phenanthrene degradation was highly variable for the different isolates. The degradation levels ranged from 2.54% (Isolate PX) to 50.66% (Isolate D1). Six of the nine isolates tested were able to effect phenanthrene degradation levels of over 30%.

There appeared to be poor recovery of naphthalene from the aqueous medium as indicated by the results of the control flasks (Table 3.6). An explanation for this was not readily apparent. It is, however, possible that naphthalene was the least soluble of the hydrocarbons tested and was not extracted from the solution by the solvent (dichloromethane). After correction with the controls, these concentrations were negligible (indicated as NEG in the table) and, thus, will not be considered for further discussion. Some biodegradation of this compound may, however, have occurred.

Measurement of the residual hydrocarbons in the controls is an indication of both the extraction efficiency and an estimate of abiotic losses from the flasks during the experimental period. The accuracy of the method is questionable as large standard errors were obtained for the replicates (Table 3.6). Hydrocarbon losses occurred each time the extract was transferred to new glassware. These losses were further compounded by the fact that significant concentrations of hydrocarbons were probably adsorbed to the fungal mycelium and were not removed by the extraction process.

It was not possible to make comparisons between the results obtained in the two bioassays ([3.5.1] and [3.6.1]).The results of the plate bioassays were highly subjective and small differences between isolates could have easily passed undetected. The results were only comparable for isolates which showed exceptional performance in the plate trials such as isolates E1, PX and FX.

The eight isolates used in the batch trial were finally identified (Table 3.7) using a

taxonomic key (Malloch, 1981) and were used as inocula in bioaugmentation trials (Chapter Four).

Table 3.7: Identification of isolates selected for soil microcosm trials (Chapter Four).

Identification code	Genus
E1	*Trichophyton*
FX	*Mucor*
PX	*Penicillium*
WG	*Graphium*
SF	*Acremonium*
C2	*Chaetomium*
D1	Unidentified basidiomycete
W1	*Chrysosporium*

CHAPTER FOUR

[4] LABORATORY EVALUATION OF *EX SITU* BIOREMEDIATION WITH MICROCOSMS

[4.1] INTRODUCTION

At the time of the study two basic methods were available for promoting the microorganisms necessary to initiate bioremediation. The first method, known as *bioaugmentation*, involves the addition of high concentrations of adapted pollutant-degrading microorganisms to the contaminated site. The second approach, *biostimulation*, is the *in situ* stimulation of indigenous soil microorganisms by the injection of soluble nutrients, microbial growth stimulants and/or the addition of ancillary organic compounds into the contaminated area. These additions should stimulate the growth of the microorganisms in the contaminated soil. If the soil contains the appropriate microorganisms, biodegradation of the contaminant molecule should proceed at an enhanced rate.

Bioaugmentation obtains its microbial activity from two sources. The first technique involves the growth of specific monocultures under controlled laboratory or fermentation plant conditions. After these cultures are "stabilised' and blended into a formulation (liquid or powder) they can be added to the contaminated area in high concentrations. Another approach is to isolate indigenous microorganisms from the contaminated site. These are then cultured in the laboratory/pilot plant and returned as a highly active liquid microbial culture back into the contaminated soil. This greatly increased inoculum potential is used to increase the rate of bioremediation.

In this study, soil microcosms were used to evaluate the bioremediation efficacy of selected physical, chemical and biological treatments on the hydrocarbon-contaminated soil. Both bioaugmentation and biostimulation bioremediation strategies were investigated with the

central aim of utilising the catabolic capabilities of the indigenous soil fungi. It was also desirable to determine whether successful bioremediation can be effected solely by the addition of low-cost soil supplements such as agricultural waste products.

In a preceding study (McGugan, 1993), microcosms (glass boxes, 30 x 30 x 25 cm) were filled with 20 kg oil-contaminated soil from the project site. This resulted in a soil depth of 15 cm. The microcosms were covered with black plastic to prevent photo-oxidative losses. The moisture levels were maintained between 50% and 70% of the water holding capacity. Each microcosm served as a plot which received a different unreplicated treatment. Seven fungal species were examined for their efficacy to bioremediate the contaminated soil. The microcosms were designed to replicate an *ex situ* soil treatment unit-type bioremediation process whereby the soil is excavated and placed into lined treatment cells on site. Dissolved mineral nutrients were sprayed over the soil surface and oxygen was delivered to the soil microorganisms by periodic tilling. From this study, it was shown that soil microcosms were useful for laboratory-scale evaluation of *ex situ* treatment methods for contaminated soil. The use of laboratory microcosms allows the simultaneous evaluation of a number of specific biological, physical and/or chemical soil treatments, while some operating parameters (e.g. temperature and moisture content) may be held relatively constant.

[4.2] BIOAUGMENTATION TRIAL 1: MATERIALS AND METHODS

The current study utilised similar microcosms to those described above with the exception that the volumes were reduced to two litre boxes which contained 1500 g soil. This allowed an increase in the number of treatments which could be assayed simultaneously and facilitated enclosure in a constant temperature incubator (Plate 4a). Temperature regulation was expected to be important in winter months as the small soil volume in the microcosms did not provide adequate thermal insulation.

[4.2.1] Soil Preparation

Contaminated soil was collected from the project site and was kept in sealed containers under refrigeration (4°C) until needed. The preceding study (McGugan, 1993) revealed that the heterogeneous contaminant concentration distribution in the soil resulted in a high degree of variation in total petroleum hydrocarbon (TPH) concentration. Therefore, before use in the microcosms, the contaminated soil was thoroughly homogenised by sieving (10 mm mesh) and mixing in a 100 l cement mixer (30 minutes, 50 r.p.m.). Longer mixing times resulted in pelleting of the soil which was undesirable. Although pelleting increased overall soil porosity, the surface area exposed to microbial attack was decreased. Rectangular PVC boxes (2240 cm^3, 14 x 20 x 8 cm) with clip-on lids were used as microcosms. Each microcosm was filled with 1.5 kg contaminated soil. Three holes (0.5cm diameter) were made in each lid and were plugged with non-absorbent cotton wool to allow gaseous exchange.

[4.2.2] Biomass Bulking of Fungal Isolates

Biomass bulking of the selected fungal species (from [3.4]) was made by a solid-state fermentation technique in polyethylene bags. This technique is routinely used for laboratory-scale biomass bulking of filamentous fungi such as *Trichoderma* sp. and *Agaricus bisporus* on a wide range of substrates (M.D. Laing, personal communication). The method is outlined below:

Barley grain was soaked in water for 24 h and then drained of excess water. The soaked grain was mixed with barley beer waste (BBW) in a 1:1 volume ratio. Barley beer waste is spent barley grains from beer brewing and was acquired from South African Breweries (Prospecton, Durban). The product had a sawdust-like consistency in its dehydrated state. Approximately 400 ml (±290 g) of the barley/BBW mixture were placed into polyethylene bags (25x 45 cm) which were then sealed with purpose-made plastic stoppers which contained cotton wool gaseous-exchange ports. The sealed bags were sterilised by autoclaving (121°C, 15 min). Inoculation of the substrate was made by aseptically

transferring a colonised potato dextrose agar (PDA) plate of the desired fungal species (diced into 5 mm cubes) into each bag. Once an initial bag was fully colonised, inoculation of other bags was made directly from this bag by aseptically transferring 50 ml of the colonised substrate to a sterile (121°C, 15 min) bag of barley/BBW. The inoculated bags were incubated (25°C) for approximately three weeks until the grain was completely colonised.

[4.2.3] Microcosm Treatments

Soil microcosms were prepared as described in Section [4.2.1]. The treatments used are listed in Table 4.1. Fungal inoculum was incorporated into the soil by thoroughly mixing the desired isolate, bulked on barley/BBW, into the soil at a concentration of 30% (w/w). The microcosms were tilled at seven-day intervals to facilitate aeration. A strict tillage duration and pattern was observed to ensure consistency of the treatment. During incubation (25°C), the moisture content of each microcosm was held at between 40% and 60% of the soil water holding capacity (WHC). This was achieved by the application of an appropriate volume of water, determined from oven-dried (105°C/24 h) soil samples, every seventh day. Soil samples (60 g) were taken immediately after each tilling for pH (Anon, 1983), total petroleum hydrocarbon (TPH) concentration [6.2.1] and microbial activity analyses [6.2]. For the latter, an ergosterol bioassay which specifically quantified the fungal biomass in the substrate was first used [6.3.1]. However, problems were encountered with this method when applied to the contaminated soil matrix. Therefore, another method, fluorescein diacetate (FDA) hydrolysis, was used for the remainder of the study [6.3.4]. Due to the problems experienced with the ergosterol bioassay, only one microbial activity measurement was made for Trials 1 and 2. The methods for residual hydrocarbon determinations and the microbial activity bioassays are discussed in detail in Chapter Six.

A 60 g sample taken from each microcosm was used as follows: 20 g for TPH determination (divided into two 10 g amounts and stored in pill vials at 4°C); 30 g for pH determination; and 10 g for microbial activity analysis. The pH and microbial activity

measurements were made immediately and the soil used was returned to the appropriate microcosms after rinsing with distilled water. Although this step would resulted in a dilution of the TPH, it was included to reduce the effect of destructive sampling on the initial soil volume. In this way, only $\pm 2\%$ of the initial soil volume was permanently removed at each sampling event. The trial was run for 50 days.

Table 4.1: Inocula used in Microcosm Trial 1.

Soil Microcosm	Inoculum
[M1]	*Trichophyton* sp.
[M2]	*Mucor* sp.
[M3]	*Penicillium* sp.
[M4]	*Graphium* sp.
[M5]	*Acremonium* sp.
[M6]	Sterile barley/BBW medium
[M7]	Non-sterile control (NSC)
[M8]	*Chaetomium* sp.
[M9]	Unidentified basidiomycete
[M10]	*Chrysosporium* sp.

[4.2.4] Results and Discussion

The non-sterile control (Microcosm 7) quantified abiotic losses, mainly through volatilisation, and the extent of intrinsic bioremediation during the trial period. The addition of sterile barley/BBW to the soil as a treatment in Microcosm 2 was also used as a control as all microcosms received this amendment which stimulated the growth of the soil microflora. Extensive fungal growth was visible in most of the inoculated microcosms and in Microcosm 6 throughout the trial period. This growth was especially noticeable on the soil surface due to the increased oxygen availability (Plate 4b).

Analysis of residual TPH in the inoculated microcosms (Fig. 4.1) showed substantial reductions during the trial period. The highest TPH reduction of 50.5% (NSC corrected, viz. a TPH reduction from 5613 mg kg^{-1} soil to 2275 mg kg^{-1} soil) was recorded with Microcosm 5 which was inoculated with an *Acremonium* sp. The second highest TPH reduction of 47.1% (NSC corrected) was effected by the addition of sterile barley/BBW medium. Other treatments effected similar or lower TPH reductions. The principal exception was the control (Microcosm 7) which resulted in a 9% reduction only. Factors that may have contributed to decreases in the biodegradation efficacy of the specific fungal inocula were poor soil bulking effected by the barley/BBW medium and loss of fungal viability due to culture on artificial growth medium.

It was accepted that the TPH method of hydrocarbon degradation analysis was not a particularly sensitive assay, especially for the higher molecular weight molecules. However, the method has many merits, especially its low cost, which made the method useful for routine use in this study. The advantages and disadvantages of the TPH method are discussed in detail in [6.2].

There appeared to be a rapid loss in the ability of the isolates to degrade the complex hydrocarbon molecules even though the cultures were maintained on hydrocarbon-supplemented agar. The final TPH concentration correlated relatively well ($r^2 = 0.665$) with the microbial activity measured midway (day 30) through the trial. This observation

was more comprehensively examined in Trials 3 and 4, where more data for the duration of the trial were collected.

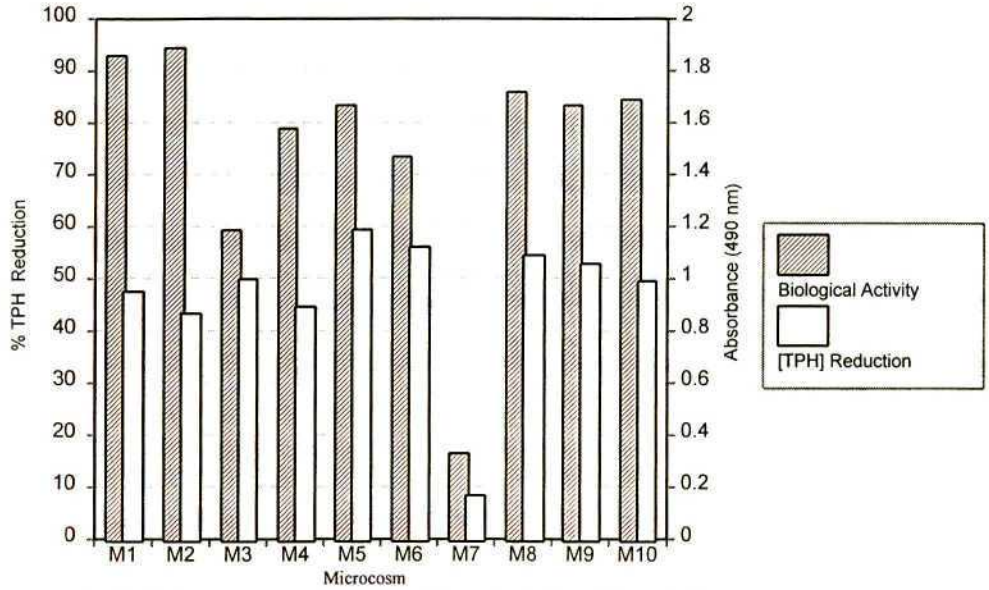

Fig. 4.1: Final TPH concentration reduction after 50 days and microbial activities (FDA) after 30 days of Microcosm Trial 1.

[4.3] BIOSTIMULATION TRIAL 2: MATERIALS AND METHODS

Soil microcosms were used to evaluate the efficacy of soil supplementation with plant- and animal-derived waste products as microbial nutrient sources for bioremediation purposes. It was desirable to determine whether comparable hydrocarbon biodegradation could be achieved by a simple and more cost-effective *in situ* biostimulation of soil fungi compared with soil inoculation.

[4.3.1] Microcosm Treatments

Microcosms were prepared as described in [4.2.1]. Six different soil treatments with organic supplements were examined (Table. 4.2). The choice of the organic supplements was based largely on cost and local availability. The agricultural/industrial waste products used were all readily available at no cost other than their transportation costs. All organic supplements were sterilised by autoclaving (121°C,15 min) before application to the soil (40%, w/w). The microcosms were tilled at seven-day intervals to facilitate aeration. A strict tillage duration and pattern was observed to ensure consistency of the treatment.

During incubation (25°C), the moisture content was held at between 40% and 60% of the soil WHC by application of appropriate volumes of water, determined from oven-dried (105°C, 24 h) soil samples, every seventh day. Soil samples (60 g) were taken immediately after each tilling event for the various analyses (pH, TPH and microbial activity) and were processed exactly as described in [4.2.3]. The trial was also run for 50 days.

Table 4.2: Soil treatments used in Microcosm Trial 2.

Soil Microcosm (M)	Treatment
[M 1]. Non-sterile control (NSC)	The soil was left untreated, i.e. received no tillage, water or organic additions.
[M 2]. Manure	Chicken manure (3.94% w/w[1] nitrogen) was air dried (ambient temperature), sieved (5mm mesh) and applied at 15% (w/w).
[M 3]. Composted pine bark	Composted pine bark (seedling tray growth medium) applied at 40% (v/v).
[M 4]. Straw	Non-sterile straw was shredded (± 5cm lengths) and applied at 40% (v/v).
[M 5]. Maize cobs	Maize cobs, milled into small chips applied at 40% (v/v).
[M 6]. Barley beer waste (BBW)	Dehydrated BBW was moistened with sterile (121°C, 15 min) distilled water and applied at 15% (w/w).

[1] Determined by Cedara Feed Laboratory, KwaZulu-Natal Department of Agriculture, P.Bag X9059, Pietermaritzburg, 3200, South Africa.

[4.3.2] Results and Discussion

All the soil supplements chosen for this trial were either agricultural or industrial waste products and are, therefore, potentially inexpensive forms of organic nutrients. The non-sterile control (Microcosm 1) was again used to quantify abiotic losses, mainly through volatilisation, and the extent of intrinsic bioremediation during the trial period. The chicken manure added to Microcosm 2 provided nitrogen, phosphorus and organic carbon as a co-metabolic substrate. Composted pine bark applied to Microcosm 3 functioned as a soil bulking agent. Composted pine bark provided little nutrients for the soil microorganisms, other than to those which possessed lignolytic enzymes, such as the white-rot fungi. Straw added to Microcosm 4 also functioned as a bulking agent and provided a more labile carbon source for the soil microorganisms than the composted pine bark. Similarly, maize cobs, used in Microcosm 5, had both bulking and nutritive effects. The BBW added to Microcosm 6 provided a substrate which contained both labile carbon and organic nitrogen. Nitrogen is present in substantial concentrations in BBW (up to 6.2% w/w) in the form of yeast residues from the brewing process.

Total petroleum hydrocarbon reduction data (mean standard error = $\pm$ 167 mg kg^{-1} soil) and FDA activity of the trial are presented in Figure 4.2.

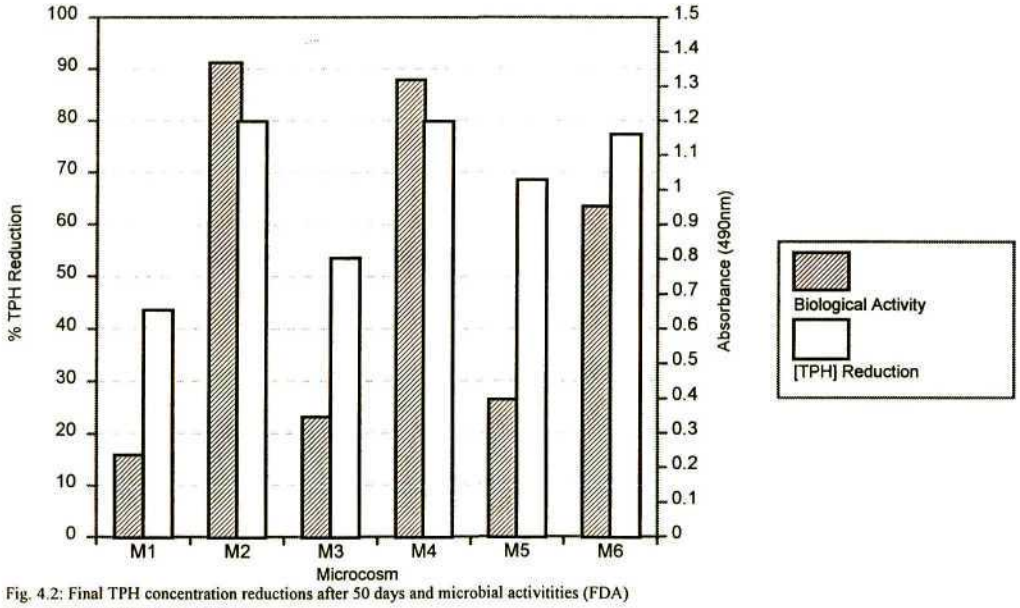

Fig. 4.2: Final TPH concentration reductions after 50 days and microbial activitities (FDA) after 30 days of Microcosm Trial 2.

From these results it was apparent that, in most cases, organic nutrient supplementation increased hydrocarbon biodegradation to levels well above the 44% TPH reduction measured in the NSC (Microcosm 1). Total petroleum hydrocarbon reduction in Microcosms 2 and 3 was as high as 35% (NSC corrected) which was equivalent to a concentration reduction from 2864 mg kg^{-1} soil to 576 mg kg^{-1} soil.

The effects of the treatments examined may be attributed to a combination of nutrient supplementation and improved aeration provided by the bulking agents and tillage. The lower percentage in TPH reduction of Microcosms 3 and 5 may be attributed to the supplements providing limited labile carbon which was rapidly depleted by the soil microorganisms. This possibility was supported by the fact that relatively low microbial activities (FDA) were detected in these microcosms (Fig. 4.2).

The high level of biodegradation recorded in Microcosm 2 was probably due to the increased concentration of nitrogen, in the form of urea, provided by the poultry manure. The recorded concentrations of nitrogen in the soil were very low (viz. $\pm$ 0.069% w/w). The addition of a lignocellulosic organic supplement to soil provides an alternative carbon source which is capable of sustaining fungal (and some bacterial) biomass in the soil for protracted periods. The correlation between TPH reduction and microbial activity, after 30 days, was higher ($r^2 = 0.756$) than Trial 1.

[4.4] GENERAL DISCUSSION

From the results of these trials it was difficult to make accurate quantitative comparisons between the two bioremediation strategies as there were large differences in the initial TPH concentrations (i.e Trial 1 = 5613 mg kg^{-1} and Trial 2 = 2864 mg kg^{-1}). Hydrocarbon reduction in the non-sterile controls of Trial 1 was 8% whereas in Trial 2 it was 40%. These differences indicate an increased toxicity of the hydrocarbon contamination at higher concentrations. This observation highlights a problem inherent in the project site where highly variable concentrations of the pollutant were present in the soil. For the full-scale site bioremediation, areas with elevated concentrations of hydrocarbons should be either

excavated and treated separately or the soil in the area should be thoroughly homogenised first by some form of mechanical mixing.

Considering the TPH results of Microcosm Trial 1 alone, only a marginal increase in hydrocarbon degradation in response to soil inoculation was recorded compared to the sterile barley/BBW treatment. Thus, there appeared to be little merit in soil inoculation considering the potential cost and time savings which could be gained if the production and application were omitted. Thus, it was decided that further experimentation would focus on biostimulation. The efficacy of potentially low-cost nutrient additions and bulking agents was, therefore, examined.

[4.5] BIOSTIMULATION TRIAL 3: MATERIALS AND METHODS

After evaluating the results of the previous trials, additional specific treatment combinations were examined, some in combination with standard agricultural fertilisers.

[4.5.1] Microcosm Treatments

The microcosms were prepared as described in [4.2.1]. The seven soil treatments used are outlined in Table 4.3. In this trial, an additional non-sterile control was made in which the soil was tilled and the water content was adjusted. This treatment was a more true indication of intrinsic bioremediation than only the non-sterile control used in the proceeding trials. The microcosms were tilled at ten-day intervals to facilitate aeration. A strict tillage duration and pattern was again observed to ensure consistency of the treatment. During incubation (25°C), the moisture content was held at between 40% and 60% of the soil WHC. This was achieved by the application of an appropriate volume of water, determined from oven-dried (105°C, 24h) soil samples, every tenth day. Soil samples (60 g) were taken immediately after each tilling event and pH, TPH and microbial activity (FDA) analyses were performed as described in [4.2.3]. The trial was run for 60 days.

Table 4.3: Treatments used in Microcosm trai 3.

Soil Microcosm	Treatment
[1] Non-sterile control (NSC)	Microcosm received no tillage, irrigation or nutrient additions throughout the experimental period.
[2] Irrigation and tillage	Received only tillage and irrigation.
[3] Straw	Non-sterile straw was shredded (± 5cm lengths) and applied at 40% (v/v).
[4] Manure	Chicken manure (nitrogen content 3.98% w/w) was air dried (at ambient temperature), sieved (5mm mesh) and applied at 40 % (w/w).
[5] Manure and straw	Shredded straw and manure (3.94% nitrogen w/w) were mixed in a ratio of 1:1 (v/v) and applied at a concentration of 40% (v/v).
[6] Nitrogen, phosphorus and potassium (NPK) fertiliser addition	Standard agricultural fertilisers [4.5.2] were added in 30 g amounts on days 0, 20 and 40.
[7] Straw and fertiliser (NPK)	Non-sterile straw was added at 40% (v/v) together with NPK as in Microcosm 6.

[4.5.2] Fertiliser Additions

The amounts of the fertilisers added to Microcosms 6 and 7 were calculated to adjust the carbon to nitrogen ratio to near optimal for fungal growth, i.e. between 10:1 and 30:1 (Griffin, 1972). Based on a total soil organic carbon content (soil fertility test, Appendix A) of 5% (w/w), the N:P:K ratio of the soil was adjusted to 3:2:1 (Griffin, 1972).

(i). Nitrogen

Nitrogen was supplied in the form of LAN (limestone ammonium nitrate, 28% w/w nitrogen). Thus 17.8g of LAN were needed 1000 g^{-1} contaminated soil to establish a C:N ratio of 10:1.

(ii). Phosphorus

Superphosphate (8.3% w/w phosphorus) was used to supply phosphate to the soil. To obtain the desired elemental nutrient ratios, the addition of 40.16 g phosphorus 1000 g^{-1} contaminated soil was required.

(iii). Potassium

Potassium was supplied in the form of muriate of potassium (50% w/w potassium). To supply the 1.66 g K required, 3.33 g muriate of potassium were added 1000 g^{-1} contaminated soil.

Thus, for the 1500 g of contaminated soil in a microcosm, the following masses of fertilisers were needed: LAN, 26.7 g; Superphosphate, 60.15 g; and muriate of potassium, 4.9 g. The fertilisers were combined, ground in a mortar and pestle and then divided into three equal masses for individual applications.

[4.5.3] Results and Discussion

Soil in Microcosm 1 served as a non-sterile control to quantify abiotic losses, mainly through volatilisation. Microcosm 2 was also a control, as all microcosms which received organic supplements also received this treatment and thus served as a measure of intrinsic bioremediation. The addition of water significantly affected microbial metabolism and the regular mixing increased microbial contact with the contaminant molecules and improved soil aeration. The addition of straw to Microcosm 3 was similar to that used in [4.3.1]. This treatment was repeated due to the different initial TPH concentrations of each microcosm trial which effected the extent of TPH attenuation. In addition, a more comprehensive evaluation of the effects of the addition of straw was needed. The treatment

used in Microcosm 4 was repeated from Trial 2 although the amount of manure added was increased (viz. from 15% to 40% w/w). The combined effect of a bulking agent (straw) and the addition of a source of organic nitrogen was examined in Microcosm 5. The addition of inorganic nutrients, in the form of slow-release agricultural fertilisers, was examined in Microcosm 6. It was expected that nitrogen loss from the manure (as added to Microcosms 4 and 5) would be rapid due to the volatilisation of ammonia. In contrast, nitrogen release from the fertilisers should have been more protracted. In Microcosm 7, the effect of a bulking agent was examined in the presence of the same fertiliser additions as Microcosm 6. Total petroleum hydrocarbon concentrations during the trial period are shown in Figure 4.3.

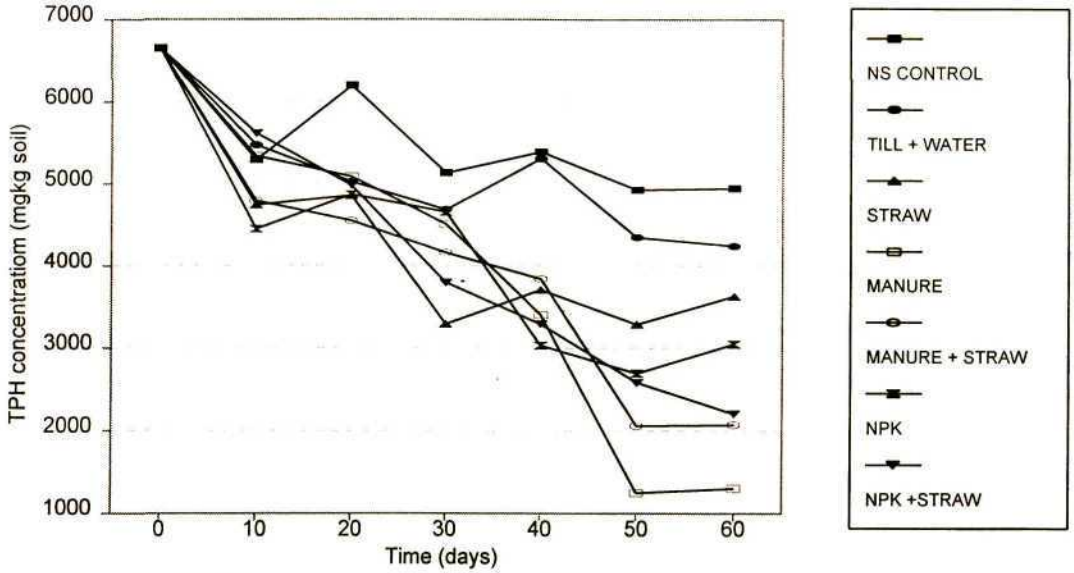

Fig. 4.3: Reductions in TPH concentrations in Trial 3 microcosms over 60 days.

All treatments which received organic supplements showed significantly higher levels of TPH reduction than the control microcosms ($Fpr < 0.001$, Appendix E). The highest level of biodegradation, 54.8% (NSC corrected), was achieved in Microcosm 4 which was supplemented with manure. The NSC (Microcosm 1) showed a 25.6% TPH reduction during the trial. The addition of water and tillage increased this figure to 36.3% (Microcosm 2). The second highest level of TPH reduction, 42% (NSC corrected), was achieved in Microcosm 5 which received manure and straw supplementation. The addition

of nitrogen appeared to have a marked effect on TPH reduction. The slightly lower TPH reduction recorded in the microcosms which received nitrogen and straw was significant (Fpr=0.001, Appendix E). The TPH concentration reduction in Microcosm 3 (straw only) was not statistically different from the other treatments although higher levels of TPH reduction were measured (see table of contrast means, Appendix E). The effect of bulking agents in this laboratory-scale study appeared to be minimal as the total soil volume was low and regular mixing of the soil facilitated oxygen diffusion throughout the matrix. Rapid initial decreases in TPH concentrations were observed in all the microcosms during the first 10 days of the trial. These were attributed to volatilisation of the lighter hydrocarbon fractions. These were especially noticeable in the two control microcosms. It was suspected that there may have been some sorption of hydrocarbons to the straw added to the microcosms. Although this sorption was not specifically quantified, it was expected to be low under the trial conditions. This contention is examined and discussed in greater detail in the general discussion [4.7]. The rates of TPH attenuation then appeared to decrease after approximately 30 days. Thereafter, the effects of nutrient additions became apparent and the TPH concentrations continued to drop but at slower rates (Fig. 4.3).

The results of microbial activities (FDA) assayed throughout the trial are shown in Figure 4.4.

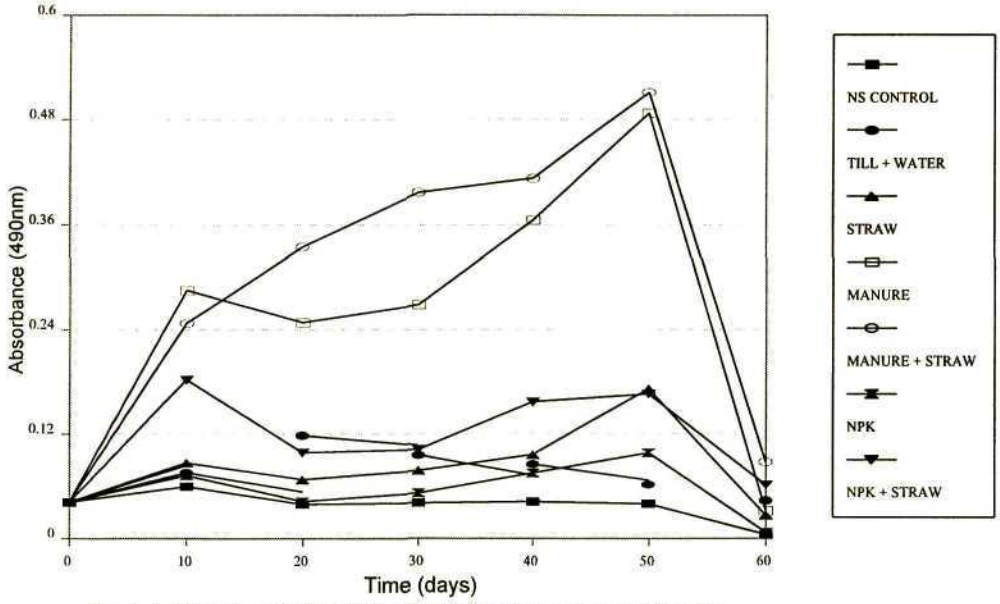

Fig. 4.4: Microbial (FDA) activities in Trial 3 microcosms over 60 days.

The addition of organic supplements to the microcosms significantly increased the microbial activities in these microcosms (Appendix F). Microbial activity in the non-sterile control (Microcosm 1) was the lowest. Tillage and the addition of water (Microcosm 2) increased the microbial activity only slightly and, by day 20, the activity began to decrease to rates similar to those of the NSC. Microbial activity in Microcosms 3 (straw addition), 6 (NPK) and 7 (NPK and straw) increased progressively until day 50 when the activities suddenly dropped to very low rates. These sudden falls could not be explained in terms of depletion of available substrate.

It was suspected that the thermal regulator of the incubator was faulty and that the temperature increased beyond physiological tolerance limits. Initial increases in temperature probably stimulated microbial activity until a threshold temperature was reached. Subsequently, enzyme inhibition occurred and eventually resulted in cell death. It is likely that microbial activity was further inhibited by a drying of the soil and an increased toxicity of the hydrocarbons that were volatilised with the increases in temperature.

The pH values in all the microcosms, with the exception of those supplemented with manure (Microcosms 4 and 5), remained relatively stable during the trial period. Only an initial small decrease of approximately 0.5 pH units was measured after ten days (Fig.4.5).

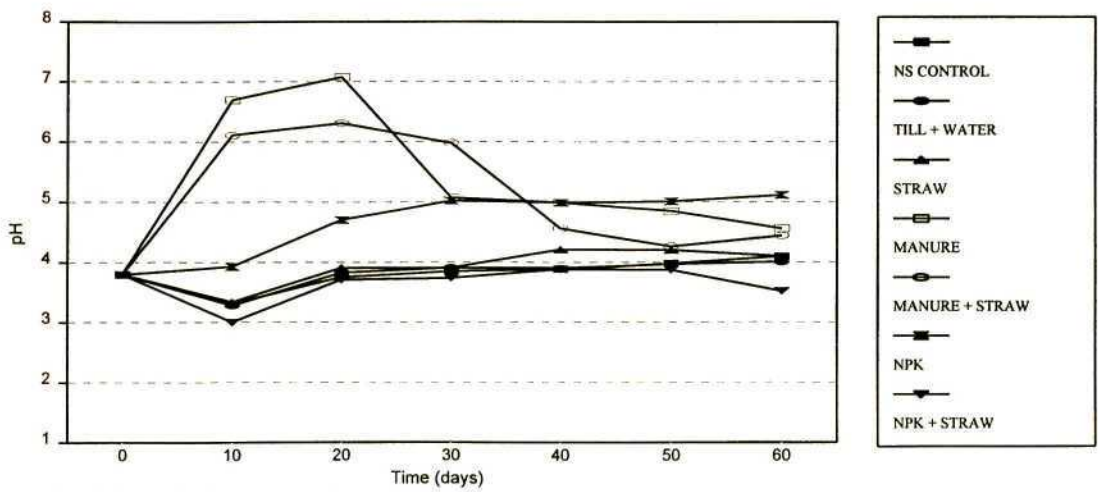

Fig. 4.5: Changes in pH values of Trial 3 microcosms over 60 days.

Over this same period, the pH changes in Microcosms 4 and 5 were 3.28 and 2.51, respectively. These changes probably resulted from the addition of NH_4^+ into the microcosms. However, NH_4^+ is a preferred nitrogen source for fungi (compared with nitrate) and is taken up rapidly. This in turn results in large pH changes (Griffen, 1972). Substantial changes in the pH values of Microcosms 4 and 5 were again observed after day 20, whereafter the values progressively decreased but remained slightly higher than the other microcosms. Full-scale bioremediation operations often rely on the use of lime to adjust the pH to near neutral; a pH that supports the greatest diversity of microorganisms (mainly bacteria). Extreme pH conditions, especially acidic ones, can also alter the mobility of soil minerals and may result in their leaching. In some contaminated soils, this may result in heavy metal leaching and possible groundwater contamination (Rangal *et al.*, 1988.). However, in the current study, the intrinsic low pH values of the contaminated soil provided a selective advantage for fungal species.

[4.6] BIOSTIMULATION TRIAL 4: MATERIALS AND METHODS

[4.6.1] Microcosm Treatments

The microcosms were prepared in the same manner as described in [4.2.1]. Three different compost media were used together with two control microcosms as shown in Table 4.4. All the microcosms were tilled at ten-day intervals with the exception of Microcosm 4 which was tilled every 14 days. A strict tillage duration and pattern was observed to ensure consistency of the soil aeration. During incubation (25°C), the moisture contents were maintained between 40% and 60% of the soil WHC. This was achieved by the application of appropriate volumes of sterile distilled water, determined from oven-dried (105°C, 24h) soil samples, every tenth day. Soil samples (60 g) for pH, TPH concentration and microbial activity (FDA) analyses were taken immediately after each tilling. These samples were analysed as described in [4.2.3]. The trial was run for 60 days.

Table 4.4: Treatments used in Microcosm Trial 4.

Soil Microcosm	Treatment
[1] Non-sterile control (NSC)	Intrinsic bioremediation (received only tillage and irrigation).
[2] Wood-chips	Pine wood-chips (± 2.5 cm x 5 cm), collected as a waste product from a wood working yard (Natal Fencing Co.), were pre-moistened with distilled water, before addition at a concentration of 40% (v/v).
[3] Wood-chips/chicken manure compost	Wood-chips and chicken manure (3.94%w/w nitrogen) were mixed in equal volumes and were added to the soil at a concentration of 40% (v/v).
[4] Spent mushroom substrate (SMS)	SMS (used for *Agaricus bisporus* culture) was added to the soil at a concentration of 40% (v/v). The soil was tilled only every 14 days.
[5] Mushroom compost	Non-sterile, uninoculated mushroom compost was added at a concentration of 40% (v/v).

[4.6.2] Results and Discussion[4.6.2]

As in the preceding three microcosm trials, the soil in Microcosm 1 quantified abiotic losses and intrinsic bioremediation in the soil. Microcosm 2 was included as a control for Microcosm 3. The combined effects of a soil bulking agent (wood-chips) and a nitrogen-rich, highly compostable, substrate (chicken manure) were examined for potential use in co-composting with the contaminated soil. The SMS used in Microcosm 4 was obtained as a waste product from a commercial mushroom farm (Daybreak Mushroom Farms). Analysis of the SMS determined the nitrogen content to be $\pm$ 1.27% (w/w)[2]. The SMS still contained active mycelium (as indicated by the elevated microbial activity, Fig. 4.7). The potential merits of SMS for bioremediation are discussed in [4.7]. Holroyd *et al.* (1992) found that the biodegradation potential of pentachlorophenol (PCP) in soil by the white-rot fungus *Phanerocheate chrysosporium* increased if the soil was left undisturbed for protracted periods. This allowed the fungal mycelium to penetrate deeper into the soil. These same principles were applied to Microcosm 4 and, hence, it was only tilled every 14 days. The compost used in Microcosm 5 was the same substrate as used in Microcosm 4 although it had not been used for mushroom cultivation. Mushroom compost contains chicken manure, horse manure, wheat straw and maize stover mixed in specific ratios, depending on the growth conditions. Typically, plant material and manure are mixed in a ratio of 3:1 (v/v). The nitrogen content of the compost used was determined to be $\pm$ 2.59% (w/w)[2]. Laine and Jorgenson (1996) reported the use of mushroom compost for bioremediation purposes. They found the substrate to be a good nutrient source and attachment surface for PCP-degrading microorganisms.The compost increased the competitive ability of the microorganisms and afforded some protection against the toxic effects of the pollutant. In addition, this type of medium is known to be rich in actinomycetes (Fermore *et al.*, 1985), many strains of which are known for their potential to degrade haloaromatic compounds (Golovleva *et al.*, 1992; Nohynek *et al.*, 1993).

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[2] Determined by Cedara Feed Laboratories.

Residual TPH concentrations for the trial period are shown in Figure 4.6.

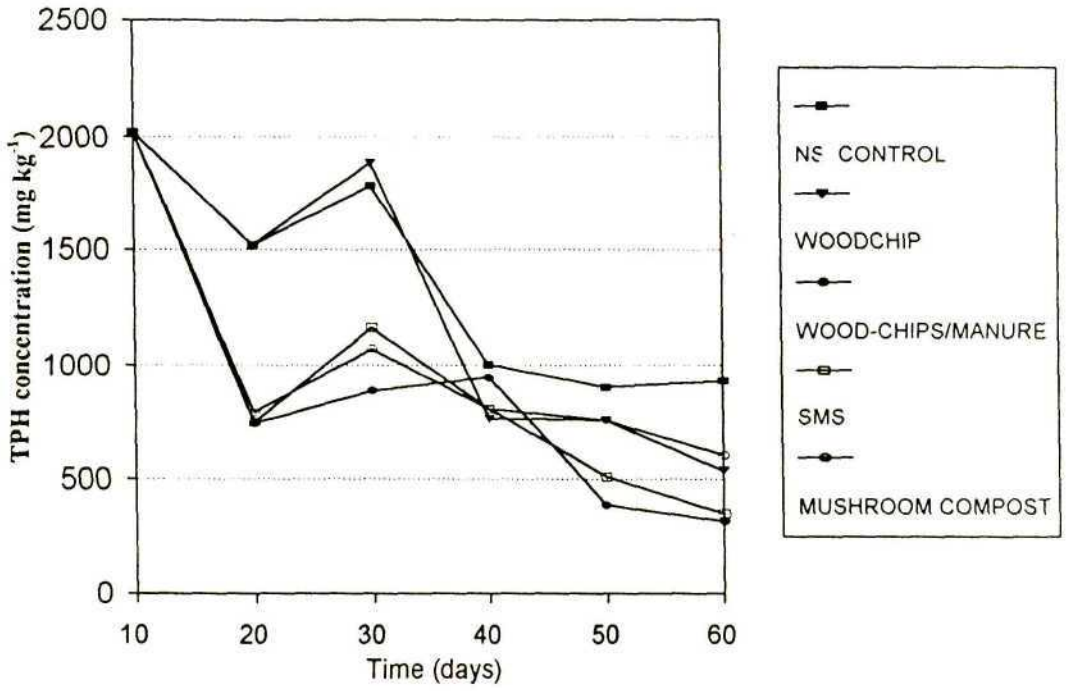

Fig. 4.6: Reductions in TPH concentration in Trial 4 microcosms over 60 days.

The soil treatment results were significantly different to the non-sterile control ($Fpr < 0.001$, Appendix G). The highest TPH concentration reduction of 34% (NSC corrected), a TPH reduction from 2019 mg kg^{-1} soil to 1665 mg kg^{-1} soil, was obtained in Microcosms 4 and 5 which received additions of SMS and mushroom compost, respectively. There was no significant difference between the TPH concentration reductions of Microcosms 4 and 5 (Appendix G). Hydrocarbon degradation in Microcosm 4 was expected to be higher than that of Microcosm 5 since it contained live fungal mycelium which resulted in significantly higher ($Fpr < 0.001$, Appendix H) microbial activity (Fig. 4.7) than Microcosm 5. It was anticipated that survival of *Agaricus bisporus* in the contaminated soil would be poor. This was confirmed by a progressive decline in microbial activity during the trial period (Fig. 4.7). However, it was assumed that biodegradation would be enhanced by the presence of lignolytic enzymes in the compost substrate. The fact that this phenomenon was not observed was attributed, largely, to the lack of sensitivity of the infra-red TPH method.

Other factors, such as depletion of available nitrogen and labile carbon in the SMS, may also have been contributing factors. The wood-chips and manure additions to Microcosm 3 were also highly effective in reducing the TPH concentrations by 28% (NSC corrected). The sole addition of wood-chips to the contaminated soil resulted in a surprisingly high TPH concentration reduction of 24% (NSC corrected).

The addition of wood-chips to the soil resulted in a highly porous matrix which increased the surface area exposed to the air and resulted in a higher degree of hydrocarbon volatilisation. Sorption of the pollutant to the wood-chips may have been instrumental in this apparent reduction. The contention that TPH losses from Microcosm 2 were largely abiotic, was supported by the fact that microbial activity in Microcosm 2 was only marginally higher than that in the NSC (Fig 4.6). In addition, the difference in microbial activity (FDA) of the wood-chips-supplemented microcosms compared with the other treatment was significantly lower ($Fpr < 0.001$; Appendix H). Differences between the treatment means of the wood-chips and the wood-chips plus manure treatment were apparent, although these were not statistically significant (Appendix H). When compared to the first three trials, trial four had a relatively low initial TPH concentration of 2019 mg kg^{-1} soil. This may account for the high biodegradation observed, as it is probable that the hydrocarbon contaminants had reduced toxicities at these lower concentrations.

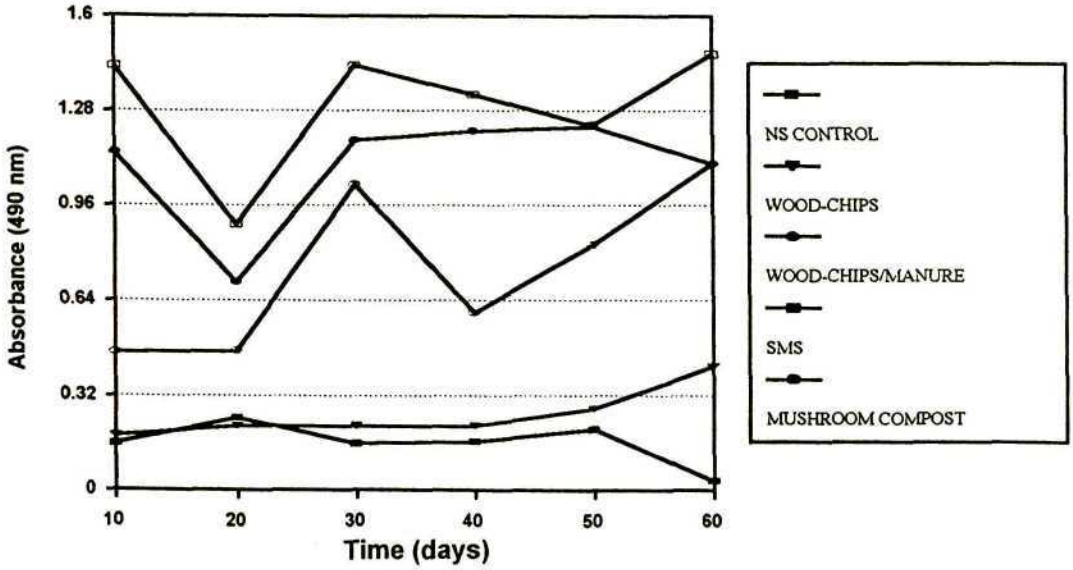

Fig. 4.7: Microbial (FDA) activities in Trial 4 microcosms over 60 days.

Elevations in pH (Fig. 4.8) of microcosms which received compost additions containing manure (viz. 3 - 5) were recorded for the first 20 days of the trial, but thereafter, the pH values decreased towards the initial starting values. These pH changes probably resulted from bacterial and fungal ammonification of the urea in the chicken manure. The highest increase in pH (from 4.1 to 5.9) occurred in Microcosm 3 which did not receive the highest addition of manure. Small decreases in the pH of the microcosms not receiving manure (viz. 1 and 2) were measured initially, but the values stabilised around the starting pH of approximately 4 after day 20.

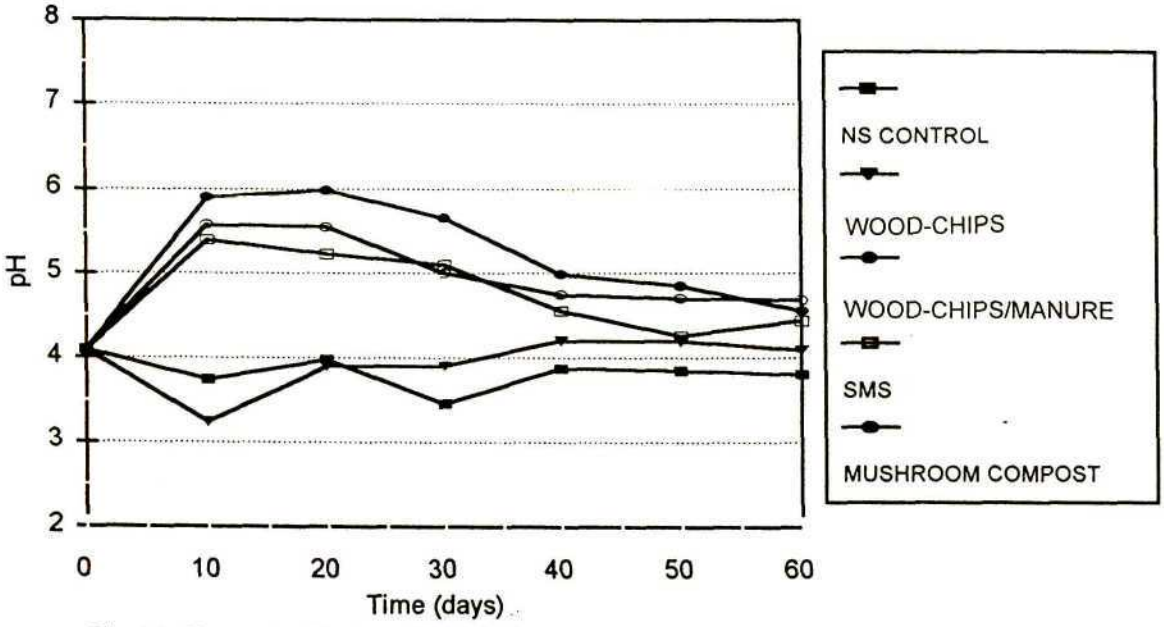

Fig. 4.8: Changes in pH values of Trial 4 microcosms over 60 days.

[4.7]. GENERAL DISCUSSION

The results of the experiments discribed in Chapters Three and Four indicated that the indigenous fungi of the contaminated site were capable of biodegrading the pollutant hydrocarbons. Contamination has been present at the site for over two decades. Thus, the indigenous microbial species had been subjected to specific selection pressures for a protracted period which has effected key enzymatic capabilities for degradation *in situ*.

Over a short bioremediation period (60 days), relatively high concentrations of the contaminant hydrocarbon were degraded under laboratory conditions. The most successful treatments in the trials achieved TPH concentration reductions 30% higher than those achievable in non-sterile controls. There appeared to be a correlation between TPH reduction and sustained high microbial activity during the trial period. These high, and sustained, rates of microbial activity were only achieved in microcosms supplemented with nitrogen. Although higher hydrocarbon attenuations were recorded in microcosms which received nitrogen in the form of chicken manure [4.5.1], it was suspected that if the trial period had been extended, the final TPH concentration reduction would have been different as the slow-release-fertiliser used would probably have sustained microbial activity in the soil for an extended period. The high microbial activity measured in the microcosms which received manure may also be explained by the high microbial activity sustained by the manure substrate *per se*, rather than any biostimulation effect of fungi in the soil.

The results also indicated that a bioaugumentation-type of approach to bioremediation would not be the most cost-effective means of remediating the site as comparable levels of biodegradation were achieved without the introduction of inoculum. However, one readily available source of fungal biomass occurs in spent mushroom substrate. After a mushroom compost has been used for two or three harvests of fruiting bodies, it is discarded. Spent mushroom substrate usually contains large amounts of viable white-rot fungal mycelium. Thus, SMS for bioremediation purposes is, potentially, a highly cost-effective soil treatment option as it obviates the time and expense associated with the production of fungal biomass. This is also a highly efficient use of the substrate as, firstly, food is produced from an agricultural lignocellulosic waste, while the SMS is ultimately disposed of and a contaminated soil may be restored (J.E. Smith, 1995, personal communication). To date, there is only one published report on the use of SMS in bioremediation (Okeke *et al.*, 1993). These researchers mixed sterile soil, contaminated with PCP, with spent sawdust cultures of three strains of *Lentinus edodes* (Shiitake mushroom) supplemented with a nutrient solution (glucose, thiamine and mineral salts). After 21 days of incubation, between 44% and 65% of the PCP had been attenuated.

The question arises: do the bioremediation data obtained from research with white-rot fungi such as *P. chrysosporium* have any relevance for *Agaricus bisporus* (the common commercial button mushroom in South Africa)? The potential certainly exists, as the ability of *Agaricus* sp. to degrade the lignin component of composted lignocellulose has long been recognised (Waksman and Nissen, 1931; Gerrits *et al.*, 1967). When grown under standard composting conditions, *A. bisporus* was shown to produce Mn dependent peroxidase (Bonnen *et al.*, 1994). Since the bioremediation capacity of these fungal systems appears to be largely dependent upon lignolytic enzyme activity, it is clear that *A. bisporus* has the potential to degrade environmental contaminants. However, definitive experiments to demonstrate this capacity have still to be undertaken. In addition, endogenous enzyme levels in SMS may not be sufficient for bioremediation purposes and some system enhancement may be necessary as it is known that low concentrations of both Mn peroxidase and laccase enzymes markedly decrease at the fruit body maturation stage which implies low activities in SMS (Buswell, 1995).

The contribution of contaminant adsorption to organic supplements was not specifically quantified as a component of abiotic TPH reduction in this study. It has been suggested by a number of researchers (Karichoff *et al.*, 1979; Means *et al.*, 1980) that the organic carbon content of soils is the single most important factor in determining the sorption of hydrophobic molecules such as PAHs. Weissenfels *et al.* (1992) found a positive correlation between the amount of soil organic carbon and the degree of PAH sorption onto soil material. With respect to PAH adsorption, soil organic carbon was the most significant factor influencing PAH sorption in the different soils examined.

Two kinetically distinct processes have been found to be associated with PAH binding onto soil material. An initial fast process is thought to represent adsorption of hydrophobic pollutants onto hydrophobic areas of soil surfaces. This is followed by a slow adsorption process which is proposed to be the migration of the hydrophobic contaminants to the less accessible sites within the soil matrix (Robinson *et al.*, 1990). Thus, longer incubation times result in the migration of an increasingly large fraction of the pollutant into the organic soil material. This process continues until the incorporation capacities of the soil organic matter

are exhausted and equilibrium is reached. Weissenfels *et al.* (1992) found, under simulated land treatment conditions (200 g soil), that the amount of extractable anthracene oil applied initially decreased by 40% within the first few hours of incubation. This demonstrated rapid and extensive sorption. However, during further exposure, the degree of PAH extractability decreased continuously indicating further adsorption at an increasingly slower rate, i.e. a 35% decrease in the amount of extractable anthracene oil over 30 days.

The fraction of incorporated pollutants represents the non-bioavailable and non-biodegradable part of contamination in soils. This immobilised component is not released by rinsing soils with water (Weissenfels *et al.*, 1992). It is expected that the opportunity for contaminants to become irreversibly bound will increase with exposure time, due to slow weathering of the soil and environmental changes causing the soil matrix to change (Kan *et al.*, 1994). Adsorption of the pollutant to the soil matrix at the project site may account for the lack of contaminant migration off the site, as indicated by analysis of groundwater samples taken from wells sunk adjacent to the site. It is believed that, due to the age of the contamination, pollutant migration and sorption onto the soil organic component had reached an equilibrium. It was thus suspected that the sorption of pollutants to the soil organic supplements applied to the soil microcosm trials were small, especially in the time frame in which the trials were run. This fraction would, however, increase with time and exposure to the materials and as the soil was mixed.

The data of many researchers (Martin *et al.*, 1978; Means *et al.*, 1980; Weissenfels *et al.*, 1992) suggested that sorption of organic pollutants to soil organic matter significantly affects biodegradability. Kan *et al.* (1994) also found that the adsorptions of PAHs (naphthalene and phenanthrene) were fast, whereas a fraction of the adsorbed pollutants desorbed only very slowly. Contact times of the pollutant with the soil were in the order of days, yet it was estimated that it might take years for complete desorption to occur. This may explain the initial large decreases in TPH concentrations typically observed in the first few days of bioremediation experiments similar to the ones seen in this study (the contribution of volatilisation having also been taken into account). This was followed by an apparent inhibition of biodegradation where relatively small decreases in TPH concentration occured

over a much longer time scale. These slower degradation rates may, in part, be explained by slow rates of desorption .

Although the precision of the TPH method is questionable, the results of the current study indicate that there was no significant difference in TPH concentration reductions in microcosms treated with SMS, compared to the uninoculated compost. The nitrogen concentration of SMS was 49% lower than the unused compost and labile carbon was also, presumably depleted. However, SMS was still able to effect levels of hydrocarbon degradation equivalent to that of the unused mushroom compost. It is, thus, felt that remediation through soil supplementation with SMS offers an extremely attractive remediation option for South Africa and is worthy of further investigation.

CHAPTER FIVE

[5] CO-COMPOSTING OF HYDROCARBON-CONTAMINATED SOIL

[5.1] INTRODUCTION

Composting is a technology which is widely used to treat solid or semi-solid organic wastes including wastewater sludges, processing wastes and municipal refuse. The primary benefits gained by composting these materials are: a reduction in the volume and water content of the waste; destruction of pathogens; reduction in odour; and stabilisation of the material to humus-like end products for ultimate disposal or use as a marketable product. In contrast to this "traditional" use of composting, the objective of combining hazardous materials with a compostable substrate is solely to convert them into innocuous end products. The process requires excavation of the soil and mixing with ancillary organic compounds and inorganic nutrients to achieve a favourable carbon to nitrogen ratio. This shift in objectives poses several important challenges for system operation. For example, operating parameters, such as treatment time, may need to be modified to ensure that the contaminant is reduced to the desired concentration. This may often require a more tightly controlled and aggressive approach to the composting process than would be used for the composting of wastewater sludges and refuse.

Composting can be defined as the accelerated degradation of heterogeneous organic matter by a mixed microbial population in a moist, warm, aerobic environment under controlled conditions (Finstein and Morris, 1975). Microorganisms such as bacteria, fungi and actinomycetes are the "digesters" in this process. The microbial species responsible for initiating the self-heating are ubiquitous in the environment and are usually present in the organic materials (Finstein and Morris, 1975; De Bertoldi *et al.*, 1982). Special inocula, in general, have not been found to be beneficial (Golueke *et al.*, 1954). The diverse microbial community at the start of the self-heating process narrows as the temperature tolerance limits for each of the different groups is exceeded. The process is termed mesophilic up to a temperature

of 38°C. Thermophilic growth starts at approximately 45°C but microbial activity reaches a peak between 50°C and 60°C. Most fungi cannot grow at temperatures above 50°C although a few can grow at temperatures up to 62°C (Tansey and Brock, 1978). The sequence leading to a narrow community structure reflects an interaction between microbial heat generation and temperature. This interaction is an overriding determinant of composting system behaviour. Evidence for supporting 60°C as the operating ceiling has been reported by numerous researchers (Bach *et al.*, 1984; McKinley and Vestal, 1984; Finstien *et al.*,1985). Microbial catabolism causes the organic material to progressively lose its identity and become stabilised so that heat generation eventually subsides and the mass cools. There is little consensus in the literature on the optimal temperature. This is largely due to the conflicting needs of different processes. These different operating temperatures can briefly be summarised as follows:

(i). Sanitisation occurs at >55°C;

(ii). Maximum biodegradation occurs at 45-55°C; and

(iii). Maximum microbial diversity occurs between 35°C and 40°C.

Composting proceeds spontaneously when the following conditions are met:

(i). The substrate and water content of the material are supportive of microbial heat generation;

(ii). The compostable mass is sufficiently large to store heat despite losses to the surroundings; and

(iii). The matrix is sufficiently porous for gaseous exchange.

Ideally, the outputs from a well-managed system are carbon dioxide, water vapour, heat, stabilised organic matter and ammonia. The compost matrix serves a multitude of purposes in the process since it: provides interfaces for microbial growth; supplies a range of nutrient sources; provides a sink for metabolic waste; provides thermal insulation; and forms a matrix for the exchange of gas and heat. Kastner and Mahro (1996) showed that the presence of the solid organic compost matrix appeared to be essential for enhanced degradation. When they separated soil/compost microflora from the organic matrix in liquid culture, the organisms exhibited a much lower degrading activity for the aromatic hydrocarbons than in the

presence of the solid organic material.

[5.2] TYPES OF COMPOSTING SYSTEMS

Composting promotes a high rate of decomposition which effectively minimises the capital and operating costs as there is less need for facility time/space to achieve a given degree of stabilisation. Large-scale composting is accomplished by one of three main systems: windrow with turning; static pile with forced aeration; and in-vessel. These strategies offer different advantages in terms of system throughput and operating costs.

[5.2.1] Windrow Composting

Windrows are the simplest of the composting processes. With relatively light engineering equipment, the compost mixture is fashioned into elongated unenclosed piles which are triangular in cross-section (approximate dimensions 1.5 - 1.8 m high x 2.4 m wide x variable length). Aeration is provided by mechanical agitation, usually by means of dedicated equipment or simply by turning the compost with a front-end loader at regular intervals.

[5.2.2] Static Pile Composting

These piles are termed "static" as they are not disturbed by mechanical agitation. Oxygen is forcibly delivered by an electric blower. As the oxygen supply to the inner regions of the pile is not dependent on passive diffusion, static piles tend to be larger than windrow composting piles. The piles are usually enclosed in canvas or plastic tarpaulins. There are two major design types for aerated static piles.

(i). **Beltsville Process:** The Beltsville pile has the approximate dimensions of: 3 m high x 4.5 - 7.5 m wide x 12 - 15 m long. Oxygen is delivered to the pile by drawing a negative pressure. The blower is activated by a timer which operates approximately 20% of the time in

any 24 h period (Finstein *et al.*, 1985).

(ii). **Rutgers Process:** The pile has the approximate dimensions of: 2 m high x 5 m wide x 14 long. A positive pressure is applied with temperature actuation of a blower. The temperature ceiling is usually set at 60°C (Finstein *et al.*, 1985).

[5.2.3] In-Vessel Composting

The material is held in a purpose-built vessel or reactor. These systems exert the highest level of control over the composting process and represent the highest level of technical sophistication of composting systems. There is great variation with respect to agitation, ventilation, vessel shape and residence time. The system may be used for either batch or continuous processing.

[5.3] PRINCIPAL FACTORS AFFECTING PROCESS DYNAMICS

The composting process in any of the systems mentioned above is governed by the same basic principles of heat and mass transfer and by biological constraints of living microorganisms. Effective system design requires an analysis of the composting process and consideration of the following 19 major controllable factors (Table 5.1).

Table 5.1: Possible controllable factors for composting (Hansen *et al.*,1989).

organic supplement	moisture control
C/N ratio	aeration
particle size	ambient temperature
percent recycled compost	retention time
mixing equipment	depth
reactor vessel size	type of process
stirring frequency	curing time
chemical pH modelling agent	inoculation
initial moisture content	bulking agents
temperature	

The most important of factors listed in Table 5.1 are discussed below.

[5.3.1] Gaseous Exchange, Ventilation and Heat Removal

Gas exchange in composts supplies oxygen and removes carbon dioxide, heat and water vapour. Common recommendations for minimum oxygen concentrations within the compost heap are between 5 % (Wilson *et al.*, 1980) and 10% (Suler and Finstein, 1977). To maintain a maximum respiration rate, the soil gas oxygen concentration should not be allowed to fall below 2% (v/v) at any point within the pile (McGinnis *et al.*, 1994). Due to the high rates of oxygen utilisation during composting, especially by thermophilic microbial respiration, the rate-limiting determinant is often the rate of oxygen diffusion through the compost matrix. At the peak of the thermophilic stage, substantially more air is required to remove heat than to supply oxygen. This may be exemplified by analysis of the complete oxidation of organic carbon to carbon dioxide and water (inlet air, 20°C; relative humidity, 50%; outlet air, 60°C; relative humidity, 100%). Calculations show that it takes 38.7 kg air (dry) to remove the heat and only 4.31 kg to replenish the oxygen, giving a ratio of 8.98:1. This is a crucial ratio as self-heating only occurs when the ratio is >1:1 (Finstein *et al.*, 1986). It has also been suggested that approximately 35% free air space is required to obtain adequate aeration for composting a wide

range of materials (Worne and Fortune, 1992).

High concentrations of oxygen are easily maintained with limited ventilation, provided that the physical structure of the material allows sufficient gaseous exchange. A blower with a delivery capacity of $< 10\ m^3\ t^{-1}h^{-1}$, actuated by a timer on a fixed schedule for only 20% of the time, typically maintains oxygen concentrations of 10-15% (v/v). In temperature feedback actuated blower systems, the blower must have a delivery capacity to meet the peak demand for heat removal of $>100\ m^3\ t^{-1}h^{-1}$ (Finstein *et al.*,1986). Ventilative heat removal is controllable and affords a practical means of maximising the decomposition rate. Other mechanisms of heat removal associated with ventilation include vaporisation and sensible heating of air. As a result of vaporisation, the material tends to dry as the composting progresses. This can often become the microbial rate-limiting factor. Depletion of labile substrate may also be considered as a microbial rate-limiting factor.

During the temperature elevation stages of composting, the ventilation should be managed to supply oxygen for aerobic respiration and to promote heat generation while minimising associated heat removal. These needs are only marginally conflicting, due to the high ratio between the volume of air needed to remove heat and supply oxygen. Hence, the mass should be ventilated at a specific rate so that deoxygenation is avoided. A suggested rate is $9m^3\ ton^{-1}\ h^{-1}$ (Finstein *et al.*, 1985). Once the desired temperature has been reached, system control shifts to an emphasis on heat removal. Under these circumstances, the oxygen demand will automatically be met by virtue of the quantitative ratio between these two factors.

[5.3.2] Carbon : Nitrogen Ratio

For the composting process to function efficiently, a favourable carbon to nitrogen ratio (C:N) must be established. The optimal C:N ratio is estimated to be in the range of 25:1 to 35:1 (Anderson, 1991; Kubota and Nakasaki, 1991). It has generally been found to be most convenient to co-compost two wastes with different C:N ratios so that the mixture falls within the optimal range.

[5.3.3] Moisture

Water is essential for microbial activity and should be present in appropriate volumes throughout the composting cycle. However, water tends to plug pores and impede gas exchange. Therefore, a moisture content must be established where the needs for available water and gas exchange are balanced. Because different materials have different water holding capacities, no exact generalisations can be made about optimal initial or time-course moisture concentrations. Moisture concentrations are, thus, a material and operation-specific parameter. Researchers are inconsistent in the terminology used. While some workers quote moisture concentrations in terms of field capacity, others refer to wet weight. Field capacity is a more desirable measurement as it accounts for the possible physical differences in the compost media. Wide ranges of moisture operating concentrations are quoted in the literature although optimal concentrations appear to be around 40% field capacity (Kamnikar, 1995) or 55% to 65% wet weight (Brown *et al.*, 1995).

[5.3.4] Porosity and Bulking Agents

If the solid matrix has insufficient structure and porosity to permit adequate gas exchange, a bulking agent may be used to impart porosity. Bulking agents increase pore space in the pile or windrow to permit adequate concentrations of moisture to be maintained but still allow free space for gaseous exchange. One approach is to recycle a small percentage of the compost (e.g. 30% v/v) as the bulking agent. In some cases, the bulking agent will have to be imported. Semi-inert bulking materials, such as wood-chips, are commonly used. These may be separated by screening the mixture at the end of the process for recycling. Dooley *et al.* (1995) examined co-composting for the bioremediation of a herbicide-contaminated soil (dicamba). Microcosms were initially prepared with 4.5% (w/w) wood-chips and 6.3% (w/w) cattle manure and mineral nutrients (300 mg kg^{-1} $N\text{-}NH_4$ and 44 mg kg^{-1} orthophosphate). They found that the biodegradation rates were highly dependent on the bulking agent ratios. The treatment concentration criteria established for the site

could be achieved in 21 to 28 days in compost with a high proportion of bulking agent. Biodegradation levels were the highest in microcosms with the highest proportion of bulking agent, i.e. 41%. Mineralisation losses in the microcosms were in the region of ±28% after 28 days compared with only 6% dicamba mineralisation in the microcosm which contained 10% (w/w) bulking agent.

Provision of a bulking agent may become a major cost factor in the treatment process especially if it has to be transported for any great distance. Special bulking agents consume much operational space on the treatment site which is often at a premium. Wood-chips are also colonised by *Aspergillus fumigatus* which poses a human health hazard as an allergen and infective agent.

[5.4] ADVANTAGES OF COMPOSTING FOR BIOREMEDIATION

There are a number of advantages in using a compost mass as a bioreactor for the degradation of hazardous compounds. The high degradation potential is primarily due to the intensity of the microbial activity within the compost matrix. The specific contaminant destruction capabilities possessed by microorganisms in a composting mass may not differ from those in soils at ambient temperature. However, the overall transformation potential for contaminants within a composting mass is worthy of consideration for a number of reasons:

(i). The elevated temperatures facilitate a higher reaction rate than is generally achievable at ambient temperatures. The elevated temperatures also, typically, result in an increased contaminant solubility and, thus, a higher mass transfer making the target molecule contaminant more available for catabolism (Williams and Keehan, 1993);

(ii). The opportunity for co-metabolism is enhanced due to the wide range of alternative substrates present. Target molecules may be catabolised by microorganisms while labile organic matter is still available within a microsite, or after labile organic material has been degraded;

(iii). The changing physico-chemical conditions within the compost mass

result in a large diversity in the microbial community which, in turn, has a diverse range of metabolic capabilities. This results in an increased range of microorganisms to which the contaminant is exposed;

(iv). The physical attributes of compost are highly favourable for the treatment of hazardous materials. The large surface area and solid phase matrix make it receptive to hydrophobic as well as hydrophilic wastes (Williams and Keehan, 1993); and

(v). Large volumes of water are vaporised at the expense of the waste. This not only reduces the mass and volume of the waste (Finstein *et al.*, 1986) but, more importantly, leachate production is reduced.

[5.4.1] Case Studies of Co-Composting for Bioremediation

The examples referred to in the following section were used as a basis for the design of the pilot-scale co-composting trial made in this study [5.7]. In each case, the system design was examined with respect to: the composition of the compost mixture; the aeration system; the contaminant molecule; engineering aspects; and the overall operational costs.

Relatively little research has been done in the field of composting hazardous matter and most reports are inconclusive and/or preliminary. To date, most research on co-composting for bioremediation has been dominated by work made on the decontamination of explosives-contaminated soils where aerated static piles are used extensively (Osmon *et al.*, 1978; Williams and Myler, 1990). However, a diverse range of organo-pollutants can be treated by compost bioremediation systems. For example, Rose and Mercer (1968) found that the insecticides diazinion, parathion and dieldrin were rapidly degraded when composted with cannery wastes. Hill and McCarty (1967) recorded significant biodegradation of chlorinated pesticides when co-composted with sewage sludge. Deever and White (1978) composted petroleum refinery wastes with sewage sludge and recorded significant reductions in grease and oil. Hunter *et al.* (1981) investigated the biodegradation of crude oil hydrocarbons,

refinery sludge and polychlorinated biphenyls in compost systems. Epstein and Alpert (1980) composted petroleum compounds (crude and No.6 oil) with paper mill pulp wastes and pharmaceutical wastes.

Williams and Myler (1990) tested co-composting for the decontamination of soil contaminated with a number of different nitroaromatics such as 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). A blower, controlled by both timer and temperature feedback systems, was used to draw air through the pile. Both mesophilic and thermophilic operating temperatures were investigated to determine whether the assumed increased microbial diversity under mesophilic conditions would effect an overall increase in contaminant degradation. The half-lives of TNT and RDX were reduced by 57% and 54% under thermophilic and mesophilic conditions, respectively. When soil loading rates were increased from 10% to 32.5% (w/w), similar results were obtained. A cost analysis indicated that if the cost of supplements could be kept below U.S. \$50 ton^{-1} (R225, 1996) the treatment would cost approximately U.S. \$100 ton^{-1} (R450, 1996). This compares favourably with incineration costs which are, typically, U.S. \$250 per ton or higher.

In recent years, co-composting has been successfully employed in large-scale operations as a low-cost biological treatment alternative. Brecheen and Patterson (1995) described a project which involved the excavation of some 2000 tonnes of jet fuel-contaminated soil. The composting materials were mixed in volume ratios of 75% contaminated soil, 20% compost and 5% turkey manure. The workers suggested that the compost component may be reduced to 10% or increased to 30%, depending on the type of soil and the specific contaminant. Once constructed into a biopile, the material was covered with a vinyl-coated nylon cover for protection from the weather and to encourage conditions conducive to microbial growth. Provided the soil remained aerobic, no further manipulations were necessary. When anaerobic conditions were detected, the pile was mechanically turned. They claimed that the soil treatment time was only one month. The soil was then backfilled into its original site. Due to the high percentage of soil used in the biopiles, the internal temperatures seldom rose above 48°C. The costs estimated for this operation were

about U.S. $5 (R23, 1996) ton^{-1} (machinery, construction of treatment area, soil sampling and analysis included). In addition, the contaminated soil did not have to be moved by road which greatly reduced the liability risks. The contracting engineers (Seymour Johnson AFB, U.S.A), claim to have used their process to remediate an estimated 200 000 tonnes of soil contaminated with petroleum products. Systems are also in the process of being developed to handle more hazardous compounds such as PCBs, trichloroethylene and benzopyrene.

Kamnikar (1992; 1995) described a similar system for the treatment of petroleum-contaminated soil. However, a more sophisticated aeration mechanism was used. The term "passive thermal-bioventing" (PTB) will be used to describe this type of co-composting system. Compost was made by mixing wood-chips and manure in a 1:1 (v/v) ratio. Diesel/petroleum-contaminated soil was then mixed with the wood-chips/manure mixture in a ratio of 4:1 (v/v) which, for the purpose of this discussion, is now designated to be compost. Wood-chips were used as a bulking agent to reduce compaction within the pile and maintain void space which is necessary for gaseous exchange. The wood-chips were also sprayed with water prior to mixing to provide an additional source of moisture for the microorganisms. A layer of compost, 45-60 cm thick, was spread out on a bituminous surface. Sections of cord-drainpipe[1] were placed at 1.5 m intervals across the width of the pile. The ends of the drain pipes extended about 35 cm beyond the pile edge. This process was repeated until three layers of equal thickness were completed. The pile was covered with 4 mm black polyethylene sheeting. Openings were cut in the sheeting to allow the ends of the pipes to protrude. Runoff and evaporation were minimised by covering the pile. Covering also enabled a more uniform heating of the pile which is essential for the optimisation of the bioremediation process. Thermocouple wires were placed within the pile during construction to monitor the temperature. The final dimensions of the constructed piles were 5 m wide, 21 m long and 1.8 m high.

[1]Cord drainpipe is perforated flexible polyethylene piping, 100 mm diameter, usually used for subsurface drainage of soil.

Elevated temperatures, approximately 20°C higher than ambient, were maintained for about two months. Thereafter, a gradual decrease towards ambient was recorded over the next three months. The highest temperature recorded in a biopile which received only sheep manure (at a soil:manure ratio of 4:1) was 54°C. This temperature was maintained for approximately 20 days. In contrast, a temperature elevation of only ±8°C was recorded in a biopile which received just NPK fertiliser (600 g m^{-3}) as a supplement. Diurnal fluctuations were not evident from the recorded temperatures. Temperature readings were consistently higher in the upper third of the pile. Some, apparently random, variations were evident along the length and width of the piles. The compost pile in which contaminated soil was mixed at a lower ratio of 3.5 parts soil to 1 part manure/wood-chips reached higher maximum temperatures (up to 5°C higher) than the other piles. Over the seven-month treatment period, the TPH concentration was effectively reduced from 2900 ppm to 15 ppm. The cost was approximately U.S. $13 per m^3 (R59, 1996).

[5.5] FUNGI IN COMPOSTING

Many fungal species from unrelated genera appear to be involved in all phases of the composting process. It is difficult to make generalisations about the fungal species composition in the different phases of composting as the species appear to be highly dependent on the specific substrates and composting conditions. Thus, the literature is often contradictory, especially with respect to discussions relating to fungi in the thermophilic phase of the process. However, some fungi appear to be isolated from most composts. Such an example is *Trichoderma* spp., which are often seen as colonisers of wood-based composts. *Trichoderma* spp., generally, have powerful cellulases and are good compost colonisers (Lynch, 1993). Other common compost colonisers are: *Geotrichum candidum*; *Aspergillus pusillus*; *Cladosporium* sp.; and *Chaetomium thermophile* (Finstein and Morris, 1975).

In comparative studies of composting municipal refuse, De Bertoldi *et al.* (1982) observed that intensive cellulose degradation during the composting process was largely through the activity of fungi. In the late phases of composting, a continuous

decrease in the numbers of bacteria was observed while, conversely, there was an increase in the numbers of cellulolytic fungi. While three different aeration strategies were examined, the microbial activity trends were all similar. During the last 22 days of a 50-day trial period (Rutgers Process) a 37% increase in the fungal biomass was recorded while a 50% decrease in the bacterial biomass was measured. De Bertoldi *et al.* (1982) explained this observation in terms of the fungi benefiting from the decreases in temperature, pH and moisture content. Due to the relatively slow metabolic rates of the Basidiomycetes, the highest levels of lignolytic fungal activity were only recorded two months after initiating the composting trial. Lignin decomposition was apparently enhanced in aerated static piles (both Beltsville and Rutgers processes) compared with turned piles. De Bertoldi *et al.* (1982) speculated that this was due to the periodic turning disturbing the growth of hyphae into the mass and thereby interfering with fungal growth. This phenomenon was observed by other workers (Holroyd *et al.*, 1992).

Bayman *et al.* (1995) compared the tolerances of some filamentous fungi to the explosive RDX to that of *P. chrysosporium*. Fungi such as *Cyathus pallidus, Cunninghamella elegans* and *Cladosporium resinae* were all able to transform RDX in culture at relatively high concentrations (100 $\mu g\ ml^{-1}$) although *P. chrysosporium* was the most effective. These filamentous fungi, among many others, are common and are major decomposers in compost systems.

Berry and Boyd (1985) identified bound residue formation or immobilisation as a potential soil detoxification and containment process for certain hazardous organic compounds. These authors indicated that the indigenous peroxidase enzymes may be stimulated to enhance the rate of bound residue formation above the natural background level. Substances involved in the formation of vetrayl alcohol free radicals were the only ones found to have a stimulatory effect. Their results indicated that mineralisation of high molecular weight PAHs may not be achievable to any significant extent in soil compost systems. However, conversion of PAHs to covalently bound soil-humus residues may be interpreted as treatment since this process transforms the chemical identity of the pollutant and effectively diminishes

its bioavailability. Results of a study by McFarland *et al.* (1992) indicated that the sole addition of maize cobs to a soil was sufficient to encourage indigenous microorganisms to mediate benzo[*a*]pyrene incorporation into the soil matrix. Thus, fungal composting can be used as an innovative low-cost soil treatment technology for a no-migration variance to land treatment of petroleum-impacted soil.

[5.6] DISCUSSION

The rapid biodegradation achievable by co-composting serves to minimise costs as it is a truism for many types of industrial activities, including large-scale composting, that time and space are functionally equivalent and will in combination affect costs. The land requirements necessary for the treatment of hazardous wastes by composting are reduced significantly when the process is used instead of landfarming. Composting, where applicable, is a productive approach to the treatment of highly concentrated waste sludges (Worne and Fortune, 1992). In the U.S.A., composting appears to have gained popularity with large governmental organisations, such as transport departments and the U.S. Military, as a simple and cost-effective treatment alternative to petroleum-contaminated soils. Bioremediation can be undertaken as an "in-house" operation as there is very little need for equipment that most of these organisations do not already have at their disposal.

Although relatively high levels of organo-pollutant degradation can be observed in laboratory-scale compost systems, problems have been encountered in full-scale projects. Maintenance of good contaminant-biomass contact and achieving effective mass transport of oxygen/water through the system have proven to be difficult (Osman and Andrews, 1978; Klausmeier *et al.*, 1982). The main factors which require optimisation are the supplements used to prepare the compost mixture.

Volatile organic contaminants may be released during composting (Kim *et al.*, 1995) which may require additional treatment equipment and increase the operating costs. In addition, if heavy metals are present in the waste materials, these may be in concentrations which are toxic to the microorganisms in the compost. The long-term

fates and the structures of the residues have to be evaluated in detail which are often difficult tasks in the light of the complexity of the compost matrix.

[5.7] PASSIVE THERMAL BIOVENTING: A PILOT-SCALE INVESTIGATION.

[5.7.1] Introduction

A pilot-scale passive thermal bioventing (PTB) treatment process was designed to specifically stimulate catabolism by indigenous fungal species in the contaminated soil. In this study, it was also important to determine whether PTB is a technology which is applicable to South African conditions, in terms of its low-cost, low-technology and low-maintenance requirements.

Oil-contaminated soil was mixed with a compostable substrate of wood-chips and poultry manure (used in Chapter Four). Thermocouples were used to monitor the temperatures within the soil pile as a function of time and position. These provided an indirect means of assessing microbial activity within the system. To realise the objective of developing a low-cost, low-maintenance remediation system, a passive ventilation system was used (Kamnikar, 1992; 1995; B. Kamnikar, personal communication). In this system, the metabolic heat generated by microbial respiration was used to initiate thermal convection in an internal pipe network of a biopile. This, in conjunction with a venturi effect of air passage over long vertical pipes, drew air into the biopile.

[5.7.2] Materials and Methods

Oil-contaminated soil (described in the General Introduction) was excavated from the project site and transported to the study site where it was placed on a polyethylene liner to protect the underlying soil from contamination. The soil was first sieved through a rolling-drum sieve (1 cm^2 mesh) to remove large clods and non-soil material. This step aided homogenisation of the contaminant oil throughout

the soil. The sieved soil was spread on the polyethylene liner to a depth of about 20 cm and was left exposed for a period of five days. This was done to maximise volatilisation of the light hydrocarbon fractions prior to initiation of the trial. Rapid decreases in TPH concentrations were detected during the first 10 days of the microcosm trials (Chapter Four). These reductions can be erroneously attributed to microbiological activity.

[5.7.3] Biopile Design and Construction

The biopile was constructed on a gentle slope ($\pm 5^{o}$) which was covered with a polyethylene liner (4 x 4 m). The base of the biopile was constructed with a 20 cm layer of composted pine bark. This provided drainage for excess moisture, thus preventing the formation of anaerobic conditions. A compostable substrate was prepared by mixing wood-chips (pine, $\pm$ 2 cm x 5 cm) with chicken manure in a 1:1 (v/v) ratio. This was homogenised in a cement mixer (80 rpm, 30 min). The mixing resulted in the release of large volumes of ammonia gas from the chicken manure. The compost was then mixed with contaminated soil ($\pm$1280 kg) in a soil:compost volume ratio of 4:1. Mixing of the soil with the compost was done by manually combining alternating layers of soil and compost. The soil/compost mixture was placed in a cone-shaped pile with a circular base with a diameter of 2 m and a height of 0.9 m.

Slotted drain pipe (polyethylene, 75 mm diameter) was used to construct a ventilation network. A lower pipe array was positioned at 300 mm above ground level. Six copper-constitan thermocouples were also located at this height (Fig. 5.1a). A second upper pipe array was placed at 600 mm above ground level with four thermocouples. An additional two thermocouples were placed along the central axis at 700 mm and 800 mm (Fig. 5.1b). Finally, the pile was covered with black polyethylene sheeting which was weighted down with bricks around the base. Holes were cut in the plastic cover to allow the ends of the pipes to protrude (Plates 5a and 5b). In total, twelve thermocouples were used to monitor the thermal environment within the biopile at 300, 600, 700 and 800 mm above ground level. Two

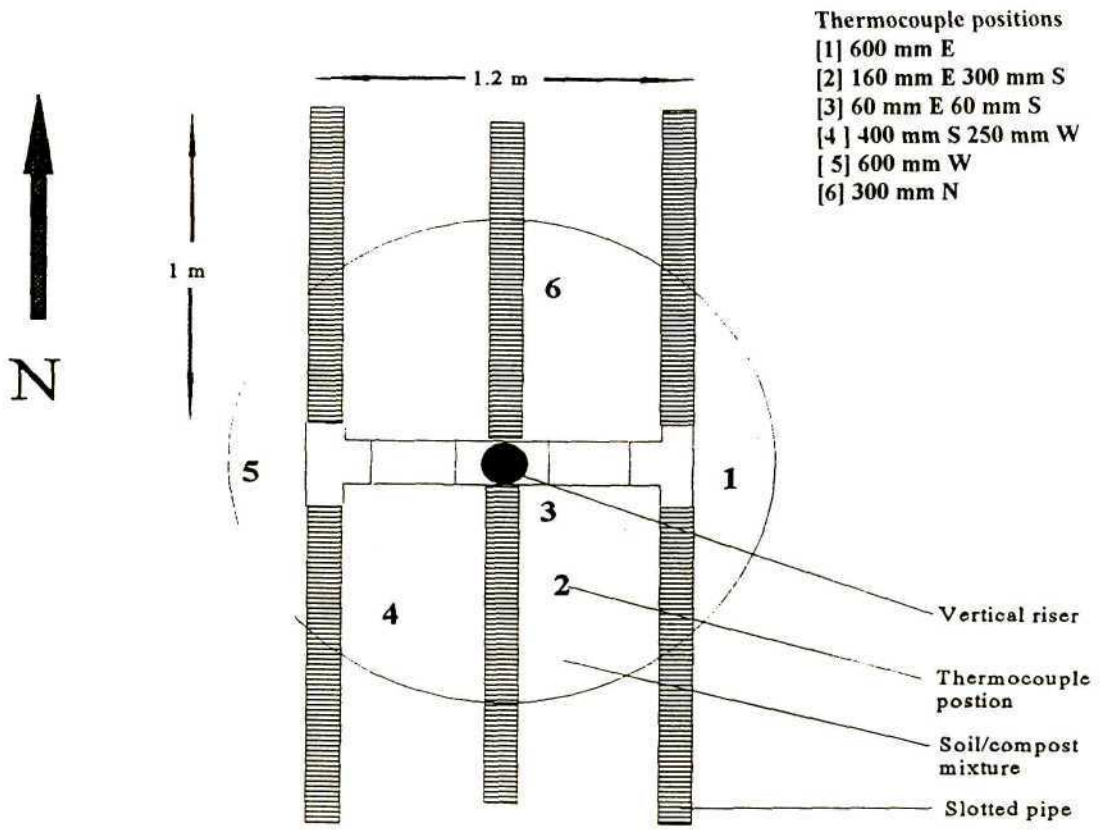

Fig 5.1a: Schematic diagram of the lower ventilation pipe array (300 mm level) with thermocouple positions indicated.

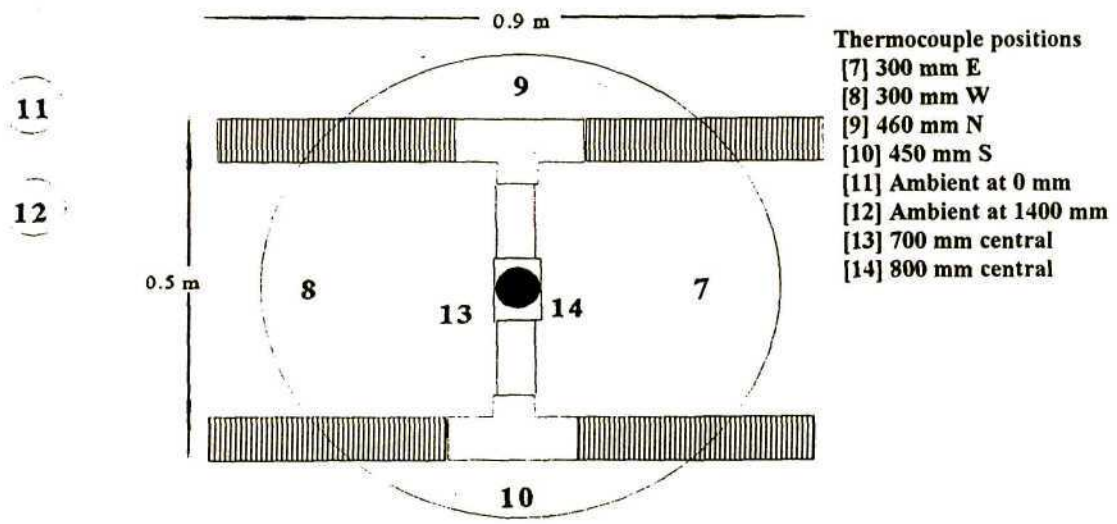

Fig 5.1b: Schematic diagram of the upper ventilation pipe array (600 mm level) with thermocouple positions indicated.

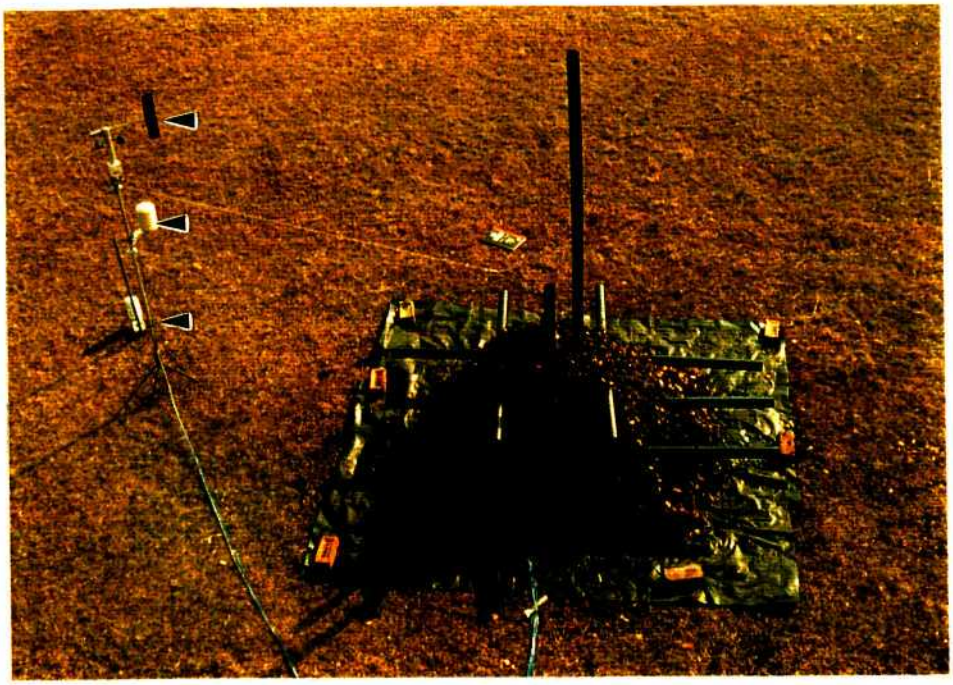

Plate 5a: Elevated view of biopile (East facing). The upper and lower ventilation pipe arrays can be seen. External environmental sensors are indicated with arrows (wind speed at 2 m, ambient temperature at 1.4 m above ground and temperature at ground level). All the blue cables were connected to the data logger.

Plate 5b: Side view of the complete biopile. The long vertical riser is connected to the lower pipe array while the short vertical riser is connected to the upper pipe array.

thermocouples, placed in radiation shields, were used to measure air temperature at 1.2 m and 300 mm above ground level. All the thermocouples were connected to a 21 Data logger (Campbell Scientific, Logan, Utaha) equipped with a data storage module (Model SM192, Campbell Scientific).

Single-ended temperature measurements were made with the internal data logger temperature used as the thermocouple reference. Measurements were made every second and averaged for each 20-minute period. All sensors were checked in the laboratory prior to use by calibration with a mercury-in-glass thermometer (accurate to 0.25°C) and a water bath, at a range of temperatures. Statistical analysis showed no differences between the thermocouples. Hence, all the thermocouple multipliers and offsets, necessary for calculating actual temperature, were the same. In addition to these temperature measurements, wind speed at a height of two metres was recorded. Twice a week, the temperature data in the data storage module were transferred to a personal computer and imported directly into a spreadsheet. Graphs for each day were created for each of the 300 and 600 mm levels and stored in a "slide show". The "slide show" enabled diurnal measurements for consecutive days to be compared on-screen by superimposition. Decisions on modifying the conditions in the biopile in an attempt to optimise the process were mainly based on these diurnal temperatures.

[5.7.4] Moisture Control

Great difficulty was initially experienced in achieving an uniform moisture content in the soil due to the hydrophobic nature of the soil as a result of the contaminant hydrocarbons. A similar problem may be anticipated for a full-scale operation. Water applied to the soil surface ponded and, eventually, became run-off. The rate of water delivery from a garden hose used to spray water on the biopile was too high. This was overcome with the installation of a circular dripper line placed over the apex of the pile at 650 mm above ground level. With this system, the appropriate volume of water could be delivered to the biopile at a much lower rate thus facilitating infiltration.

[5.7.5] Soil Analyses

Soil samples for analysis of TPH [6.2.1], pH (Anon., 1983) and nitrogen content (Appendix I) were made by horizontally auguring into the centre of the biopile at 450 mm above ground level. These samples were taken every ten days and were stored at 5°C in sealed plastic vials until needed. Samples for TPH determinations were first air dried (25°C, 48 h) and then sieved (0.5 mm mesh) to remove organic material before analysis. This helped to reduce the dilution effects of the soil supplements. In addition to the indirect thermal measurement of microbial activity, fluorescein diacetate hydrolysis [6.3.4] was also used. Samples for the FDA analysis were collected every seven days by horizontally auguring into the centre of the pile to three depths (viz.: at the surface; 30 cm; and 80 cm) at 450 mm above ground level. These samples were analysed immediately. The soil moisture content was maintained between 30 and 50% (w/w). Adjustments to soil moisture were based on calculations of the moisture content of oven dried samples (55°C, 48h) and, after day 110, from a moisture probe's measurements.

[5.8] RESULTS AND DISCUSSION

To optimise the composting process, it was necessary to make alterations to the compost mixture and ventilation system during the trial. For the purposes of this discussion, each biopile modification will be referred to as a treatment. The initial biopile conditions were thus Treatment 1. Twenty-three days after initiating Treatment 1, the temperatures within the biopile had decreased to near ambient and it was decided to modify the biopile conditions (Treatment 2).

For Treatment 2, the biopile was dismantled and the manure content of the compost was increased to provide a manure:wood-chips ratio of 2:1. Thus, the total manure content of the soil/compost mixture was 35% (v/v). Fresh chicken litter (less than 30 days old) was used for this treatment. This manure had a pungent ammonia gas odour and evidence of thermophilic microbial activity was present as temperatures

within the manure were as high as 55°C.

Elevated temperatures were maintained for approximately 22 days. A third treatment was made 110 days after the initiation of Treatment 1. For Treatment 3, design modifications were made to the ventilation system. The long vertical riser was removed and a low-power blower (0.50 kW) was used to introduce air into the base of the pile. The blower ran continuously as its air delivery rate was insufficient to contribute to heat loss via ventilation but increased the oxygen concentration within the pile. All other conditions remained the same as in Treatment 2. At this stage, a moisture content probe (Theta probe ML1, Delta-T Devices, Cambridge, UK) was installed.

[5.8.1] Hydrocarbon Degradation

A final TPH concentration reduction of 68% was measured at the end of Treatment 3, i.e. 130 days after initiating the trial (Fig. 5.2). An initial rapid TPH decrease of 2251 mg kg^{-1} soil (43% reduction) was detected during the first 30 days of the trial. Thereafter, the rate of TPH reduction was gradual with a loss of 1360 mg kg^{-1} soil over the next 70 days.

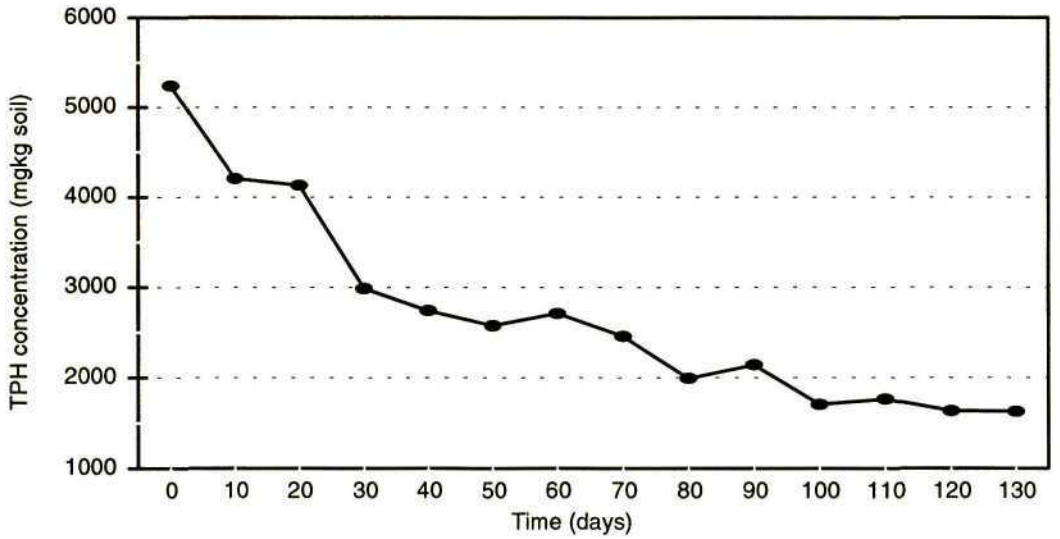

Fig. 5.2 : Residual TPH concentration in the biopile over 130 days.

The curve of TPH concentration reduction versus time (Fig. 5.2) was similar to those obtained in the Microcosm trials [4.5] and [4.6]. The rapid rate of TPH disappearance was attributed to both volatilisation of the light hydrocarbon fractions and sorption of pollutant molecules to the organic supplements. The addition of fresh organic material provided additional hydrophobic binding sites and, thus, disrupted the sorption equilibrium already established in the soil. However, sorption to the new sites would have been rapid (Kan *et al.*, 1994). Therefore the contribution of sorption to abiotic hydrocarbon losses should mostly have occurred in the early stages of the trial (i.e. the first 30 days). Sorption still occurred over the rest of the trial period but at a much lower rate. Conversely, after day 30, TPH reduction may have become rate-limited due to slow desorption.

The light hydrocarbon fractions, which are subject to volatilisation, are also readily degraded by microorganisms. This factor would have also contributed to the initial decrease in soil TPH concentration. At the start of the trial, the soil was highly aerated before soil compaction had begun to reduce the air-filled porosity. The parameters which control aeration and metabolic heat are mutually dependent. The fact that neither oxygen nor substrate were limiting in the initial stages resulted in high levels of metabolic heat. As a result, the highest average temperature (43°C) was recorded at the 300 mm level during the first 30 days of the trial. This heat facilitated the efficient functioning of passive aeration. Higher levels of oxygen in the system, in turn, resulted in higher levels of microbial activity which generated more metabolic heat.

Hydrocarbon disappearance in the biopile was further examined by GC analysis (Appendix D) of the residual hydrocarbon fractions (Soxhlet-extracted) from samples taken from the centre of the biopile. Figure 5.3a is a GC trace of an extract of a sample taken before bioremediation while Figure 5.3b was after 130 days treatment.

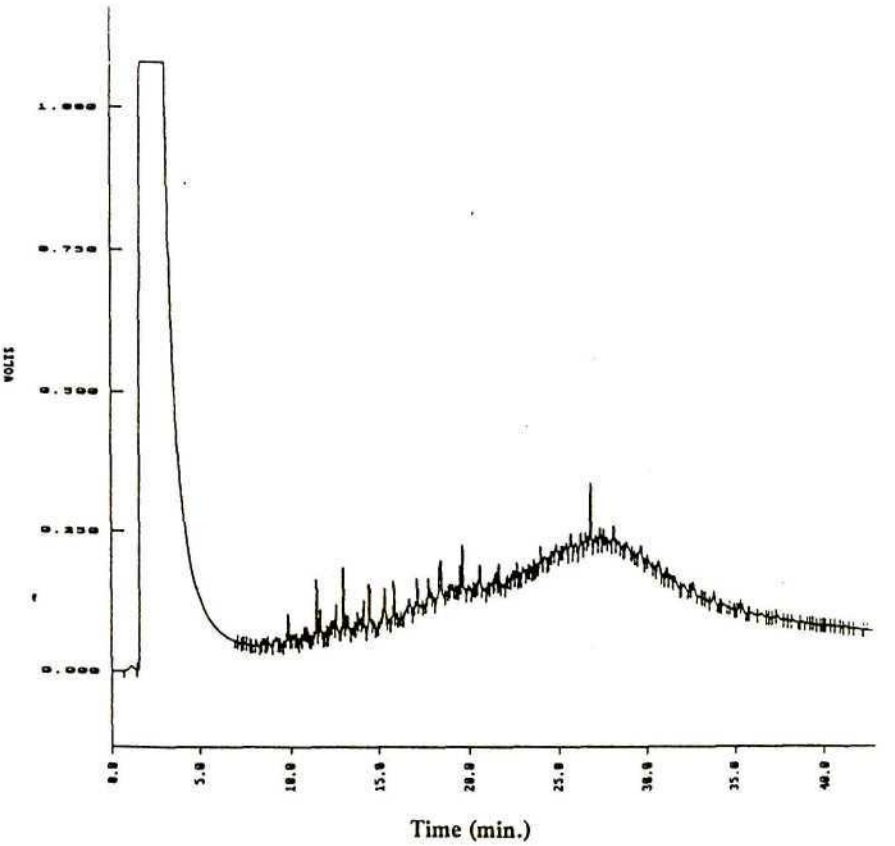

Fig. 5.3a: Gas chromatogram of residual Soxhlet-extracted hydrocarbons from a soil sample before initiation of bioremediation.

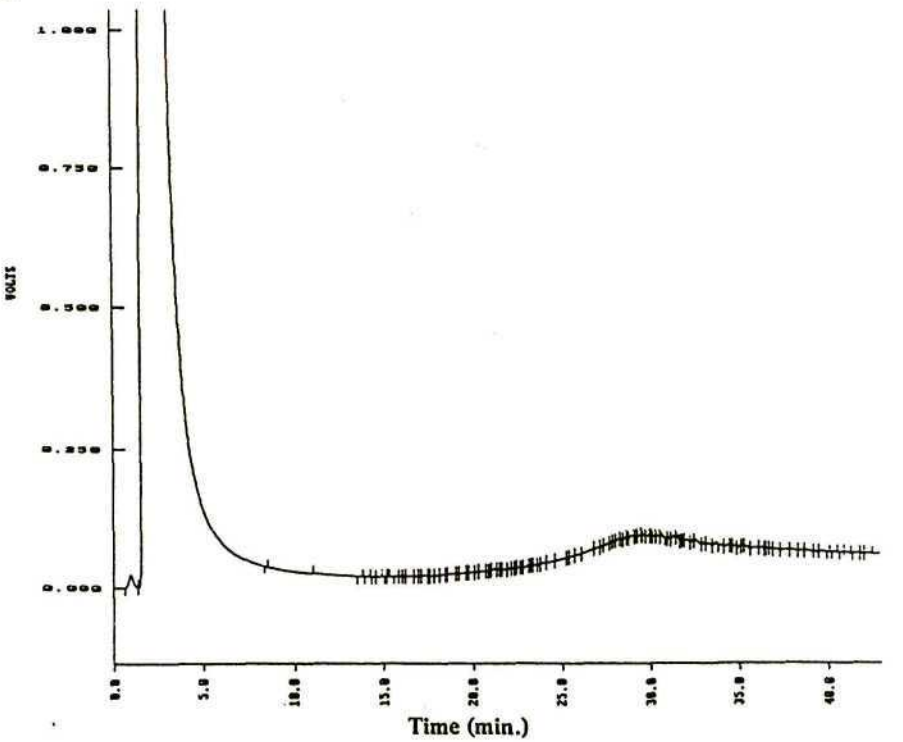

Fig. 5.3b: Gas chromatogram of residual Soxhlet-extracted hydrocarbons from a soil sample after 130 days of bioremediation

after 130 days of bioremediation

The effect of bioremediation on the residual contaminant hydrocarbons is clearly visible. The initial 190 peaks detected were reduced to 108, with all the major compounds either volatilised, catabolised or transformed to intermediates which were not resolved from the baseline. The near disappearance of all major peaks $> C_{22}$ during the relatively short bioremediation period tends to indicate that many of these compounds may have been sequestered into the humic component of the soil/compost rather than having undergone complete mineralization. These compounds were not extracted with carbontetrachloride from the humic complexes formed.

[5.8.2] The Thermal Environment

For the experimental site, the winds were usually southerly and the wind speed varied from calm conditions to 15 m s^{-1}. On average, the wind speed was less than 6 m s^{-1}. The air temperature varied between 4.5°C and 39°C during the 130-day trial period.

(i). Biopile Treatment 1

The temperatures rose rapidly in the biopile until a peak was reached after approximately six days. The average temperatures, at each level, for the three treatment periods are presented graphically in Figure 5.4. During the first treatment run, a maximum temperature of 43.9°C was recorded at thermocouple position 4 (see Figs. 5.1a and b for location of the thermocouples) on day seven. Comparable temperatures were recorded in a full-scale biopile with similar hydrocarbon contaminant concentrations and soil/compost ratios (Kamnikar, 1995).

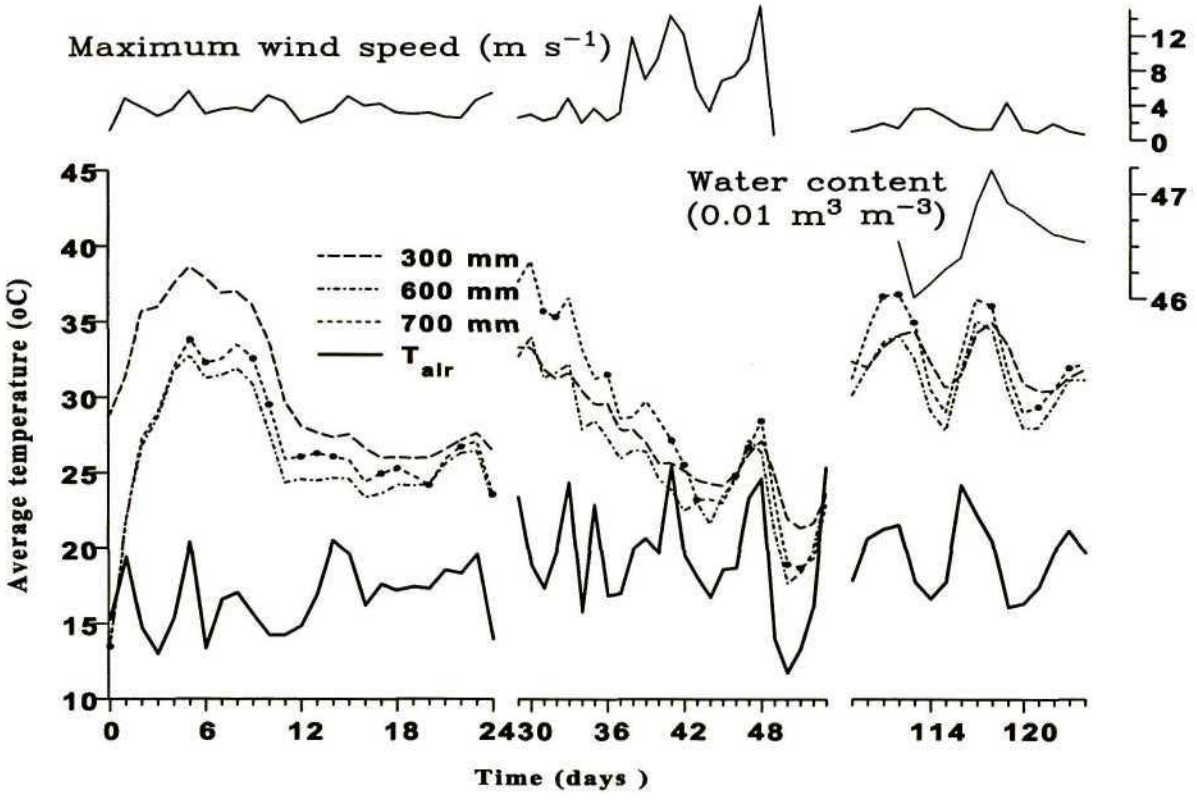

Fig. 5.4: Average temperatures at three levels in the biopile, average air temperature and average wind speed during the trial period. The curve for water content over the last 15 days (measured by the Theta probe) is also included.

The influence of the external temperatures on the temperatures within the biopile were investigated. The amplitude of the diurnal temperature variation was not the same at all positions in the biopile. The diurnal variations in temperature at five positions within the biopile and air temperature variations for 10 consecutive days (days 3 to 13) are shown in Figure 5.5. In particular, the smallest variation consistently occurred at thermocouple position 4 at the 300 mm level. The thermocouple positions which measured small diurnal variations also had elevated temperatures compared to the other locations at the same level. Elevated temperatures were also maintained for longer at these locations.

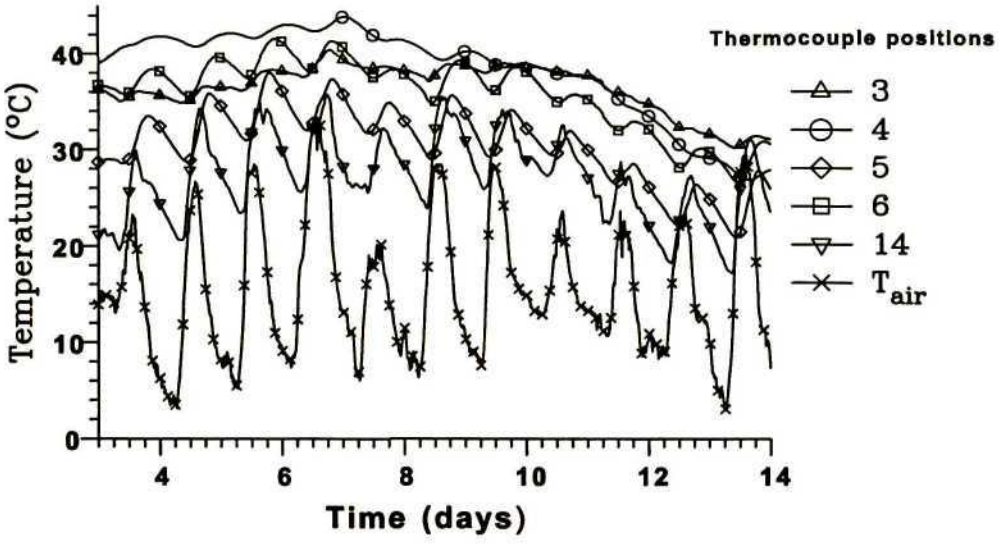

Fig. 5.5. Diurnal variation in the biopile (at 300 mm) and air temperature over 14 days (location of thermocouples shown in Fig. 5.1.a).

There was an increased diurnal variation at the 600 mm level (Fig.5.6) compared to the 300 mm level.

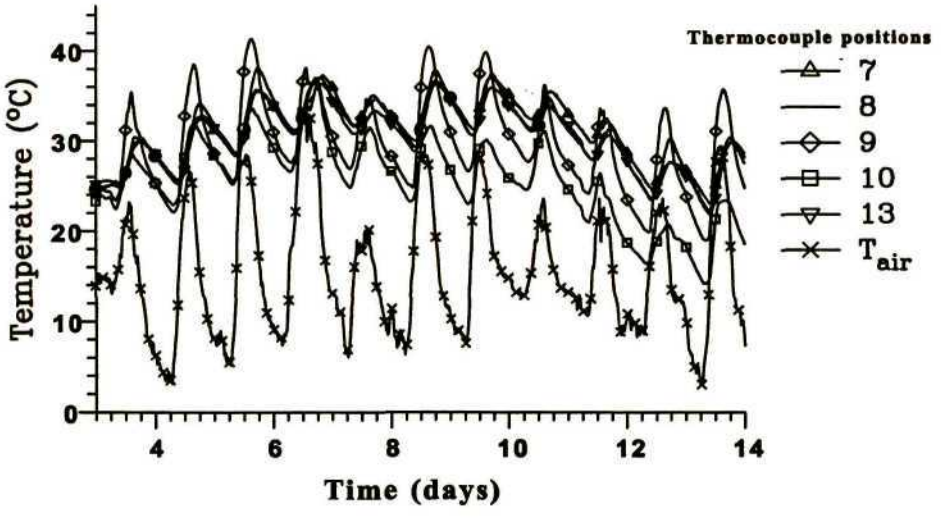

Fig. 5.6. Diurnal variation in the biopile (at 600 mm) and air temperature over 14 days (location of thermocouples shown in Fig. 5.1.b).

This variation, although less pronounced, was also apparent at positions located near to the outside of the biopile at the 300 mm level (viz. positions 1, 5 and 6).

These observations serve to illustrate the impacts which the external environmental conditions had on the biopile. Thermocouples located nearer to the central axis of the biopile recorded less diurnal variations due to the increased volume of insulative soil/compost mass around them. This insulative mass also served to reduce the loss of heat generated by the microorganisms in the compost matrix. This effect is also illustrated in Figure 5.7 where higher temperatures and smaller diurnal variations in temperature were recorded by a thermocouple at position 4, compared with other thermocouples at 300 mm.

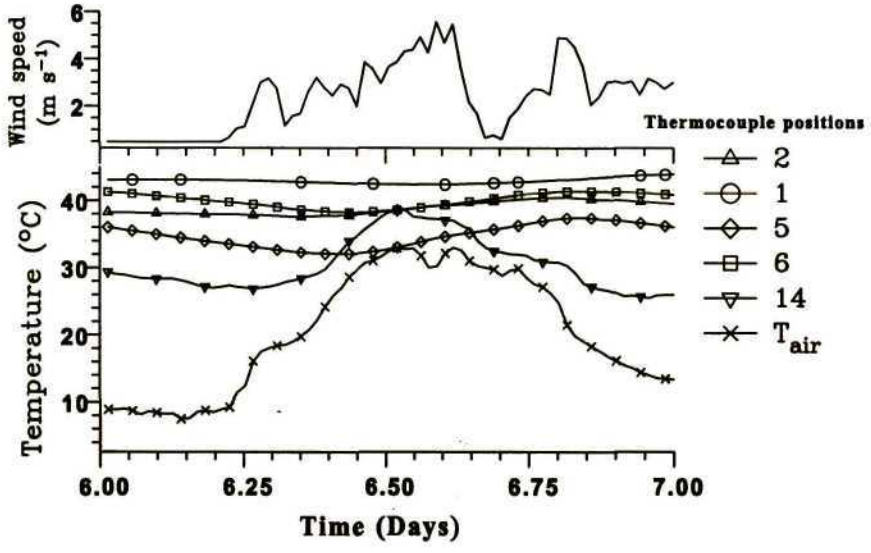

Fig. 5.7: Diurnal temperature variations at four positions in the biopile (at 300 mm), the air temperature and wind speed for 24 h (locations of thermocouples shown in Figs. 5.1a and b)

A summary of the temperature variations on day 6 is presented in Table 5.2.

Table 5.2: Diurnal variation of air temperature and two thermocouple positions (300 mm level) in the biopile for day 6*, Treatment 1.

Thermocouple position	Average temperature °C	Diurnal variation
Central [4]	42.8	1.7°C
Perimeter [1]	33.2	7°C
Air	20.5	25°C

*The highest wind speed recorded on this day was 5.53 m s^{-1}.

The rate of change in temperature, i.e. δ temperature/δ time at the 300, 600, 700 and 800 mm level above ground for days 3 to 5 (Fig. 5.8) showed that the greatest change was at the 800 mm level and the least at the 300 mm level. The minimum air temperature appeared to result in pronounced temperature changes at the 600 mm and 700 mm levels, particularly in the early- to mid-morning hours.

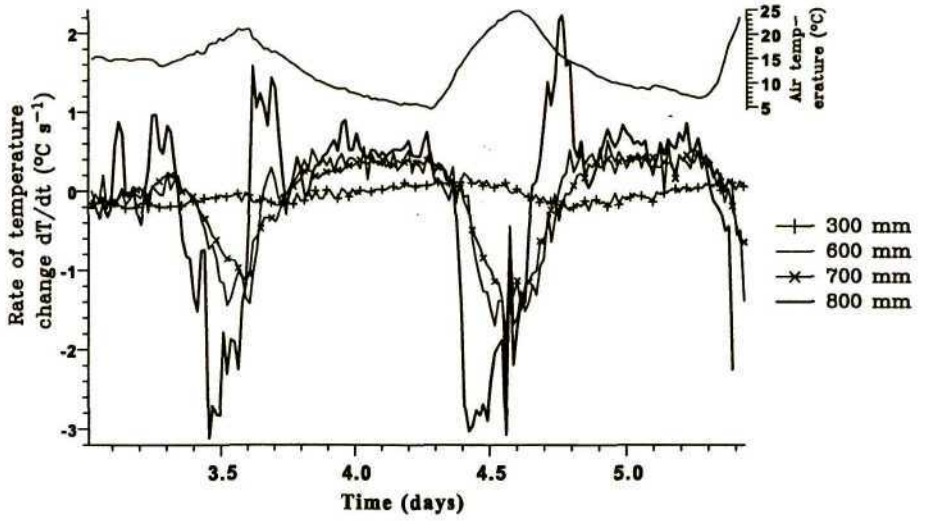

Fig.5.8: Rates of temperature change at four levels in the biopile over two days. The air temperatures for these days (top curve) show the magnitude of the rate of temperature change which was associated with the minimum air temperature. The 800 mm level, however, was also influenced by the maximum air temperature.

Temperature changes at 800 mm were greatly affected by the minimum and maximum air temperatures.

The spatial variation of temperature with time was further examined by three-dimensional (fishnet projection) graphic representations of local time and height above the ground. Temperature data triplet sets were generated with a bivariate 70 by 70 grid interpolation of the biopile temperature data for days 3 to 15 (Fig. 5.9). An inverse distribution method (weighting of 3) was used for mathematical grid interpolation. This projection showed that the temperatures at 300 mm were maintained compared to the other heights.

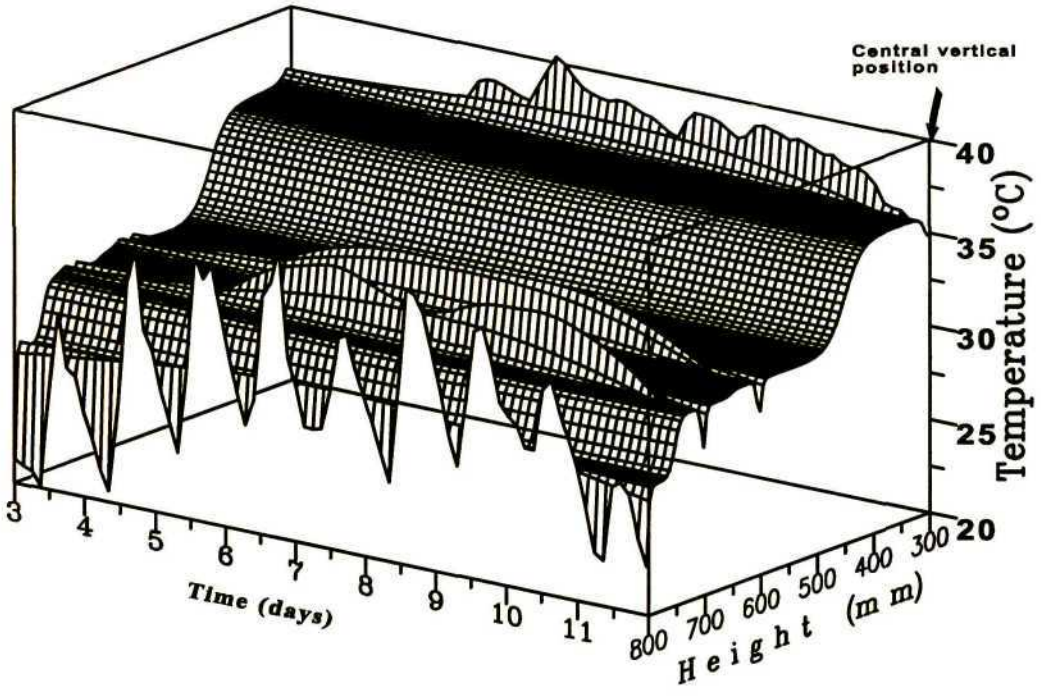

Fig. 5.9: Fishnet projection of the spatial and temporal variations in the biopile temperatures for days 3 to 12 at the central vertical position where height on the y axis refers to the vertical height measured from the ground surface. The projection was based on temperatures measures at thermocouple positions 3, 8, 13 and 14 (locations of thermocouples shown in Figs. 5.1a and b).

Given that the air temperature varied from one hour to the next, and differently on different days, the consistently high temperatures recorded at 300 mm were probably due to increased microbial activity. At the 800 mm height above ground, the influence of the air temperature variation was indicated by the increases in the compost temperature. This influence was also apparent at the 300 mm height after day seven.

A similar three-dimensional (fishnet projection) plot was made of local time and position at the 300 mm level measured as the distance eastward from the centre (Fig. 5.10). An inverse distribution method with a weighting factor of three was again used. This projection repeatedly showed that there were locations within the biopile at which the microbial activity was elevated. This increased activity was maintained for more than seven days.

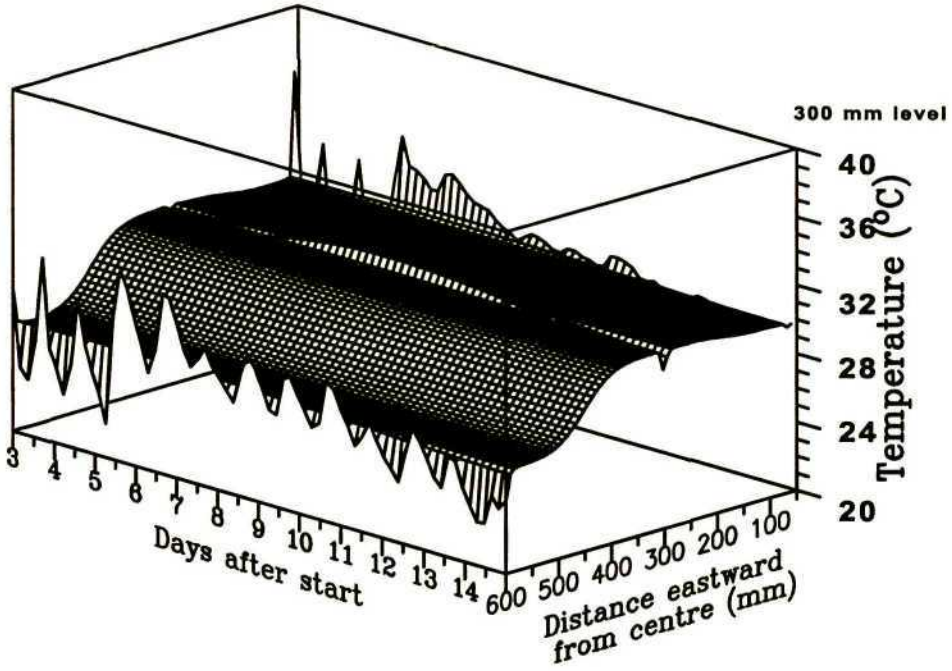

Fig. 5.10: Fishnet projection of the spatial and temporal variations in the biopile for days 3 to 15 at the 300 mm level where the y axis refers to the distance measured eastwards from the centre of the biopile. The projection was based on temperatures measured at positions 1, 2 and 3 (thermocouple locations are shown in Fig. 5.1a).

(ii). Biopile Treatment 2

Fresh manure, which had already begun to compost naturally, was used for this treatment. Thermophilic microbial activity in the manure resulted in initial rapid temperature increases in the biopile to as high as 53.6°C (position 8). These high temperatures, however, were not maintained, and there was a gradual decrease over the next 16 days (Fig. 5.4). Between days 45 and 49 there was a small increase in temperature within the biopile which coincided with elevated air temperatures and high wind speeds of 15.41 m s^{-1}. However, the negligible effect of wind speed was illustrated by data collected on days 42 and 43. On day 42, the wind speed reached a maximum of 14.32 m s^{-1}. This coincided with a 6°C increase in average air temperature although this had little effect on the temperatures at both the 300 and 600 mm levels (Fig. 5.4). By the end of the treatment period (day 50), all the temperatures recorded within the biopile were still higher than the air temperature. Average temperatures of 25.0±2.0°C and 21.2±1.2°C were recorded at the 300 mm and 600 mm levels, respectively, while the average air temperature was 13.9°C.

(iii). Biopile Treatment 3[2]

During the third treatment run, the temperatures recorded were lower than those in the previous two treatments and, as a result, the temperatures within the biopile tended to show greater diurnal variations (Fig 5.4). There are a number of possible explanations for these lower average temperatures. Firstly, the pile was not opened in order to make changes to its configuration, as was done at the start of Treatment 2. Thus, the compost which had undergone compaction was not re-aerated. Secondly, a large proportion of the labile carbon in the compostable substrate was presumably already depleted. It was suspected that the air pressure output of the blower was insufficient to aerate the pile. However, the forced aeration used during Treatment 3 appeared to have a dramatic effect on the average temperature trends at each level. Compared with the preceding treatment periods, the

[2] Note, due to electrical failure, the data storage module only collected readings for 15 days during Treatment 3.

temperatures did not undergo the same declines toward ambient temperatures and remained elevated although they were strongly influenced by the environmental temperatures (Fig 5.4). These observations seemed to indicate that oxygen was limiting in the preceding treatment periods.

The possible influence of windspeed and air temperature on temperatures within the biopile were investigated. Graphic representations of data collected on day 6 (Fig. 5.7) and days 34 to 37 (Fig. 5.11, i.e. days with elevated wind speeds) are discussed here. The influences of the wind speed and the air temperature were minimal at the central position 3 (300 mm level). The influences were, however, more pronounced near to the surface of the pile while the highest temperatures were recorded at position 6 (Fig. 5.7). These results again demonstrated that the higher the temperature in the biopile, the less significant was the influence of the external environment.

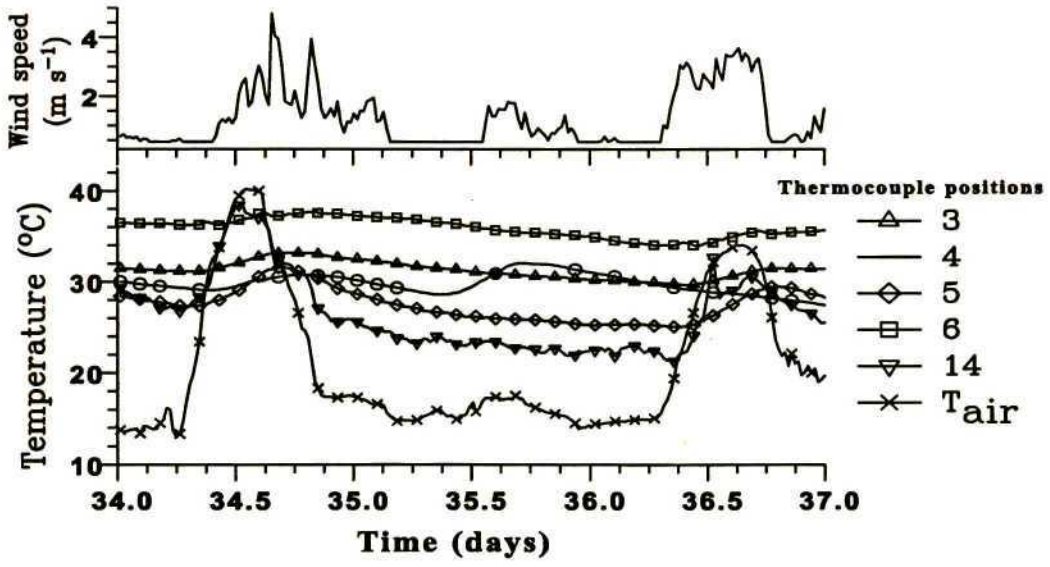

Fig. 5.11: Diurnal temperature variations at five positions in the biopile for three consecutive days of which day 34 was the hottest of the study. At this point, the biopile temperature was the highest at position 6, whereas for days 6/7 (Fig. 5.7) the highest temperature was at position 4 (thermocouple locations indicated in Figs. 5.1a and b).

Given that the sun was on the north side of the biopile, the highest temperature should have been recorded on the north side at the 800 mm level. The fact that this was not the case for days 3 to 15 (Fig. 5.6) was an indication that in the initial stages of the experiment, the elevated temperatures and spatial variations of temperature reflected spatial differences in microbial activity in the biopile.

[5.8.3] Microbial Activity (FDA)

For all three treatments, microbial activity (Fig. 5.12) at the surface and at a depth of 20 cm was highly variable over the 130 days of the trial. This was largely attributed to the alternating wetting and drying occurring on the surface. Microbial activity at a depth of 80 cm in the biopile appeared to be less variable and a more gradual increase was measured until day 60, whereafter the activity began to decrease. Increases in microbial activity were recorded at the initiation of each treatment. Overall, higher levels of microbial activity were measured in the biopile than were measured in any of the microcosm trials (Chapter Four).

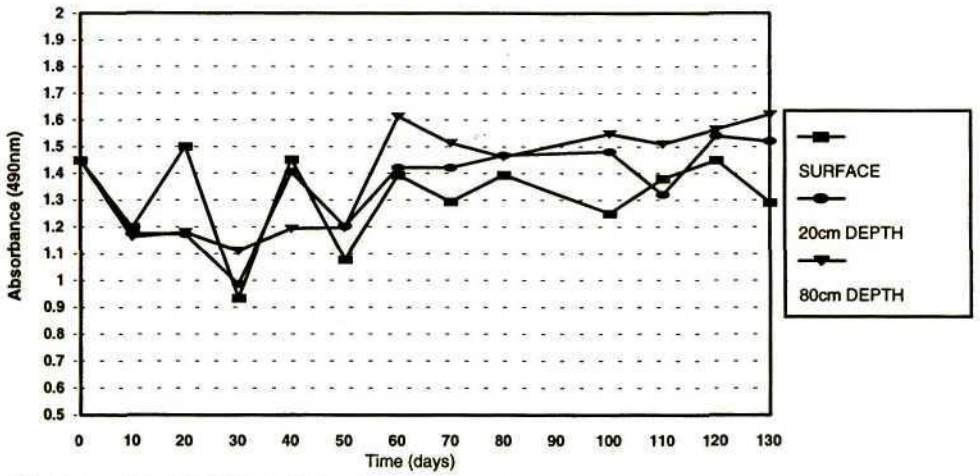

Fig. 5.12: Microbial activity (FDA) in the biopile, at three horizontal sample depths, over 130 days.

The highest microbial activities (Fig. 5.12) were recorded during Treatment 3 at the central region of the pile (80 cm horizontal depth, 450 mm level). This implies that, although

thermophilic conditions were not present, a high degree of microbial activity was still achieved with mesophilic microorganisms.

[5.8.4] Soil pH

There was a gradual change in pH within the biopile from the initial pH of 4.2 to 7.4 by day 75. Thereafter, only a small pH drop (0.8 pH units) was recorded for the remainder of the trial (Fig. 5.13). This pH range was well within the range recommended for composting by other researchers (Hunter *et al.*, 1981; Kubota and Nakasaki, 1991). It was, however, expected that the addition of ammonium ions to the soil (in the chicken manure) would have had a greater influence in increasing the pH. The fact that this was not observed was probably due to a combination of the initial low soil pH and the anaerobic formation of organic acids from the degradation of both the compost and the contaminant hydrocarbons.

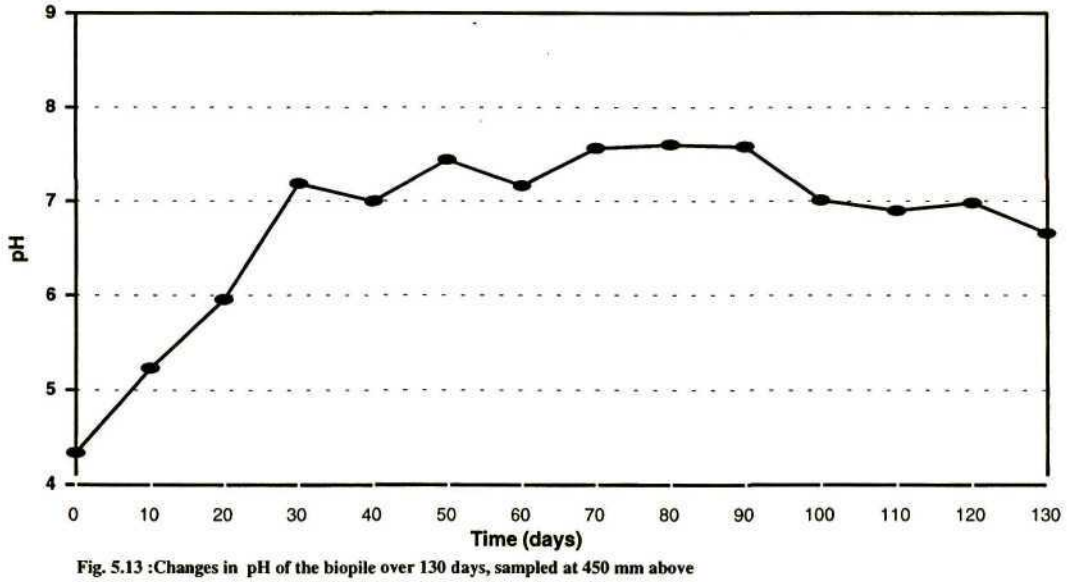

Fig. 5.13 :Changes in pH of the biopile over 130 days, sampled at 450 mm above ground.

[5.8.5] Soil Nitrogen Content.

The changes in the carbon to nitrogen ratio, as a result of manure additions, were calculated from soil nitrogen content analyses (Appendix I). The initial soil C:N ratio (before the addition of manure) was ± 1300:1 but this decreased to ± 320:1 after the manure additions at the start of Treatment 2. The ratio then increased to ±450:1 by the end of Treatment 3. Although these ratios were substantially higher than the optimal C:N ratio of 35:1 which is recommended for composting (Anderson, 1991), they were realistic for co-composting , in terms of the amount of manure added to the contaminated soil.

[5.9] GENERAL DISCUSSION

Temperature is a definitive parameter in the composting process. Although elevated temperatures were measured in the biopile, thermophilic conditions, which largely define composting, were not. This could be accounted for by, firstly, the high percentage of soil used in the compost mixture may have resulted in sub-optimal nutrient balances in the biopile. There was probably insufficient labile carbon to support thermophilic microbial activity. The C:N ratio was higher than the optimal ratio recommended for composting. Secondly, the thermal data suggested that the biopile volume was too small and resulted in excessive metabolic heat losses. The relatively small size of the compost heap did not provide adequate insulation to retain microbial metabolic heat which is needed to facilitate the natural succession of microorganisms through to the thermophilic stage. This heat loss had the additional impact of preventing the effective functioning of the passive ventilation system. The small biopile size also resulted in external environmental temperatures exerting a significant influence on the temperatures within the biopile (especially at the 600 mm level).

These observations, however, were not unexpected and a major objective of this pilot study was to determine whether the central region of the biopile was indicative of the conditions within a full-scale biopile. The data obtained here supported the assumption that a central core of approximately 500 cm^3 provided such a zone. The advantages of the small biopile in a research context were that it was a rapid and inexpensive means of obtaining

data on the suitability of a particular substrate for use in co-composting. As it is easy to construct a biopile with manual labour, a number of piles may be set up in a small area. This provides convenient access and costly monitoring equipment may be protected from theft and possible damage. For future studies, however, it is recommended that the total volume should be increased to at least 3 m^3. A layer of insulative material, such as straw, should be placed between the biopile surface and the covering plastic. Straw would be an ideal material as it is inexpensive and provides good insulation but still facilitates gaseous exchange.

While attainment of thermophilic conditions in PTB is desirable, there are some distinct advantages of "mesophilic composting". With a mesophilic temperature ceiling of 37°C, a much greater community diversity is anticipated which suggests that decomposition of a wider range of industrial chemicals is possible (Williams and Myler, 1990). High levels of mesophilic microbial activity were recorded throughout the trial period (Fig. 5.12). Due to the high soil content of a bioremediation biopile, it is unlikely that true thermophilic conditions are achievable. Accepting this factor, it can be assumed that oxygen is the rate-limiting factor. If forced aeration is to be used effectively in such a biopile, a blower actuated by soil gas oxygen concentration, rather than temperature, is appropriate. Thus, blowers are restarted when the soil gas concentration approaches 2% (v/v). Intermittent blower operation also optimises biopile remediation with respect to energy utilisation and system maintenance costs (McGinnis *et al.*, 1994).

The composition of the compost mixture often requires optimisation within the constraints of what is locally available and is economically feasible. Criteria for selecting operating conditions are driven by the overall economics of the treatment process. This is not necessarily a straightforward evaluation. For example, a higher cost supplement may prove more economical to use than a less effective, but cheaper, alternative since soil throughput may be higher with the former. A similar situation exists with the soil percentage. Contaminants may be transformed at higher rates with lower soil percentages but the system throughput may be greater with higher soil percentages and acceptance of lower rates of transformation. It is, however, advantageous to minimise the amount of material to

be handled and disposed. A cost analysis (Lowe *et al.*, 1989) indicated that soil volume plays a greater role in controlling the overall economics of bioremediation by composting than the transformation kinetics. There are two concerns in selecting an effective compost/soil mixing ratio that are in conflict with one another. There should be sufficient manure present to facilitate the spontaneous self-heating process. While one would wish to maximise this addition, the volume must be minimised to limit the pile volume. Large piles require larger areas of land which are often not readily available for on-site operations. In addition, the operating costs rise dramatically when large volumes of manure have to be transported to the treatment site.

A substrate worthy of examination for use in PTB is municipal sewage sludge (MSS). Much research has been conducted on the composting of MSS for use in co-composting of hazardous compounds as: it has an intrinsically highly active microbial population; its supply of labile carbon and other nutrients (especially nitrogen) can sustain a high level of microbial activity; and its high buffering capacity could reduce the detrimental effects of highly acidic or alkaline pollutants (Finstein *et al.*, 1986). Mixing MSS with a hazardous waste will also decrease the effective concentrations of toxic constituents through dilution and sorption mechanisms.

There is a possibility that observed decreases in the TPH concentrations in contaminated soils supplemented with composts are increased by sorption onto the organic matter of the compost. It is well known that sorption of organic compounds increases with the organic content of soil (Means *et al.*, 1980; Hassett and Bandward, 1989). Therefore, adsorption effects have to be expected with composts. Biodegradation might be wrongly inferred from the data obtained from organic solvent extraction and subsequent analysis (Hsu and Bartha, 1976; Eschenbach *et al.*, 1994). To distinguish between these processes, Kastner and Mahro (1996) applied a humic acid extraction, by alkaline hydrolysis, to compost-amended PAH-contaminated soil, prior to extraction with organic solvents. They found that significant decreases in PAH concentrations over a 100-day trial period were not due to sorption. These results contradicted the findings of Ryan *et al.* (1991) whose study of

diesel-contaminated soil identified a gap in the carbon balance of the diesel degradation which indicated that some sorption of the contaminant had occurred. These findings tend to indicate that the extent of sorption varies according to the soil and/or compost type. The type of compost also affects the amount of humic compounds which are able to bind with soil particles.

The extent of sorption was not examined in this study although it was expected that it would be relatively low due to the highly weathered physical state of the contaminant which was already strongly adsorbed to the soil matrix. It is possible for a pollutant to become irreversibly bound, without chemically reacting with the soil matrix, if some physical alteration takes place. Such alterations include configuration changes to the organic matter as a consequence of changes in ionic strength, pH, relative humidity or coagulation of mineral/organic soil particles (Kan *et al.*, 1994). A number of these changes may have occurred in the study soil. These include changes in ionic strength and pH as a result of the chicken manure additions, and an increase in the relative humidity under composting conditions. Sorbed compounds are suggested to have greatly reduced bioavailabilities and, thus, the toxicological threat they pose to the environment is significantly reduced (Berry and Boyd, 1985). However, the long-term stability of these complexes and their final fates are unknown.

It is felt that there may be some advantages to adsorption of the pollutant onto the bulking agent. The wood-chips could behave as a "sponge" for the pollutant while providing moisture, nutrients and a support matrix for organisms such as white-rot fungi, thus facilitating co-metabolic degradation of the adsorbed pollutants. The possible merits of such bound residue formation as a no-migration pollutant remediation option was discussed in [5.5]. The final fate of the contaminant in the wood-chips should, however, be examined. This is also true for the entire compost for which the final toxicity should be determined.

The cost of a full-scale PTB biopile has been estimated in Table 5.3.

Table 5.3: Cost estimates for the passive thermal bioventing of 50 tons of contaminated soil (from the study site) over a maximum treatment period of five months. The biopile dimensions: 2.5 m (high); 4 m (wide); and 15 m (long).

Item	Cost
Black PVC pipes(125 mm diameter): 150 m @ R18 m^{-1}	R2700
Pipe joiners: 22 @ R86 each	R1892
Irrigation dripper lines: 50m @ R10 m^{-1}	R500
Black polyethylene sheeting: 1.5 m x 30 m @ R1.33 m^{-1}	R40*
Front-end loader hire (JCB): 10 hours @ R105 h^{-1}	R1050*
Hydrocarbon degradation analysis: 25 TPH analyses @ R100 per sample and 10 GC analyses @ R250 per sample	R5000*
Road transport: 6 tons manure and 3 tons woodchips, 50 km @ R0.35 km^{-1}	R165*
Manual labor: 3 persons @ R75 day	R225*
Temperature probes: 3 probes with a hand-held volt meter	R300
Treatment of the first 50 tons contaminated soil	Approximately R230 ton^{-1}
Treatment of the next 50 tons* contaminated soil	Approximately R140 ton^{-1}

Note, the second treatment cost given in Table 5.3 (R140), is based only on the figures marked with asterisks as these costs will be incurred with each treatment run, whereas all other equipment is reusable. The overall cost will vary with differing soil types, contaminant concentrations and molecules. Changes in these factors affect the amount of organic additions needed and, possibly, the overall treatment time.

While there are few comparable figures available for the cost of soil bioremediation in South Africa, estimates from a recent Water Research Commission report (Pearce *et al.*,

1995) indicate that the cost of landfarming petroleum hydrocarbon contaminated soil is between R250 and R350 ton^{-1}. Once optimised, PTB offers an organo-pollutant treatment process which costs up to 50% less than accepted treatment methods and has many additional advantages. The technology is simple and requires little maintenance. The system does not require sophisticated mechanical aeration mechanisms or a power supply. Such equipment is expensive and is prone to theft and vandalism, a problem commonly encountered in other bioremediation operations conducted in this country (H.G. Snyman, personal communication). Thus, PTB biopiles may be established in remote areas and are relatively self-contained. No operator is required, other than someone to periodically take temperature measurements and soil samples for water and TPH analyses. The biopiles can be constructed with non-specialised machinery such as bulldozers and front-end loaders; equipment which most civil engineering companies or transport companies own. The ventilation pipes used are standard drainage pipes. Once purchased, the pipes may be used repeatedly as their configurations may be easily adapted to suit any design modification or pile size.

CHAPTER SIX

[6] ESTIMATION OF BIOREMEDIATION EFFICACY

[6.1] INTRODUCTION

Throughout the course of this study, two important parameters were used extensively to evaluate the efficacy of the various bioremediation treatments, namely, the total petroleum hydrocarbon (TPH) concentration in soil samples and soil microbial activity. Selection of an appropriate analytical method should be based on a number of criteria such as: accuracy; reproducibility; sample preparation/analysis time; and cost (in terms of both capital equipment, human resources and consumables). This chapter discusses some of the merits and problems associated with current methods and describes the methods used in this research programme.

[6.2] ANALYSIS OF HYDROCARBON BIODEGRADATION

Analyses are required to evaluate the extent of contamination and the progress or efficacy of remedial treatments. These analyses range from simple assessment to confirm the presence or absence of specific molecules to determinations of concentrations of contaminants. The least specific and most general analytical approach involves some form of total petroleum hydrocarbon measurement. This is in contrast with analysis which focuses on selected target compounds.

Regardless of production mode, most petroleum hydrocarbon products are exceptionally complex materials with a wide range of physical and chemical properties. Petroleum, diesel or related fuel products may contain over a thousand individual constituents with boiling point distributions in the order of hundreds of degrees Celsius. Several chemical classes are usually represented, including paraffins, olefins, aromatics, heteroaromatics and hydrocarbons which

contain oxygen, nitrogen and sulphur (Riser-Roberts, 1992). Another significant characteristic of the products is that their compositions are highly variable. This is primarily in terms of the relative amounts of the various hydrocarbons which they contain. The relative compositions of these products may change dramatically once they are released into the environment. Processes such as volatilisation, dissolution and abiotic/biotic degradation are responsible for these changes. These factors, and others, make petroleum product residue analysis in soil a formidable analytical challenge. Analytical methods which are broad in scope are required and, where target compound analysis is involved, there is also a need for high degrees of analytical selectivity and specificity. Compounds must be detected in the presence of numerous potential interferences and, considering the toxicity of many petroleum constituents, high sensitivity is needed. Detection limits in the region of 1 - 10 mg l^{-1} must be routinely achieved. In the light of the complexity of petroleum products, analysis is often reduced to the measurement of indicator parameters. The simplest method for TPH determination is the EPA Method 503 which involves the gravimetric measurement of solvent-extractable constituents of the sample (Block *et al.*, 1990). A modification of this method includes a silica "clean-up" step to remove polar-solvent extractable components which are naturally present in most soils. This method is useful for measuring gross contamination by relatively high boiling point hydrocarbon fractions. There may, however, be some loss of low boiling point components, which volatilise during the solvent elimination step. This method has largely been replaced by infra-red (IR) spectrophotometry methods such as the EPA Method 418.1 for TPH content. Petroleum hydrocarbons with $-CH_2-$ groups in the compound, namely n-paraffins, exhibit strong absorbance at a wavelength of 2930 cm^{-1}. Total petroleum hydrocarbon concentration is calculated by comparing absorbance peak areas of samples with those generated by a standard hydrocarbon mixture containing a fixed ratio of aromatic and paraffinic hydrocarbons. This method also requires the silica "clean-up" step. Although EPA Method 418.1 has widespread regulatory acceptance, it does not provide the quantitative information often required for biological treatment process monitoring (Block *et al.*, 1990).

Capillary gas chromatography with flame ionisation detection (FID) has been found to be a successful alternative to the IR technique. Obtaining reliable quantitative information of petroleum contamination by either IR or GC(FID) techniques requires the selection of an analytical standard which is representative of the contaminant present in the sample. The hydrocarbon calibration mixture used for IR is usually standard and consists of a 3:2:2 (v/v) mixture of *n*-hexadecane, *iso*-octane and chlorobenzene. However, with the high resolution of complex hydrocarbon products offered by GC(FID), it is often a difficult task to provide a suitable standard.

[6.2.1] Total Recoverable Petroleum Hydrocarbons: Materials and Methods

In the current study, quantification of the total recoverable petroleum hydrocarbons was made by a modification of EPA Method 418.1 (U.S. EPA, 1982):

(1). 2 g contaminated soil and 2 g anhydrous sodium sulphate (Na_2SO_4) were placed in a 30 ml amber glass vial;

(2). Carbon tetrachloride (10 ml) was added before sealing with a teflon-lined screw cap. The vial was then placed on a vortex mixer for 15 seconds;

(3). The vial was then placed into a sonicating bath (Whaledent Biosonic) for 30 minutes. After 15 minutes, the vial contents were remixed (vortex) and placed back in the sonicator for the remaining 15 minutes;

(4). The solvent was transferred to a clean dry vial containing 1.0 g activated Florosil™ (Sigma) and 6% water (w/w). The sealed vial was shaken for one minute and then allowed to stand overnight at ambient temperature. This is the silica "clean-up" step to remove interfering humic materials.

(5). The extract was finally filtered through a Whatman GF/C glass fibre filter. The filtrate was diluted to 10 ml in a volumetric flask; and

(6). The absorbance was determined with a Fourier Transform Infra-red Spectrophotometer (Nicolet DX V5.17, KBr cell, 10 mm path length). The sample was scanned at wavelengths between 2760 and 3070 cm^{-1} and an integration value for the absorbance peak area was generated.

(i). Calibration mixture

The reference hydrocarbon mixture was made up as follows:

(1).15.0 ml n-hexadecane, 15.0 ml *iso*-octane and 10 ml chlorobenzene were pipetted into a 15 ml glass-stoppered bottle. Stock standards were made from this by pipetting 1.0 ml into a 200 ml volumetric flask. This was diluted to volume with carbon tetrachloride. A series of five working standards (viz.:10; 100; 250; 500; and 1000 ppm) was made from the stock standard;

(2). A calibration curve was derived by determining the absorbance of each standard at 2930 cm^{-1}. A calibration plot of absorbance versus mg petroleum hydrocarbons 100 ml^{-1} solution was generated with Apex™ chromatography software; and

(3). The concentration of petroleum hydrocarbons in each extract was determined by comparing the response with the calibration plot.

(ii). Calculations

A linear equation ($y = 1.192\ x + 1.363$) was generated by the Apex™ software.

Standards used for the calibration curve gave a high correlation coefficient of 0.998. The equation was rewritten as:

$x = (y - 1.363/1.192)50$

Where: x = hydrocarbon concentration of sample (kg^{-1} soil)

y = peak area

The actual hydrocarbon concentration in mg kg^{-1} soil was then calculated by multiplying x by a factor of 50 which compensated for the cell pathlength, sample size and dilution factor. This formula was programmed into a spreadsheet which then automatically calculated TPH concentrations in mg kg^{-1} from the infra-red absorbance values as they were entered.

[6.2.3] Gas Chromatography of Soil Extractions: Materials and Methods

Capillary GC(FID) analysis was made on the residual hydrocarbons from a number of Soxhlet-extracted (Appendix C) soil samples. The samples were taken at the beginning and end of the biopile experiment (5.7).

The GC (Varian 3600) conditions were as follows (Anon, 1983):

Split injector at 240°C ;

FID detector at 300°C;

Carrier gas, helium, at 30 ml min^{-1};

Oven conditions: 50°C to 320°C at a ramp rate of 10°C min^{-1} which was held at 320°C for 15 min, total run time of 43 minutes;

Column: J&W DB5, length 30 m, i.d. 0.326 mm, and film thickness of 1.0.μm.

Sample injection volume: 200μl

A solvent blank was run after every two samples to ensure "ghost" peaks from preceding injections were not causing any interference.

A hydrocarbon mixture of the following carbon-chain lengths and concentrations (mg l^{-1}) were used as a qualitative standard:
C_{18}, 2.6; C_{22}, 2.5; C_{23}, 2.5; C_{25}, 2.2, C_{28}; 2.1; C_{34}, 2.6; and C_{38}, 3.1.

[6.2.4] Results and Discussion

In the current study, it was only necessary to observe hydrocarbon biodegradation trends in response to the various soil treatments. The final fates of specific hydrocarbon molecules in the soil were not determined. Thus, the TPH method was used for routine hydrocarbon concentration determinations (Chapters Four and Five). A major advantage of the TPH approach is that instrumentation costs are modest and extensive training in analysis, which would be costly, are not required. Excellent reproducibility can also be obtained. There are, however, some important limitations. Measurement accuracy may vary widely, depending on the products involved and the extent of the molecules post-release which may have occurred. Volatile compounds may be lost in the solvent concentration step and a low recovery of heavy distillates, which may be poorly soluble in the solvent (typically trichlorotrifluoromethane), may be responsible for low accuracy. In addition, the detector response is only obtained for those compounds which have a $-CH_2-$ group. As a consequence, the method has a poor sensitivity for aromatic hydrocarbons. The hydrocarbon mixture used for calibration has a constant composition whereas the relative compositions of petroleum products and their residues are highly variable and may introduce some uncertainty into the measurements.

At the opposite extreme of indicator parameter monitoring are the direct measurements of specific components in contaminated soil and water. The most widely used of these are U.S. EPA 500, 600 and 8000 (Potter, 1989). A common feature of the methods is that they often utilise GC with packed columns. The use of GC-mass spectrometry (GC/MS) is emphasised in these methods as this instrument is well known for its superior sensitivity and ability to

specifically detect organic compounds. Two sets of methods (610 and 8100, 8015) use FID. This detector gives a nearly universal response to hydrocarbons (Tong and Karasek, 1984) but offers no selectivity, and identification is based on chromatographic separation. The heavier fractions in the hydrocarbon mixture elute very slowly from the GC column. As a result, post analysis conditioning, which can substantially increase the analysis time, is usually required. When high resolution capillary columns are used, "humps", which represent envelopes of unresolved compounds, are observed. This poor resolution is due to the large number of compounds that cannot be resolved on a single column and so identification of target compounds in these regions of the trace is difficult. This phenomenon was observed with GC(FID) analysis of soil samples in the current study (evident in the chromatograms shown in Figs. 5.3a and 5.3b). Reproducibility of the results was poor when samples were injected with an autosampler. This factor, together with the long sample run time of 43 min (including solvent blanks), made routine use of GC as an analytical tool impractical for the study. Thus, only selected samples were examined by this method.

Quantitative results may vary widely due to poor chromatographic separation and relatively low hydrocarbon recoveries in solvent extracts. This leads to the conclusion that fundamental changes in the TPH analytical approach are required. Solvents or mixtures of solvents which more effectively extract high molecular weight aromatic compounds need to be identified. Chemical group separations, into more homogenous groups by liquid chromatography, are needed prior to GC analysis. These group-type separations are, however, time consuming and labour intensive. One possible solution for analysis of contaminated soils is the use of flash thermal desorption (FTD). With FTD, solid samples are heated very rapidly which results in the desorption of the hydrocarbons into the inlet of a GC FID. Flash thermal desorption has been shown to be applicable for the analysis of a wide range of hydrocarbons (up to C_{30}) in solid samples (Crist *et al.*, 1985). A distinct advantage of FTD is the ease of sample preparation.

[6.3] MEASUREMENT OF MICROBIAL ACTIVITY

During this study, it was necessary to have a means of monitoring microbial activity in the contaminated soil. In this way, the response of soil microorganisms to the various treatments could be evaluated. It was also possible to determine if correlations existed between microbial activity data and the other parameters measured. As the emphasis of this study was on the fungal component of the indigenous microflora, a method had to be found for the selective estimation of fungal population densities *in situ*. Determination of fungal biomass within solid substrates is an intrinsic problem in fields of biotechnology as diverse as ecology, plant pathology and food science. Fungi in solid matrixes, such as food and soil, are often quantified by counting colony-forming units (CFU) although it is generally recognised that this method provides a poor estimation of fungal biomass (Pitt, 1984). Hence, there was a need for a method whereby the fungal biomass present in the soil could be quantified by means other than the number of colonies produced by propagules on a solid agar medium. Some of these problems have been highlighted in Chapters Two and Three of this thesis.

[6.3.1] Ergosterol Bioassay: Introduction

Methods exist whereby the growth of fungi can be indirectly monitored by the measurement of fungal-specific molecules which can be related to mycelial dry mass. In this regard, compounds such as glucosamine, chitin and ergosterol are commonly used. The ergosterol assay is generally considered to be the most superior (Steitz *et al.*, 1979; Matcham *et al.*, 1985; Nout *et al.*, 1987) especially when an estimation of live biomass is to be made (West *et al.*, 1987). Ergosterol (ergosta-5,7,22-trien-3β-ol) is the predominant sterol component of most fungi and is either absent or only a minor constituent in most higher plants. A rapid ergosterol assay has been developed for use with high-performance liquid chromatography (HPLC) and is more sensitive than the chitin assay for monitoring growth in solid substrate cultures (Matcham

et al., 1985). Martin *et al.* (1990) found that the method could detect fungal biomass in amounts as little as 2 mg (wet weight) while the lowest detection limit of chitin was 100 mg. Chitin analysis also necessitates more specialised equipment such as ion-exchange chromatography which requires an amino acid analyser. Colorimetric methods for chitin determinations require only standard equipment but involve many steps and are subject to more interferences (e.g. extraneous hexosamines) than chromatographic methods (Wu and Stahmann, 1975). West *et al.* (1987) compared the ergosterol, diaminopimelic acid (DAP) and glucosamine contents of soil as a means of monitoring changes in microbial populations in response to various soil treatments. They found that only ergosterol could be quantitatively related to actual biomass. Other than the work reported by West *et al.*(1987), ergosterol measurement in soil is rarely used.

The concentrations of both ergosterol and chitin in fungal mycelium have been found to vary with age, growth rate and developmental stage (Matcham *et al.*, 1985; Martin *et al.*, 1990). Ergosterol concentration was also found to be strongly influenced by the composition of the substrate and the degree of aeration (Nout *et al.*, 1987). Matcham *et al.* (1985) found the ergosterol content of *Agaricus bisporus* to be 2 - 2.7 $\mu g\ mg^{-1}$ dry weight (DW). Steitz *et al.* (1979) obtained similar estimates for fungi such as *Alternaria alternata* (3.8 - 4.4 $\mu g\ mg^{-1}$ DW) and *Aspergilius flavus* (2.3 - 3.3 $\mu g\ mg^{-1}$ DW). Nout *et al.* (1987) found that the ergosterol content of *Rhizopus oligosporus*, when grown in synthetic laboratory medium, varied with age from 0.2% (w/w) in fresh cultures to 2.4% (w/w) in older cultures. Although ergosterol concentration varies between fungal species, the variation within a species is also large. Thus, the method cannot be applied as a taxonomic parameter.

[6.3.2] Ergosterol Bioassy: Materials and Methods

The ergosterol bioassay method used in this study was a modification of the method described by Martin *et al.* (1990):

(1). 20 g of soil were placed in a 150 ml glass amber bottle to protect the ergosterol from UV oxidation;

(2). 20 ml of reagent grade methanol (10% distilled water) were added and the bottle was then shaken vigorously for 30 seconds. The bottle was placed into an ultra-sonication bath (Whaladent Biosonic), which contained iced water, for 10 minutes;

(3). The sonicated mixture was then filtered through Whatman GF/C paper into a 40 ml amber vial.

(4). The volume of the extract was reduced under a gentle stream of nitrogen to approximately 1 ml. The extract was then diluted to 2 ml and placed in an Eppendorf centrifuge tube and centrifuged (80 000 r.p.m.x g, 10 min);

(5). The extract was finally filtered through a disc filter (nitrocellulose, 0.2μm, Sartorius) to remove residual particulate matter; and

(6). Samples (200 μl) were injected into an HPLC (Waters) fitted with an autosampler (Microsep, M23). The UV detector (Waters 486, tunable absorbance detector) was set at 280 nm. A Nova-Pak C18 (3.9 x 150 mm) column was used. Methanol was used as the mobile phase (diluted 10% - 60% v/v with distilled water). The ergosterol standards (2 mg; 10 mg; 50 mg; and 100 mg, Sigma) were eluted after approximately 8 minutes.

For an improved ergosterol bioassay, Salmanowicz and Nyland (1988) recommended saponification (refluxing in KOH) of the extract which aids release of the ergosterol esters. In the current study, this step was omitted as it increased sample preparation time by five hours. Martin *et al.* (1990) also found that omission of this step allowed faster processing of samples

while no significant interference was recorded.

[6.3.3] Fluorescein Diacetate Bioassay: Introduction

Stubberfield and Shaw (1990) conducted a study to establish how favourably two simple microbial activity assay methods, fluorescein diacetate (3',6'-diacetylfluorescein [FDA]) hydrolysis and idophenyl nitrophenyl tetrazolium chloride (INT) reduction, compared to the well established method of ATP extraction. Adenosine triphosphate assays of samples correlated significantly with both the FDA ($r=0.98$) and INT assays ($r=0.99$). The FDA and INT assays also correlated directly with each other ($r=0.972$). In view of the observed correlations, the FDA and INT assays are preferable as they are technically much simpler and offer benefits in both cost and time reductions.

These two simple assays allow quantitative estimation of microbial activity to be made based upon the enzymatic hydrolysis of a chromogenic substrate to a coloured end product. The fluorescein formed from FDA hydrolysis may be extracted and quantified spectrophotometrically with an absorbance peak at approximately 490 nm. The extent of product formation serves as an index for microbial activity. In the tetrazolium assay, the activity of the succinate dehydrogenase complex bound to intracellular membranes is measured. Idophenyl nitrophenyl tetrazolium chloride acts as an artificial electron acceptor and is reduced to form extractable visible deposits of INT-formazan (Norton and Firestone, 1991).

Swisher and Carroll (1985) used the FDA assay to estimate fungal biomass on fir needles. The rate of enzymatic hydrolysis of FDA was shown to correlate with fungal biomass. The hydrolysis of FDA is, in part, due to free enzymes (e.g. esterases, acylases and lipases). Consequently, the assay is most applicable for the determination of decomposer activity in a sample, rather than its absolute biomass (Stubberfield and Shaw, 1990). Thus, this method is ideally suited for the determination of microbial activity in the degradation of pollutant

molecules *in situ*. Reed *et al.* (1989) examined the FDA method to determine if a soil supported a microbial community capable of utilising a pesticide (thiocarbamate) as an energy source. This is a useful method to determine the effect a contaminant has on "adapted" microbial populations rather than as a "first-encounter" response. Maximum pollutant concentration tolerance levels of the indigenous microorganisms may also be determined. Stubberfield and Shaw (1990) concluded that the FDA method is well suited to assay heterotrophic activity. In contrast, the INT method is better suited for measuring population densities.

A concentration of 50% (v/v) acetone is needed to terminate hydrolysis of FDA. Acetone destroys the cell phospho-lipid membranes, thus facilitating extraction of the fluorescein formed in the microbial cells (Schnürer and Rosswall, 1982). One disadvantage of using acetone to terminate the reaction is that it is known to extract humic substances (Stevenson, 1982) which may cause excessive background absorbance. Fluorescein is a polar compound and is partially adsorbed by organic matter present in the media. The degree of this adsorption will, therefore, depend on the type and amount or organic matter present (Inbar *et al.*, 1991). Non-biological hydrolysis of FDA may occur at high and low pH values (Schnürer and Rosswall, 1982).

[6.3.4] Fluorescein Diacetate Bioassay: Materials and Methods.

In the current study, a modification of the method described by Inbar *et al.* (1991) was used:

(1). A 2.0 mg l^{-1} FDA (Sigma) stock solution was made by dissolving 0.2 mg FDA in 96% acetone and diluting to 100 ml. The solution was stored in an amber glass bottle at -4°C;

(2). Two grams of soil were placed into a 150 ml Erlenmeyer flask which contained 20.0 ml 60 mM potassium phosphate buffer (pH 7.6; 8.7g KH_2PO_4 and 1.3g K_2HPO_4 l^{-1} distilled

water);

(3). 0.2 ml of stock solution was added to the flask. Each treatment was replicated three times with an additional replicate serving as a blank to correct for background absorbance (i.e. no FDA stock was added to the blank, only 0.2 ml of acetone). The flasks were sealed with Parafilm™;

(4). The flasks were then placed in a controlled environment incubator (25°C) shaker (90 rpm) (New Brunswick Scientific) for 60 minutes. The reaction was terminated by the addition of 20.0 ml of acetone (i.e. 50% v/v) to each flask;

(5). The contents of the flasks were filtered through Whatman No.1 filter paper;

(6). The filtrates were collected in 100 ml Erlenmeyer flasks which were then covered with Parafilm™ and immediatly stored (4°C); and

(7). The concentration of fluorescein which formed was determined spectrophotometrically (Milton Roy Spectronic 301) by comparing absorbance values measured at 490 nm against a standard curve. The standard curve was produced by measuring the absorbance values of a range of fluorescein dye standards (viz. 0, 100, 200, 300, 400 and 500 $\mu g\ l^{-1}$). These values are arbritary unless they are related back to the actual fungal biomass. Therefore, in this study, readings were not converted to mg l^{-1} fluorescein but were left simply as absorbance values from which relative comparisons between treatments could be made.

[6.3.5] Results and Discussion

The ergosterol bioassay is an appropriate means for monitoring fungal activity in soil as one is able to specifically quantify fungal biomass. After a series of experimental measurements, it

was concluded that the ergosterol bioassay was unsuitable for the type of soil samples used in the study. Solvent (methanol) extraction of ergosterol from fungal propagules in the soil resulted in the simultaneous removal of light hydrocarbon fractions and some humic compounds. These molecules bind to active sites on the C_{18} HPLC column. As a result, ergosterol was not retained by the column and was simultaneously eluted with the solvent peak. This phenomenon became apparent after only three to four sample elutions. A clean-up stage, of passing the sample through a Sepak Plus™ C_{18} cartridge filter (Waters) and elution with methanol, was explored but was rejected as this proved too expensive and time consuming for the numbers of samples which were to be processed. The possibility of determining ergosterol concentrations in the soil with a UV-spectrophotometer (Beckman DU-600) was then explored. Standards were scanned over a range from 190 nm to 300 nm. The ergosterol peak was obtained at 280 nm which is the suggested wavelength for HPLC UV detection (Martin *et al.*, 1990). However, corresponding ergosterol peaks in actual soil samples were masked by background absorbance generated by other soil components. This interference was still apparent when distilled water was added (up to 50% v/v) to the methanol to increase its polarity. From these observations it was concluded that retention of ergosterol on the C_{18} column was essential for its resolution.

Although the FDA bioassay is less specific, it is used routinely by researchers to monitor fungal activity (Swisher and Carroll, 1985; Inbar *et al.*, 1991; Boehm and Hoitink, 1992). The FDA hydrolysis assay has the advantage of being simple, rapid and sensitive and is especially useful for comparative studies of microbial activity in natural habitats. The FDA bioassay was found to be ideal for the purposes of this study. By repeating a similar scan as described above, from 190 nm to 600 nm (Beckman DU-600), the fluorescein peak at 490 nm did not experience the same degree of interference as the ergosterol peak. This was also due, in part, to the fluorescein peak being substantially larger than the peaks created by the interfering compounds at this wavelength. It should be noted that only background absorbances below 0.05 are acceptable (Schnürer and Rosswall, 1982). By using the FDA method, up to 50

samples may be processed in a three-hour time span whereas it would only be possible to process six ergosterol bioassay samples in the same period. More recently, a FDA analogue, 2',7'-dichloro-dihydro-fluorescein diacetate, succinimidyl ester (Molecular Probes, Oregon, U.S.A.) became available and provides a more sensitive bioassay for fungal activity and is, therefore, worthy of further investigation for application in this type of study.

Other more sophisticated methods exist for the *in situ* monitoring of the physiological state of specific microorganisms. Reverse transcription (RT)-PCR-based approaches have been applied to *Pseudomonas putida* in bioreactors (Selvaratnam *et al.*, 1995) and during the remediation of naphthalene-contaminated soils by the same species (Flemming *et al.*, 1993). Methods that use this type of mRNA analysis have distinct advantages over other approaches used with white-rot fungi, such as the quantification of ergosterol or PCR-based quantification of fungal DNA (Bogan *et al*, 1996c). Firstly, tailoring of PCR primers allows species specificity which is not possible with ergosterol measurements. Secondly, mRNA quantification data are superior in that they provide an insight of the physiological status of the fungus rather than merely quantifying biomass. This method will, thus, provide a more informative assessment of the bioremediation process.

CHAPTER SEVEN
[7] GENERAL DISCUSSION

South Africa is a semi-arid country with only a small percentage of arable surface area. With its high population growth, the country's two most important resources, namely groundwater and top soil, will require increasingly vigilant environmental conservation efforts to ensure their sustainability. As a result, bioremediation will have an important role to play in the restoration and protection of contaminated soil and water. Although bioremediation is not unknown in South Africa, little of its potential has been realised (Pearce and Oellermann, 1994). The main reason for the lack of full-scale bioremediation projects in South Africa is probably the lack of legal incentive. Relevant environmental legislation is virtually absent and/or is often poorly enforced.

In the U.S.A., ever-tightening environmental legislation is possibly the principal driving force behind soil clean-up operations and, in most cases, the costs of site remediation are substantially lower than possible litigation costs. It is also accepted by U.S. companies that a relatively large percentage of their profits must be spent on environmental protection and pollution remediation. Soil treatment technologies costing less then U.S. \$60 m^{-3} (R270, 1996) are generally considered cost effective (C.A. du Plessis, personal communication). As a result, the annual U.S. bioremediation market is currently worth an estimated U.S \$175 million and will be worth between U.S. \$350 and \$600 million by the year 2000. It has been estimated that the global bioremediation market will be worth about U.S. \$1.3 billion by the turn of the century (Glass, 1995).

In South Africa, industries responsible for environmental pollution are either unaware or unperturbed by the potential hazards related to their activities. Situations exist where heavy manufacturing industries have been provided with tax incentives to create employment and are thus given a "scheduled industry" status. As a result, these industries are often able to continue

their operations with little regard to their environmental impacts and are, to a large extent, protected from prosecution (C. Albertyn, personal communication). Due to ineffective laws and poor enforcement of legislation, it is undoubtedly more cost-effective for certain industries to periodically discharge their effluents to sewer. These industries then pay the meagre fines imposed on them, rather than investing larger sums of money in waste treatment plants or addressing the problems at the process/manufacturing stage. Such discharges are frequently observed at municipal sewage works in South Africa where the treatment process is adversely affected by sudden influxes of industrial effluents (P. Gaydon, personal communication).

Many contaminated sites are known to exist in South Africa, the majority of which are of an industrial nature. The principal contaminants identified can be placed in the following decreasing frequency of occurrence: hydrocarbons (mainly petroleum products); landfill leachate; inorganic salts; heavy metals; wood treatment chemicals; biocides; explosives; other organics; and radioactive waste (Pearce *et al.*, 1995). South Africa's long coastline (approximately 3000 km) which is adjacent to one of the world's major shipping routes is particularly vulnerable to marine pollution. In 1993, it was estimated that approximately 120 million tonnes of Middle East oil exports passed the Cape of Good Hope. This oil represents a major risk to ecologically sensitive coastal areas (Pearce *et al.*, 1995).

A recent survey made by the CSIR (Pearce *et al.*, 1995) evaluated the extent of use and acceptance of bioremediation in South Africa. The survey identified 28 sites in South Africa where bioremediation had been used. For each site, the treatment was reported to be cost effective. Landfarming and soil vapour extraction were the most well known and most frequently used bioremediation technologies. Full-scale bioremediation has had most application with the petrochemical industry. In 1994, the South African oil industry agreed in principle that *"No liquid hydrocarbon shall be disposed of to a landfill site, waste dump or Class 1/A site. Liquid hydrocarbons shall be recovered and be reused whether before or after processing as conditions require. Polluted soil shall be bioremediated, whether in situ or at an*

approved location, or treated in another acceptable manner so as to render it acceptable to the environment" (Camp, 1994).

The survey was by no means a conclusive estimation of the extent of environmental contamination in South Africa although it provided a good indication of the types of problems which are faced. Only 50% of the questionnaires were returned and often inadequate details of the sites were provided. These findings were indicative of the situation which currently exists in South Africa:

(i). Information on the nature and extent of contaminated land is scarce;
(ii). There is reluctance by industry to pool information;
(iii). The survey was conducted on a voluntary participatory basis;
(iv). Industries were reluctant to disclose information due to its sensitive nature and, perhaps, fears of facing future litigation;
(v). Inadequate detail was known about the sites due to unreliable and infrequent monitoring;
(vi). A legal framework defining clean-up procedures or the specification of concentration targets for hazardous compounds in soil, has not yet been formulated. Thus, target concentrations in completed projects were set mainly by the Department of Water Affairs and Forestry, local authorities, the specific industry or the client themselves; and
(vii). There are few companies capable of implementing bioremediation and these companies are not well known to industry.

There appears to be a shortage of trained personnel in this field in South Africa. Projects are currently being undertaken largely by non-specialised civil engineering companies which lack knowledge of the biological aspects of bioremediation. Collaboration between scientists and engineers from multidisciplinary backgrounds, as is needed for successful bioremediation, is

not readily practised. Interactions with consulting companies give the impression that they are extremely ambitious in the pursuit of securing the market niche rather than being able to render a reliable service. This may have potentially detrimental effects on the bioremediation industry in South Africa.

There is currently a contention in the U.S.A. that the EPA is overspending on environmental clean-up operations due to unrealistically stringent toxicity limits. Some Superfund sites are into their fourth and even sixth year of operation but are unable to reach the "impossible" contaminant concentration target levels set for site closure by EPA regulators (Ames, 1995). Collectively, such sites have become massive sinks of the EPA budget. Although the U.S. has greater experience in the field, much can be learnt from the European Community (EC). The EC has had the advantage of learning from the problems the U.S. bioremediation industry had to first overcome. As a result, it appears that the EC has formulated a more flexible and progressive approach to pollution control and the use of bioremediation. This approach is exemplified in the following points (Kavanaugh *et al.*, 1995):

(i). Present and future land use is given specific consideration in remedy selection, i.e. remedy selection is driven by site-specific clean-up goals;
(ii). There is no use of presumptive remedies;
(iii). Cost effectiveness is considered explicitly; and
(iv). Innovative technology is more easily permitted.

There are important lessons to be learnt from developed nations for individuals currently drafting environmental legislation in South Africa. Pollutant tolerance levels should be related to factors such as bioavailability and mobility (which are often interrelated) rather than just final contaminant concentrations in the soil or water. An acceptable balance needs to be drawn between the poor environmental legislation that is found over most of the African continent and the strict guidelines set by regulatory bodies such as the U.S. EPA.

By selecting relevant technologies and finding ways of applying them, South Africa is in a position to take advantage of the enormous advances that have resulted in the field of bioremediation over the last 25 years. The work undertaken in this study identified the potential of passive thermal bioventing (PTB) as a relevant bioremediation technology. In the current study, PTB proved to be effective in reducing the concentrations of contaminants in petroleum hydrocarbon-compromised soils at both laboratory- and pilot-scale. Passive thermal bioventing fulfils the most pertinent criterion in South Africa, that is cost. Even if a highly cost-effective remediation technology is currently available for a particular contaminated site, remediation may not be performed as there is little or no legal incentive. With the current environmental legislative and economic situation in South Africa, PTB may serve an important role in soil clean-up operations. Although more efficient methods, with shorter treatment times and the ability to remediate to lower contaminant concentrations, exist their degree of sophistication and cost make them unrealistic for South African conditions.

Passive thermal bioventing is applicable for the types of soil contamination which have already been handled by landfarming operations in South Africa. However, PTB has the specific advantage of minimising VOC emissions due to the biopile being enclosed. Landfarming operations often encourage the volatilisation of VOCs and there is no specific legislation controlling these types of emissions in South Africa (M. Kidd, personal communication).

It is speculated that PTB is most effective where contamination has been present for protracted periods. Many such sites exist in South Africa, particularly in railway yards, transport/trucking depots, wood treatment yards, beneath petrol filling stations and at oil refineries. All these examples share a common factor in that contamination has usually built up over a number of years to unacceptably high levels which may pose a significant toxicological threat to ground/surface waters. In these soils, specific microbial communities, which

catabolise the contaminants as carbon and energy sources, may "evolve". It is these adapted/selected indigenous microorganisms, particularly the fungal species, which thermal bioventing aims to stimulate. Biostimulation is effected by alleviating some of the rate-limiting factors through the provision of, for example, nitrogen, oxygen and co-metabolic substrates, in an environment which is conducive to contaminant degradation.

Innovations in other fields of biotechnology, such as molecular biology, will soon have an immense impact on bioremediation. Currently, methods such as gene probe hybridisation, mRNA analysis and bioluminescent reporter technology provide accurate determinations of catabolic activities for specific bacteria or overall populations in response to changing environmental conditions (Sanseverino *et al.*, 1994). Recombinant DNA technology facilitates the systematic isolation and expression of microbial metabolic activities under novel conditions. This allows the deliberate and directed alteration of microorganisms which may then be applied as agents of bioremediation. This approach is time consuming and, aside from an academic exercise, is often only advantageous where the natural constraints on genetic recombination and selection will not foster the evolution of desired species. Despite considerable success in the laboratory, recombinant DNA approaches to toxic and hazardous waste treatment have enjoyed very little success (King *et al.*, 1992b). At present, the greatest impediment to the successful application of recombinant DNA technology is a lack of detailed information concerning the ecology of released organisms. Public acceptance of the release of genetically manipulated microorganisms is still an issue that has to be addressed. However, one promising area for continued study is the production of cell-free enzyme extracts, such as the lignolytic enzymes produced by white-rot fungi, for application *in situ*. The potential impact of recombinant DNA technology on bioremediation is enormous and is comparable to advances that have been effected through the application of such techniques in the fields of medicine and agriculture.

LITERATURE CITED

Alexander, M. 1981. Biodegradation of chemicals of environmental concern. Sci. 211: 32-138.

Alleman, B.C., Logan, B.E. and Gilbertson, R.L. 1993. A rapid method for screening fungi for resistance to toxic chemicals. Biodegrad. 4: 25-129.

Ames, B. 1995. Does current cancer risk assessment harm health? Key note address, The Third International Symposium for *In-Situ* and On-Site Bioreclamation, San Diego, CA., U.S.A.

Anderson, J.P.E. and Domsch, K.H. 1975. Measurement of bacterial and fungal contributions to respiration of selected agricultural and forest soils. Can. J. Microbiol. 21: 314-322.

Anderson, J.G. 1991. Treatment of Wastes by Composting. In: Microbiology of Landfill Sites. Senior, E. (Ed.). CRC Press Inc, FL, U.S.A., pp. 59-77.

Anonymous. 1983. The Determination of Hydrocarbon Oils in Waters by Solvent Extraction, Infrared Absorption and Gravimetry. In: Methods for the Examination of Waters and Associated Materials Series. HSMO, London.

Arthur, M.F., O'Brien, G.K., Marsh, S.S. and Zwick, T.C. 1989. Cited by Hoeppel, R.E., Hinchee, R.E. and Arthur, M.F. 1991. Bioventing soils contaminated with petroleum hydrocarbons. J. Indust. Microbiol.8: 141-146.

Atlas, R.M. 1978a. Microorganisms and petroleum pollutants. Bioscience 28: 387-391.

Atlas, R.M. 1978b. Stimulated petroleum biodegradation. CRC Crit. Rev. Microbiol. 5: 371-386.

Atlas, R.M. 1981. Microbial degradation of petroleum hydrocarbons: An environmental perspective. Microbiol. Rev. 45: 180-209.

Atlas, R.M. 1988. Biodegradation of hydrocarbons in the environment. In: Environmental Biotechnology: Reducing Risks From Environmental Chemicals Through Biotechnology (Vol. 45). Omenn, G.S. (Ed.). Plenum Press, NY., U.S.A., pp. 211-222.

Atlas, R.M. and Bartha, R. 1972. Biodegradation of petroleum in seawater at low temperatures. Can. J. Microbiol. 18: 1851-1855.

Aust, S.D. 1990. Degradation of environmental pollutants by *Phanerochaete chrysosporium.* Microbial Ecol. 20: 197-209.

Bach, P.D., Shoda, M. and Kubota, H. 1984. Cited by Finstein, M.S., Miller, F.C. and Strom, P.E. 1986. Waste Treatment Composting as a Controlled System. In: Biotechnology Vol 8: Microbial Degradations. Rehm, H.J. and Reed, G. (Eds.). VCH Publishers, NY., U.S.A., pp. 363-398.

Bartha, R. 1986. Biotechnology of petroleum pollutant biodegradation. Microbial Ecol. 12: 155-172.

Bartha, R. and Atlas, R.M. 1977. The microbiology of aquatic oil spills. Adv. Appl. Microbiol. 22: 255-266.

Bartha, R. and Bossert, I. 1984. The Treatment and Disposal of Refinery Wastes. In: Petroleum Microbiology. Atlas, R.M. (Ed.). MacMillan, NY., U.S.A., pp. 435-473.

Battermann, G. and Meier-Lohr, M. 1995. Nitrate as an electron acceptor *in situ* of an abandoned refinery site bioremediation. Pre-print of Proceedings of The Third International Symposium for *In Situ* and On-Site Bioreclamation, San Diego. CA., U.S.A.

Bayman, P., Ritchey, S.D. and Bennett, J.W. 1995. Fungal interactions with the explosive RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine). J. Indust. Microbiol. 15: 418-423.

Berry, D.F. and Boyd, S.A. 1985. Decontamination of soil though enhanced formation of bound residues. Environ. Sci. Technol. 19: 1132-1133.

Blackburn, J.W. and Hafker, W.R. 1993. The impact of biochemistry, bioavailability and bioactivity on the selection of bioremediation techniques. Trends in Biotechnol. 11: 328-333.

Block, R.N., Clark, T.P. and Bishop, M. 1990 . Biological Treatment of Soils Contaminated by Petroleum Products. In: Petroleum Contaminated Soils (Vol. 3). Kostecki, P.T. and Calabresse, E.J. (Eds.). Lewis Publishers Inc., Michigan, U.S.A., pp. 167-176.

Bogan, B.W., Lamar, R.T. and Hammel, K.E. 1996. Flourene oxidation in vivo by *Phanerochaete chrysosporium* and in vitro during manganese peroxidase-dependent lipid peroxidation. Appl. Environ. Microbiol. 62: 1788-1792.

Bogan, B.W., Schoenike, B., Lamar, R.T. and Cullen, D. 1996a. Manganese peroxidase mRNA and enzyme activity levels during bioremediation of polycyclic aromatic hydrocarbon-contaminated soil with *Phanerochaete chrysosporium.* Appl. Environ. Microbiol. 62: 2381-2386.

Bogan, B.W., Schoenike, B., Lamar, R.T. and Cullen, D. 1996b. Expression of *lip*

genes during growth in soil and oxidation of anthracene by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 62: 3697-3703.

Boehm, M.J. and Hoitink, H.A.J. 1992. Sustenance of microbial activity in potting mixes and its impact on severity of *Pythium* root rot of Poinsettia. Phytopathology 82: 259-262.

Bonnen, A.M., Anton, L.H. and Orth, A.B. 1994. Lignin-degrading enzymes of the commercial button mushroom, *A. bisporus*. Appl. Environ. Microbiol. 60: 960-965.

Bos, R.P., Theuws, J.L.G., Jongeneelen, F.J. and Henderson P.T. 1988. Mutagenicity of bi-, tri- and tetracyclic aromatic hydrocarbons in the "taped plate" assay and in the conventional salmonella mutagenicity assay. Mutat. Res. 204: 203-206.

Bossert, I. and Bartha, R. 1984. The Fate of Petroleum Hydrocarbons in Soil Ecosystems. In: Petroleum Microbiology. Atlas, R.M. (Ed.). MacMillan Publishing Company, London. pp. 399-434.

Bossert, I. and Compeau, G.C. 1995. Cleanup of petroleum hydrocarbon contamination in soil. In: Microbial Transformation and Degradation of Toxic Organic Chemicals. Young, L.Y. and Cerniglia, C.E. (Eds.). Wiley and Sons Publication, N.Y., U.S.A. pp. 77-125.

Boyd, S.A. and Shelton, D.R. 1984. Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge. Appl. Environ. Microbiol. 47: 272-277.

Bragg, J.R., Prince, R.C. and Atlas, R.M. 1992. Bioremediation for shoreline cleanup following the 1989 Alaska oil spill. Exxon Company, Houston, TX, U.S.A.

Brown, K.W., Thomas, J.C. and Seago, O.J. 1995. Degradation of explosive propellants by in-vessel composting. BioCycle 39: 56-58.

Braun, S. and Vecht-Lifshitz, S.E. 1991. Mycelial morphology and metabolite production. Trends in Biotechnol. 9: 63-68.

Brecheen, T. and Patterson, R. 1995. Successful bioremediation with compost. BioCycle 36: 57-59.

Brodkorb, T.S. and Legge, R.L. 1992. Enhanced biodegradation of phenanthrene in oil tar-contaminated soils supplemented with *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 54: 3117-3121.

Buening, M.K., Wislocki, P.G., Levin, W., Yagi, H., Thakker, D.R., Akagi, H., Koreeda, M., Jerina, D.M. and Conney, A.H. 1978. Cited by Launen, L., Pinto, L., Wiebe, C., Kiehlmann, E. and Moore, M. 1995. The oxidation of pyrene and benzo[a]pyrene by non-basisdiomycete soil fungi. Can. J. Microbiol. 41: 477-488.

Bumpus, J.A. 1989. Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 55: 154-158.

Bumpus, J.A. and Aust, S.D. 1987. Biodegradation of DDT [1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane] by the white-rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 53: 2001-2007.

Bumpus, J.A., Tien, M., Wright, D. and Aust, S.D. 1985. Oxidation of persistent environmental pollutants by a white-rot fungus. Sci. 288: 1434-1436.

Burdsall, H.H. and Eslyn, W.E. 1974. A new *Phanerochaete* with a *Chrysosporium* imperfect stage. Mycotaxon 1: 123-133.

Buswell, J.A. 1995. Potential of spent mushroom substrate for bioremediation purposes.

Mushroom News 43: 28-34.

Cacciatore, D.A. and McNeil, M.A. 1995. Principles of soil bioremediation. BioCycle 36: 61-64.

Camp, C.R. 1984. Cited by Pearce, K., Snyman, H., van Heerden, H., Greben, H. and Oellermann, R.A. 1995. Bioremediation Technology for the Treatment of Contaminated Soil in South Africa. Final Report to the Water Research Commission. WRC Report Number 543/1/95.

Cantwell, S.G., Lau, E.P., Watt, D.S. and Fall, R. 1978. Biodegradation of acyclic isoprenoids by Pseudomonas species. J. Bacteriol. 135: 324-333.

Cerniglia, C.E. 1984. Microbial metabolism of polycyclic aromatic hydrocarbons. Adv. Appl. Microbiol. 30: 31-71.

Cerniglia, C.E., Campbell, W.L., Fu, P.P., Freeman, J.P. and Evans, F.E. 1990. Stereo selective fungal metabolism of methylated anthracenes. Appl. Environ. Microbiol. 56: 661-668.

Cerniglia, C.E. and Gibson, D.T. 1979. Oxidation of benza[*a*]pyrene by the filamentous fungus *Cunninghamella elegans*. J. Biol. Chem. 254: 12 174- 12 180.

Cerniglia, C.E. and Gibson, D.T. 1980. Fungal oxidation of benzo[*a*]pyrene and (±) trans 7,8-dihydrobenzo[*a*]pyrene: evidence for the formation of a benzo[*a*]pyrene 7,8-diol-9,10-epoxide. J. Biol. Chem. 225: 5159-5163.

Cerniglia, C.E., Kelly, D.W., Freeman, J.P. and Miller, D.W. 1986. Microbial metabolism of pyrene. Chem. -Biol. Interact. 57: 203-216.

Cerniglia, C.E., Hebert, R.L., Szanislo, P.J. and Gibson, D.T. 1978. Fungal

transformation of naphthalene. Arch. Microbiol. 117: 135-143.

Cerniglia, C.E. and Perry, J.J. 1973. Crude oil degradation by microorganisms isolated from the marine environment. Z. Allg. Mikrobiol. 13: 299-306.

Chakrabarty, A.M. 1976. Plasmids in Pseudomonads. Annu. Rev. Genet. 10: 7-30.

Chang, H.-M., Joyce, T.W., Campbell, A.G., Gerrard, E.D., Huynh, V.B. and Kirk, T.K. 1985. Fungal Decolorization of Bleach Plant Effluents. In: Recent Advances in Bleach Plant Effluents No.9. Hicuchi, T., Chang, H.-M. and Kirk, T.K. (Eds.). Uni Publishers, Tokyo. pp 257-268.

Chang, P.C. and Blackwood, A.C. 1968. Simultaneous production of three phenazine pigments by *Pseudomonas aeruginosa* Mac 436. Can. J. Microbiol. 15: 439-444.

Cobet, A.B. and Guard, H.E. 1973. Effect of a bunker fuel on the beach bacteria flora. Proc. Joint Conference on the Prevention and Control of Oil Spills, API, Washington DC. p. 185.

Colell, E.B. 1971. The Ecological Effects of Pollution and Marine Life. Cambridge University Press, Cambridge, U.K. p. 321.

Compeau, G.C., Mahaffey, W.D. and Patras, L. 1990. Full-Scale Bioremediation of Contaminated Soil and Water. In: Environmental Biotechnology for Waste Treatment. Sayler G.S., Fox, R. and Blackburn, J. (Eds.). Plenum Press, NY., U.S.A. pp. 91-109.

Conant, B., Akindunni, F.J. and Gillham, W. 1995. Effect of well-screen placement on recovery of vertically stratified contaminants. Ground Water 33: 445-456.

Cooney, J.J., Silver, S.A. and Beck, E.A. 1985. Factors influencing hydrocarbon

degradation in three freshwater lakes. Microbial Ecol. 11:127-137.

Coutts, D.A.P., Senior, E. and Balba, M.T.M. 1987. Multi-stage chemostat investigation of inter-species interactions in a hexanoate-catabolizing microbial association isolated from anoxic landfill. J. Appl. Bact. 62: 251-260.

Crawford, R. 1981. Lignin Biodegradation and Biotransformation. John Willey, NY., U.S.A. p.130.

Cripps, C., Bumpus, J.A. and Aust, S.D. 1990. Biodegradation of azo and heterocyclic dyes by *P. chrysosporium*. Appl. Environ. Microbiol. 56: 1114-1118.

Crist, W.A., Ellis, J., De Leeuw, J. and Schenck, P.A. 1985. Flash thermal desorption as an alternative solvent extraction for the determination C_8 to C_{35} in oil shales. Anal. Chem. 58: 258-261.

Cserjesi, A.J. and Johnson, E.L. 1972. Methylation of pentachlorophenol by *Trichoderma vigatum*. Can. J. Microbiol. 18: 45-49.

Davis, J.S. and Westlake, D.W.S. 1978. Crude oil utilization by fungi. Can. J. Microbiol. 25: 146-156.

De Bertoldi, M., Vallini, G., Pera, A. and Zucconi, F. 1982. Comparison of three windrow compost systems. BioCycle 23: 45-50.

De Klerk, H. and Van der Linden, A. C. 1974. Bacterial degradation of cyclohexane: Participation of a co-oxidation reaction. Antonie van Leeuwenhoek 40: 7-15.

Deever, W.R. and White, R.C. 1978. Cited by Wilson, G.B. Sikora, L.J. and Parr, J.F. 1983. Composting of Chemical Industrial Wastes Prior to Land Application. In: Land

Treatment of Hazardous Wastes. 1983. Parr, J.F., Marsh, P.B. and Kla, J.M. (Eds.). Noyes Data Corporation, NJ., U.S.A. pp.263- 273.

Dibble, J.T. and Bartha, R. 1979. The effect of environmental parameters on the biodegradation of an oily sludge. Appl. Environ. Microbiol. 31: 729-739.

Dineen, D., Slater, P.H., Holland, J. and Clendening, D.L. 1990. *In Situ* Biological Remediation of Petroleum Hydrocarbons in Unsaturated Soils. In: Petroleum Contaminated Soils. Vol. 3. Kostecki, P.T. and Calabresse, E.J. (Eds.). Lewis Publishers Inc., Michigan, U.S.A. pp.177-187.

Donnelly, P.K. and Fletcher, J.S. 1993. Potential Use of Mycorrhizal Fungi as Bioremediation Agents. In: Bioremediation through Rhizosphere Technology. Anderson, T.A. and Coats, J.R. (Eds.). 1994. ACS, Chicago, U.S.A., pp. 93-99.

Dooley, M.A., Taylor, K. and Allen, B. 1995. Composting of herbicide contaminated soil. Pre-print of Proceedings of The Third International Symposium for *In Situ* and On-Site Bioreclamation, San Diego. CA., U.S.A.

Dupont, R.R. 1992. Application of Bioremediation Fundamentals to the Design and Evaluation of *In Situ* Soil Bioventing Systems. In: Proceedings of the 85th Annual Meeting and Exhibition of the Air and Waste Management Association. pp 79-114.

Eaton, D.C. 1985. Mineralization of polychlorinated biphenyls by *Phanerochaete chrysosporium*: A lignolytic fungus. Enzyme Microb. Technol. 7: 194-196.

Englert, C.J., Kenzie, E.J. and Dragun, J. 1993. Bioremediation of Petroleum Products in Soil In: Principles and Practices for Petroleum Contaminated Soils. Calabrese, E.J. and Kostecki, P.T.(Eds.). Lewis Publishers, NY., U.S.A. pp. 111-115.

Epstein, E. and Alpert, J.E. 1980. Cited by Willson, G.B. Sikora, L.J. and Parr, J.F.

1983. Composting of Chemical Industrial Wastes Prior to Land Application. In: Land Treatment of Hazardous Wastes. 1983. Parr, J.F., Marsh, P.B. and Kla, J.M. (Eds.). Noyes Data Corporation, N.J., U.S.A. pp. 263-273.

Eschenbach, A., Kastner, M., Bierl, R., Schaefer, G. and Mahro, B. 1994. Evaluation of a new and more effective method to extract polycyclic aromatic hydrocarbons from soil samples. Chemosphere 28: 683-692.

Evans, W.C. and Fuchs, G. 1988. Anaerobic degradation of aromatic compounds. Ann. Rev. Microbiol. 42: 289-317.

Fedorak, P.M., Semple, K.M. and Westlake, D.W.S. 1984. Oil degrading capabilities of yeasts and fungi isolated from coastal marine environments. Can. J. Microbiol. 30: 365-571.

Fermore, T.R., Randle, P.E. and Smith, J.F. 1985. Compost as a Substrate and its Preparation. In: The Biology and Technology of the Cultivated Mushroom. Flegg, P.B., Spencer, D.M. and Wood, D.A. (Eds.). John Wiley and Sons, Ltd., Chichester, U.K. pp. 81-109.

Fernando, T., Bumpus, J.A. and Aust, S.D. 1990. Biodegradation of TNT (2,4,5-trinitrotoluene) by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 56: 1666-1671.

Field, F.A., Baten, H., Boelsma, F. and Rulkins, W.H. 1996. Biological elimination of polycyclic aromatic hydrocarbons in solvent extracts of polluted soil by the white rot fungus *Bjerkandera* sp. strain BOS55. Environ. Technol. 17: 317-323.

Field, F.A., Boelsma, F., Baten, H. and Rulkens, W.H. 1995. Oxidation of anthracene in water/solvent mixtures by the white-rot fungus, *Bjerkandera* sp. strain BOS55. Appl.

Microbiol. Biotechnol. 44: 234-240.

Field, J.A., De Jong, E., Gumersindo, F-G. and De Bont, J.A.M. 1992. Biodegradation of polycyclic aromatic hydrocarbons by new isolated of white-rot fungi. Appl. Environ. Microbiol. 58: 2219-2226.

Field, J.A., De Jong, E., Gumersindo, F-G. and De Bont, J.A.M. 1993. Screening for lignolytic fungi applicable to biodegradation of xenobiotics. Trends in Biotechnol. 11: 44-49.

Finstein, M.S., Miller, F.C. and Strom, P.E. 1986. Waste Treatment Composting as a Controlled System. In: Biotechnology Vol 8: Microbial Degradations. Rehm, H.J. and Reed, G. (Eds.). VCH Publishers, NY., U.S.A. pp. 363-398.

Finstein, M.S., Miller, F.C., Strom, P.F., MacGregor, S.T. and Psarianos, K.M. 1983. Composting ecosystem management for waste treatment. Biotechnol. 1: 347-353.

Finstein, M.S., Miller, F.C., Strom, P.F., MacGregor, S.T. and Psarianos, K.M. 1985. The Rutgers Strategy for Composting: Process Design and Control. EPA Project Summary EPA/600/S2-85/059.

Finstein, M.S. and Morris, M.L. 1975. Microbiology of solid waste composting. Adv. Appl. Microbiol. 19: 127-131.

Fleming, J. T., Sanserverino, J. and Sayler, G.S. 1993. Quantitative relationship between naphthalene catabolic gene frequency and expression in predicting PAH degradation in soils at town gas manufacturing sites. Environ. Sci. Technol. 27: 1068-1074.

Fletcher, M.A, Baker-Lee, C., Avila, O. and Munnecke, D. 1994. Microbial

remediation of polycyclic aromatic hydrocarbon from manufactured gas plant sites. Pre-print of Proceedings of Seventh International IGT Symposium on Gas, Oil and Environmental Biotechnology, Colarado Springs, CO.

Frankland, J.C. 1982. Biomass and Nutrient Cycling by Decomposer Basidiomycetes. In: Decomposer Basidiomycetes. Frankland, J.C., Hedger, J.W. and Swift, M.J. (Eds.). Cambridge University Press, Cambridge, U.K. pp. 241-261.

Gerrits, J.P.G., Bels-Koning, H. and Muller, F.M. 1967. Changes in compost constituents during composting, pasteurisation and cropping. Mushroom Sci. 6: 228-243.

Gibson, D.T. 1982. Microbial degradation of hydrocarbons. Environ. Toxicol. Chem. 5: 237-250.

Glass, D.J. 1995. International activities in bioremediation: Growing markets and opportunities. Key note address, The Third International Symposium for *In-Situ* and On-Site Bioreclamation, San Diego, CA., U.S.A.

Glenn, J.K. and Gold, M.H. 1983. Decolorization of several polymeric dyes by the lignin degrading basisidomycete *P. chrysosporium*. Appl. Environ. Microbiol. 45: 1741-1747.

Gold, M.H., Wamishi, H. and Valh, K. 1989. Cited by Aust, S.D. 1990. Degradation of environmental pollutants by *Phanerochaete chrysosporium*. Microbial Ecol. 20: 197-209.

Golovleva, L.A., Zaborina, O., Evtushenko, I. and Baskunov, B. 1992. Degradation of polychlorinated phenols by *Streptomyces rochei* 303. Biodegrad. 2: 201-208.

Golueke, C.G., Card, B.J., and MaGauhey, P.H. 1954. Cited by Finstein, M.S., Miller, F.C. and Strom, P.E. 1986. Waste Treatment Composting as a Controlled System. In: Biotechnology Vol 8: Microbial Degradations. Rehm, H.J. and Reed, G. (Eds.). VCH

Publishers, NY., U.S.A. pp. 363-398.

Griffin, D.M. 1972. The Ecology of Soil Fungi. Chapman and Hall Publishers, London. pp. 29-43.

Griffiths, R.P., McNamura, T.M., Cladwell, B.A. and Morita, R.Y. 1981. A field study on the effects of the dispersant Corexit 9527, on glucose uptake by marine microorganisms. Marine Environ. Res. 5: 16-20.

Guthrie, M.K., Regen, T.R., Paczkowski, M.T., Cushing, R. and Sharp, D. 1995. Bioventing in soil piles to augment landfarming of hydrocarbon-impacted soil. Pre-print of Proceedings of The Third International Symposium for *In Situ* and On-Site Bioreclamation, San Diego, CA., U.S.A.

Haemmerli, S.D., Leisola, M.S.A., Sanglard, D. and Fiechter, A. 1986. Oxidation of benzo[*a*]pyrene by extracellular lignase of *Phanerochaete chrysosporium*: Veratyl alcohol and stability of lignase. J. Biol. Chem. 261: 6900-6903.

Hammel, K.E., Kalyanaraman, B. and Kirk, T.K. 1989. Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]dioxins by *Phanerochaete chrysosporium* ligninase. J. Biol. Chem. 261: 16948-16952.

Hansen, R.C., Keener, H.M. and Hoitink, H.A.J. 1989. Poultry manure composting: An exploratory study. Transactions of the ASAE 32: 2151-2158.

Hasset, J.J. and Banwart, B.L. 1989. The Sorption of Nonpolar Organics by Soils and Sediments. In: Reactions and Movement of Organic Chemicals in Soils. Sawhney, B.L. and Brown, K. (Eds.). 1989. SSSA and ASA, Madison, Wisconsin. pp. 31-44.

Heitkamp, M.A. and Cerniglia, C.E. 1989. Polycyclic aromatic hydrocarbon degradation

by *Mycobacterium* sp. in microcosms containing sediment and water from a pristine ecosystem. Appl. Environ. Microbiol. 55: 1968-1973.

Heitkamp, M.A., Franklin, W. and Cerniglia, C.E. 1988. Pyrene degradation by a *Mycobacterium* sp.: Identification of ring oxidation and ring fission products. Appl. Environ. Microbiol. 54: 2556-2565.

Herbes, S.E. and Schwall, L.R. 1978. Microbial transformation of polycyclic aromatic hydrocarbons in pristine and petroleum contaminated sediments. Appl. Environ. Microbiol. 35: 306-316.

Hill, D.W. and McCarty, P.L. 1967. Anaerobic degradation of selected chlorinated hydrocarbon pesticides. J. Water Poll. Cont. Fed. 39: 1259-1277.

Hinchee, R.E., Downey, D.C. and Coleman, E.J. 1987. Cited by Hoeppel, R.E., Hinchee, R.E. and Arthur, M.F. 1991. Bioventing soils contaminated with petroleum hydrocarbons. J. of Indust. Microbiol. 8: 141-146.

Hoeppel, R.E., Hinchee, R.E. and Arthur, M.F. 1991. Bioventing soils contaminated with petroleum hydrocarbons. J. Indust. Microbiol. 8: 141-146.

Holroyd, M.L., Caunt, P. and Charley, R.C. 1992. Accelerated fungal processing for degradation of recalcitrant organics in soils. BioTal Ltd. Technical Report (D217).

Horvath, R.S. and Alexander, M. 1970. Cometabolism of *n*-chlorobenzene by an *Arthrobacter*. Appl. Microbiol. 20: 254-258.

Hsu, T.S. and Bartha, R. 1976. Hydrolyzable and non-hydrolyzable 3.4-dichloroaniline-humus complexes and their respective rates of biodegradation. J. Agric. Food. Chem. 24: 118-122.

Huddleston, R.L. 1979. Solid waste disposal: Landfarming. Chem. Eng. 26: 119-124.

Hulbert, M.H. and Krawiec, S. 1977. Cometabolism: A Critique. In: Bioremediation of Petroleum Contaminated Sites. CRC Press, FL, U.S.A. pp. 35-63.

Hunter, J.V., Finstein, M.S., Suler, D.J. and Bopal, R.R. 1981. Cited by Finstein, M.S., Miller, F.C. and Strom, P.E. 1986. Waste Treatment Composting as a Controlled System. In: Biotechnology Vol 8: Microbial Degradations. Rehm, H.J. and Reed, G. (Eds.).VCH Publishers, NY., U.S.A. pp. 363-398.

Huttermann, A., Trojanowski, J. and Loske, D. 1988. Cited by Morgan, P., Lee, S.A., Lewis, T., Sheppard, A.N. and Watkinson, R.J. 1993. Growth and biodegradation by white-rot fungi inoculated into soil. Soil Biol. Biochem. 25: 279-287.

Inbar, Y., Boehm, M.J. and Hoitink, H.A.J. 1991. Hydrolysis of fluorescein diacetate in sphagnum peat container media for predicting suppressiveness to damping-off caused by *Pythium ultimum.* Soil. Biol. Biochem. 23: 479-483.

Ingram, L.O. and Buttke, T.M. 1994. Effects of alcohols on microorganisms. Adv. Microb. Physiol. 25: 4031-4036.

Jensen, V. 1975. Cited by Bossert, I. and Bartha, R. 1984. The Fate of Petroleum Hydrocarbons in Soil Ecosystems. In: Petroleum Microbiology. Atlas, R.M. (Ed.). MacMillan Publishing Company, London. pp. 399-434.

Jobson, A., Cook, F.D. and Westlake, D.W.S. 1972. Microbial utilization of crude oil. Appl. Microbiol. 23: 1082-1089.

Johnson, P.C., Kemblowski, M.W. and Colthart, J.D. 1990. Quantitative analysis for

the clean-up of hydrocarbon-contaminated soils by *in situ* bioventing. Groundwater 28: 413-429.

Jones, J.G. and Edington, M.A. 1968. An ecological survey of hydrocarbon-oxidising microorganisms. J. Gen. Microbiol. 52: 281-390.

Jones, J.G., Knight, M. and Byrom, J.A. 1970. Cited by Bossert, I. and Bartha, R. 1984. The Fate of Petroleum Hydrocarbons in Soil Ecosystems. In: Petroleum Microbiology. Atlas, R.M. (Ed.). MacMillan Publishing Company, London. pp. 435-473.

Kamnikar, B. 1992. Bioremediation of contaminated soil. Pol. Eng. 24: 50-53.

Kamnikar, B. 1995. The Biomound Environment: A Field Evaluation. Special Report to the U.S. EPA.

Kan, A.T., Fu, G. and Tomson, M.B. 1994. Adsorption/desorption hysteresis in organic pollutant and soil/sediment interaction. Environ. Sci.Technol. 28: 859-867.

Karickhoff, S.W., Brown, D.S. and Scott, T.A. 1979. Sorption of hydrophobic pollutants on natural sediments. Water Res. 13: 241-248.

Kastner, M. and Mahro, B. 1996. Microbial degradation of polycyclic aromatic hydrocarbon in soils affected by the organic matrix of compost. Appl. Microbiol. Biotechnol. 44: 668-675.

Kaufman, D.D. and Plimmer, J.R. 1972. Approaches to the Synthesis of Soft Pesticides. In: Water Pollution Microbiology. Mitchell, R. (Ed.). Wiley Interscience, NY., U.S.A. pp. 173-203.

Kavanaugh, M., Kordes, B., Veul, M. and Reed, S. 1995. Pre-print of the proceedings

of The Third International Symposium for *In-Situ* and On-Site Bioreclamation, San Diego, CA., U.S.A.

Keck, J., Sims, R.C., Coover, M., Park, K. and Symons, B. 1989. Evidence for co-oxidation of polynuclear aromatic hydrocarbons in soil. Water Res. 12: 1467-1476.

Kennedy, D.W., Aust, S.D. and Bumpus, J.A. 1990. Comparative biodegradation of alkyl halide insecticides by the white rot fungus *Phanerochaete chrysosporium* (BKM-F-1767). Appl. Environ. Microbiol. 56: 2347-2353.

Keshavarez, T., Eglin, R., and Walker, E. 1990. The large scale immobilisation of *Pennicillium chrysogenum*: Batch and continuous operation in an lift reactor. Biotechnol. Bioeng. 36: 763-770.

Kim, J.Y., Park, J.K., Emmons, B. and Armstrong, D.E. 1995. Survey of volatile organic compounds at a municipal solid waste co-composting facility. Water Environ. 67: 1044-1051.

King, B.R. , Long, G.M. and Sheldon, J.K. 1992a. Surface Bioremediation of Soils and Sludges. In: Practical and Environmental Bioremediation. Lewis Publishers, London. pp. 71-81.

King, B.R. , Long, G.M. and Sheldon, J.K. 1992b. Future Trends in Development: The Road Ahead. In: Practical and Environmental Bioremediation. Lewis Publishers, London, p.127

Kirk, P.W. 1969. Isolation and culture of lignicolous marine fungi. Mycologia 61: 174-177.

Kirk, P.W. and Gordon, A.S. 1988. Hydrocarbon degradation by filamentous marine

higher fungi. Mycologia 80: 776-782.

Kirk, P.W. and Kohlmeyer, E. 1979. Marine Mycology, The Higher Fungi. Academic Press, NY., U.S.A. p. 690.

Kirk, T.K. 1984. Degradation of Lignin. In: Microbial Degradation of Organic Compounds. Gibson, D.T. (Ed.). Dekker, NY., U.S.A. pp. 339-436.

Klausmeier, R.E., Jamison, E.I. and Tatyrek, A. 1982. Cited by Williams R.T. and Keehan, K.R. 1993. Hazardous and Industrial Waste Composting. In: Science and Engineering of Composting: Design, Environmental and Utilisation Aspects.. Hoitink, H.A. and Keener, H.M. (Eds.). Renaissance Publications, OH., U.S.A. pp. 363-382.

Klug, M.J. and Markovetz, A.J. 1967.Thermophilic bacteria isolated on *n*-tetradecane. Nature (London) 215: 1082-1083.

Koenigs, J.W. 1972. Production of extracellular hydrogen peroxide and peroxidase by wood-rotting fungi. Phytopath. 62: 100-110.

Kohler, A., Jager, H., Willerhausen, A. and Graf, H. 1988. Extracellular ligninase of *Phanerocheate chrysosporium* Burdsall has no role in the degradation of DDT. Appl. Microbiol. Biotechnol. 29: 618-620.

Kohlmeyer, J. 1980. Marine fungi from Martinique. Can. J. Bot. 59: 1314-1321.

Kowalenko, G.C. 1978. Organic Nitrogen, Phosphorus and Sulfur in Soils. In: Soil Organic Matter. Schnitzer, M. and Khan, S.U. (Eds.).Elsevier Scientific Publ. Co., NY., U.S.A. pp. 95-135.

Kubota, H. and Nakasaki, K. 1991. Accelerated thermophilic composting of garbage.

BioCycle 32: 66-68.

Laine, M.M. and Jorgensen, K.S. 1996. Straw compost and bioremediated soil as inocula for the bioremediation of chlorophenol-contaminated soil. Appl. Environ. Microbiol. 62: 1507-1513.

Lamar, R.T. and Detrich, D.M. 1990. *In Situ* depletion of pentachlorophenol from contaminated soil by *Phanerochaete* spp. Appl. Environ. Microbiol. 56: 3093-3100.

Lamar, R.T., Larsen, M.J. and Kirk, T.K. 1990. Sensitivity to degradation of pentachlorophenol by *Phanerochaete* spp. Appl. Environ. Microbiol. 56: 3519-3526.

Launen, L., Pinto, L., Wiebe, C., Kiehlmann, E. and Moore, M. 1995. The oxidation of pyrene and benzo[*a*]pyrene by non-basidiomycete soil fungi. Can. J. Microbiol. 41: 477-488.

Leahy, J.G. and Colwell, R.R. 1990. Microbial degradation of hydrocarbons in the environment. Microbial Rev. 54: 305-315.

Lees, Z.M. 1991. A Preliminary Investigation to Determine the Feasibility of *In Situ* Bioremediation of an Oil-Contaminated Soil. BSc. Honours Thesis, University of Natal, Pietermaritzburg, South Africa.

Lees, Z.M. 1997. The Bioremediation of an Oil-Contaminated Soil: A South African Case Study. Ph.D. Thesis, University of Natal, Pietermaritzburg, South Africa.

Levin, W., Wood, A.W., Yagi, H., Jerina, D.M. and Conney, A.H. 1976. Cited by Launen, L., Pinto, L., Wiebe, C., Kiehlmann, E. and Moore, M. 1995. The oxidation of pyrene and benzo[*a*]pyrene by non-basidiomycete soil fungi. Can. J. Microbiol. 41: 477-488.

Lowe, W., Williams, R.T. and Marks, P.J. 1989. Composting of explosives contaminated soil technology. USATHAMA Report No. CETHA-TE-CR-90027.

Lowery, C.E., Forster, J.W. and Jurtshuck, P. 1968. Cited by Klug, M.J. and Markovetz, A.J. 1971. Utilization of aliphatic hydrocarbons by microorganisms. Adv. Microb. Physiol. 5: 1-39.

Lundquist, K., Kirk, T.K. and Connors, W.J. 1977. Fungal degradation of kraft lignin and lignin sulfonate prepared from synthetic ^{14}C-lignins. Arch. Microbiol. 112: 291-296.

Lynch, J.M. 1993. Substrate Availability in the Production of Composts. In: Science and Engineering of Composting: Design, Environmental and Utilisation Aspects. Hoitink, H.A. and Keener, H.M. (Eds.). Renaissance Publications, OH., U.S.A. pp. 24-35.

Lynch, J. and Genes, B.R. 1993. Land Treatment of Hydrocarbon Contaminated Soils. In: Principals and Practices for Petroleum Contaminated Soils. Calabrese, E.J. and Kostecki, P.T.(Eds.). Lewis Publishers, NY., U.S.A. pp. 409-422.

Malloch, D. 1981. Moulds: Their Isolation, Cultivation and Identification. University of Toronto Press, Canada.

Manilal, V.B. and Alexander, M. 1991. Factors affecting the microbial degradation of phenanthrene in soil. Appl. Microbiol. Biotechnol. 35: 401-405.

Martin, F., Delauruelle, C. and Hilbert, J.L. 1990. An improved ergosterol assay to estimate fungal biomass in ectomycorrhizas. Mycol. Res. 94: 1059-1064.

Martin, J.P., Para, A.A. and Haider, K. 1978. Influence of intimate association with humic polymer on biodegradation of ^{14}C-labelled organic substances in soil. Soil Biochem.

10: 483-486.

Masaphy, S., Levanon, D., Henis, Y., Venkateswarlu, K. and Kelly, S.L. 1996. Evidence for cytochrome P-450 and P-450 mediated benzo(a)pyrene hydroxylation in the white rot fungus *Phanerochaete chrysosporium.* FEMS Microbiol. Letters 135: 51-55.

Matcham, S.E., Jordan, B.R. and Wood, D.A. 1985. Estimation of fungal biomass in a solid substrate by three independent methods. Appl. Microbiol. Biotechnol. 21:108-112.

McFarland, M.J., Qiu, J.L., Sims, M.E., Randolph, M.E. and Sims, R.C. 1992. Remediation of petroleum impacted soils in fungal compost bioreactors. Water Sci. Tech. 25: 197-206.

McGill, W.B. 1977. Soil Restoration Following Oil Spills: A Review. In: Bioremediation of Petroleum Contaminated Sites. Riser-Roberts, E. (Ed.). 1992. CRC Press. FL., U.S.A. pp. 35-63.

McGinnis, D., DuPont, R., Everhart, K. and Laurent, G. 1994. Evaluation of field soil pile bioventing systems for the remediation of PCP contaminated surface soils. Environ. Tech. 15: 729-739.

McGugan, B.R. 1993. Fungal Bioremediation of an Oil-Contaminated Soil. Bsc. Honours thesis, University of Natal, Pietermaritzburg, South Africa.

McGugan, B.R., Lees, Z.M. and Senior, E. 1995. Bioremediation of an Oil-Contaminated Soil by Fungal Intervention. In: Bioaugmentation for Site Remediation. Hinchee, R.E. (Ed.). Battele Press, Columbus, OH., U.S.A., pp. 149-156.

McGugan, B.R. and Ramsden, P.J. 1992. The Isolation and Identification of Oil-Hydrocarbon-Degrading Fungi From an Oil-Contaminated Soil. Unpublished Report,

University of Natal, Pietermaritzburg, South Africa.

McKenna, E.J. 1977. Biodegradation of polynuclear aromatic hydrocarbons by soil and water microorganisms. 70th Annual Meeting of American Institute of Chem. Eng., N.Y., U.S.A.

McKinley, V.L. and Vestal, J.R. 1984. Biokinetic analyses of adaptation and succession: Microbial activity in composting municipal sewage sludge. Appl. Environ. Microbiol. 47: 933-941.

Means, J.G., Wood, S.G., Hasset, J.J. and Banwart, B.L. 1980. Sorption of polynuclear aromatic hydrocarbons by sediments and soils. Environ. Sci. Technol. 14: 1524-1528.

Mersch-Sundermann, V., Rosenkranz, H.R. and Klopman, G. 1992. Structural bias of the genotoxicity of polycyclic aromatic hydrocarbons. Mutagen. 7: 211-218.

Mileski, G.J., Bumpus, J.A., Jurek, M.A. and Aust, S.D. 1988. Biodegradation of pentachlorophenol by the white-rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 54: 2885-2889.

Miller, J.D. and Whitney, N.J. 1981. Fungi of the Bay of Fundy III: Geofungi in the marine environment. Mar. Biol. 65: 61-68.

Miller, R.M. and Hinchee, R.E. 1990. Cited by Hoeppel, R.E., Hinchee, R.E. and Arthur, M.F. 1991. Bioventing soils contaminated with petroleum hydrocarbons. J. of Indust. Microbiol. 8: 141-146.

Miyoshi, M. 1895. Cited by Klug, M.J. and Markovetz, A.J. 1971. Utilization of aliphatic hydrocarbons by micro-organisms. Adv. Microb. Phys. 5: 1-39.

Mohr, D.H. and Merz, P.H. 1995. Application of a 2D air flow model to soil vapour extraction and bioventing studies. Ground Water 33: 433-444.

Montemango, C.D. and Irvine, R.L. 1989. Cited by Cacciatore, D.A. and McNeil, M.A. 1995. Principles of soil bioremediation. BioCycle 36: 61-64.

Morgan, P., Lee, S.A., Lewis, T., Sheppard, A.N and Watkinson, R.J. 1993. Growth and biodegradation by white-rot fungi inoculated into soil. Soil Biol. Biochem. 25: 279-287.

Morgan, P., Lewis, S.T. and Watkinson, R.J. 1991. Comparison of abilities of white-rot fungi to mineralize selected xenobotic compounds. Appl. Microbiol. Tech. 34: 693-696.

Mueller, J.G., Chapman, P.J. and Pritchard, P.H. 1989. Action of a fluoranthene-utilising bacterial community on polycyclic aromatic hydrocarbon components of creosote. Appl. Environ. Microbiol. 55: 3085-3090.

Mulkins-Phillips, G.J. and Stewart, J.E. 1974. Effects of four dispersants on biodegradation and growth of bacteria on crude oil. Appl. Microbiol. 28: 547-552.

Nash, J., Downey, D. and Traver, R. 1987. Cited by Hoeppel, R.E., Hinchee, R.E. and Arthur, M.F. 1991. Bioventing soils contaminated with petroleum hydrocarbons. J. of Indust. Microbiol. 8: 141-146.

Nelson, D.W. and Sommers, L.E. 1972. A simple digestion procedure for estimation of total nitrogen in soils and sediments. J. Environ. Qual.1: 423-425.

Nohynek, L.J., Haggblom, M.M., Palleroni, N.J., Kronqvist, K., Nurmiaho-Lassila, F. and Salkinoja-Salonen, M.S. 1993. Characterization of *Mycobacterium fortuitum* strain

capable of degrading polychlorinated phenolic compounds. Syst. Appl. Microbiol. 16: 126-134.

Norton, J.M. and Firestone, M.K. 1991. Metabolic status of bacteria and fungi in the rhizosphere of *Ponderosa* pine seedlings. Appl. Environ. Microbiol. 57: 1161-1167

Nout, M.J.R., Bonants-van Laarhoven, T.M.G., De Jongh, P. and De Koster, P.G. 1987. Ergosterol content of *Rhizopus oligosporus* NRRL 5905 grown in liquid and solid substrates. Appl. Microbiol. Biotechnol. 26: 456-461.

Nyns, E.J., Auquiere. J.P. and Wiaux, A.L. 1968. Taxonomic value of the property of fungi to assimilate hydrocarbons. Antonie van Leeuwenhoek 34: 441-457.

Okeke, B.C., Smith, J.E., Paterson, A. and Watson-Craik, I.A. 1993. Aerobic metabolism of pentachlorophenol by spent sawdust culture of "shiitake" mushroom (*Lentinus edodes*) in soil. Biotech. Let. 15: 1077-1080.

Osman, J.L. and Andrews, C.C. 1978. Cited by Williams, R.T. and Keehan, K.R. 1993. Hazardous and Industrial Waste Composting. In: Science and Engineering of Composting: Design, Environmental and Utilisation Aspects. Hoitink, H.A. and Keener. H.M. (Eds.). Renaissance Publications, OH, U.S.A. pp. 363-382.

Osmon, J.L., Andrews, C.C. and Tatyreh, A. 1978. Cited by Finstein, M.S., Miller, F.C. and Strom, P.E. 1986. Waste Treatment Composting as a Controlled System. In: Biotechnology Vol 8: Microbial Degradations. Rehm, H.J. and Reed, G. (Eds.). VCH Publishers, NY., U.S.A. pp. 363-398.

Otjen, L., Blanchette, R., Effland, M. and Leatham, G. 1987. Assessment of 30 white rot basidiomycetes for selective lignin degradation. Holzforschung 41: 343-349.

Oudot, J., DuPont, J., Haloui, S. and Roquebert, M.F. 1993. Biodegradation potential of hydrocarbon-assimilating tropical fungi. Soil. Biol. Biochem. 25: 1167-1173.

Oudot, J., Fusey, P., Abdellouahid, D.E., Haloui, S. and Roquebert, M.F. 1987. Capacites degradatives de bacteries et de champignons isoles dun sol contamine par un fuel. Can. J. Microbiol. 3: 232-243.

Pakula, R. and Freeman, A. 1996. A new continuous biofilm bioreactor for immobilised oil-degrading filamentous fungi. Biotechnol. Bioeng. 49: 20-25.

Parbery, D.G. 1971. Biological problems in jet aviation fuel and the biology of *Amorphotheca resinae*. Mat. Organ. 6: 161-208.

Parks, K.S., Sims, R.S., DuPont, R.R., Doucette, W.J. and Mathews, J.E. 1990. Fate of PAH compounds in two soil types: Influence of volatilization, abiotic loss and biological activity. Environ Toxicol. Chem. 9: 187-195.

Pearce, K. and Oellermann, R.A. 1994. Cited by Pearce, K., Snyman, H., van Heerden, H., Greben, H. and Oellermann, R.A. 1995. Bioremediation Technology for the Treatment of Contaminated Soil in South Africa. Final Report to the Water Research Commission. WRC Report Number 543/1/95.

Pearce, K., Snyman, H., van Heerden, H., Greben, H. and Oellermann, R.A. 1995. Bioremediation Technology for the Treatment of Contaminated Soil in South Africa. Final Report to the Water Research Commission. WRC Report Number 543/1/95.

Perry, J.J. 1979. Microbial co-oxidations involving hydrocarbons. Microbial. Rev. 43: 59-72.

Perry, J.J. 1984. Microbial Metabolism of Cyclic Alkanes. In: Petroleum Microbiology. Atlas, R.M.(Ed.). MacMillan Publishing Co., NY., U.S.A. pp. 61-98.

Perry, J.J. and Cerniglia, C.E. 1973. Cited by Oudot, J., DuPont, J., Haloui, S. and Roquebert, M.F. 1993. Biodegradation potential of hydrocarbon-assimilating tropical fungi. Soil. Biol. Biochem. 25: 1167-1173.

Pettyjohn, W.A. and Houndslow, A.W. 1983. Bioremediation of Petroleum Contaminated Sites. Riser-Roberts, E. (Ed.). 1992. CRC Press, Inc. FL, U.S.A. pp. 35-57.

Pfaender, F.K. and Bartholomew, G.W. 1982. Measurement of aquatic biodegradation rates by determining heterotrophic uptake of radio-labelled pollutants. Appl. Environ. Microbiol. 44: 159-164.

Pierson, L.S. and Thomashow, L.S. 1992. Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 38-84. Mol. Plant-Microbe Interact 5: 330-339.

Pinholdt, Y., Struve, S. and Kjoller, A. 1979. Cited by Bossert, I. and Bartha, R. 1984. The Fate of Petroleum Hydrocarbons in Soil Ecosystems. In: Petroleum Microbiology. Atlas, R.M. (Ed.). MacMillan Publishing Company, London. pp. 435-473.

Pirnik, M.P. 1977.Microbial oxidation of methyl branched alkanes. Crit. Rev. Microbiol. 5: 413-422.

Pitt, J.I. 1984. The significance of potentially toxigenic fungi in foods. Food Technol. Aust. 36: 218-219.

Pothuluri, J.V., Freeman, J.P., Evans, F.E. and Cerniglia, C.E. 1993. Biotransformations of fluorene by the fungus *Cunninghamella elegans*. Appl. Environ. Microbiol. 59: 1977-1980.

Potter, T.M. 1989. Analysis of Petroleum Contaminated Soil and Water: An Overview. In: Petroleum Contaminated Soils (Vol.2). Calabrese, E.J. and Kostecki, P.T.(Eds.). Lewis Publishers, NY., U.S.A. pp. 97-109.

Radtke, C., Cook, W.S. and Anderson, A. 1994. Factors affecting antagonism of the growth of *Phanerochaete chrysosporium* by bacteria isolated from soils. Appl. Microbiol. Biotechnol. 41: 274-280.

Rangal, C.M.P., Frantz, J.H. and Bastiani, M. 1988. Landfarming for treatment and disposal of oily wastes originating in the south petrochemical complex. Water Sci. Tech. 20: 221-230.

Rayner, A.D.M. and Boddy, L. 1988. Fungal Decomposition of Wood: Its Biology and Ecology. John Wiley and Sons. Inc., NY., U.S.A. p. 342.

Reed, J.P., Kruegar, H.R. and Hall. F.R. 1989. Fluorescein diacetate hydrolysis for determination of accelerated degradation of thiocarbamate herbicides. Bull. Environ. Contam. Toxicol. 43: 929-934.

Riser-Roberts, E. 1992. Indigenous Microorganisms in Biodegradation. In: Bioremediation of Petroleum Contaminated Sites. CRC Press, Inc. FL, U.S.A. pp. 35-57.

Robinson, K.G., Farmer, W.S. and Novak, J.T. 1990. Availability of sorbed toluene in

soils for biodegradation by acclimated bacteria. Water Res. 24: 345-350.

Rose, W.W. and Mercer, W.A. 1968. Cited by Wilson, G.B. Sikora, L.J. and Parr, J.F. 1983. Composting of Chemical Industrial Wastes Prior to Land Application. In: Land Treatment of Hazardous Wastes. 1983. Parr, J.F., Marsh, P.B. and Kla, J.M. (Eds.). Noyes Data Corporation, NJ., U.S.A. pp. 263- 273.

Ruckdeschel, G. and Renner, G. 1986. Effects of pentachlorophenol and some of its known and possible metabolites on fungi. Appl. Environ. Microbiol. 51: 1370-1372.

Ryan, J.R., Loehr, R.C. and Rucker, E. 1991. Bioremediation of organic contaminated soils. J. Haz. Mat. 28: 159-169.

Salmanowicz, B. and Nyland, J.-E. 1988. High performance liquid chromatography determination of ergosterol as a measure of ectomycorrhiza infection of Scots pine. Euro. J. Phytopath. 18: 291-298.

Sanseverino, J., Flemming, J.T., Heitzer, A., Applegate, B.M. and Sayler, G.S. 1994. Applications of Environmental Biotechnology to Bioremediation. In: Remediation of Hazardous Waste Contaminated Soils. Wise, D.L. and Trantolo, D.J. (Eds.).Marcel Dekker Inc., NY., U.S.A. pp. 97-123.

Savage, G.M. and Golueke, G. 1986. Major cost elements of composting. BioCycle 27: 33-35.

Schnürer, J. and Roswall, T. 1982. Flourescein diacetate hydrolysis as a measure of total microbial activity in soil and litter. Appl. Environ. Microbiol. 43: 1256-1261.

Selvaratnam, S., Schoedel, B.A., McFarland, B.L. and Kupla, C.F. 1995. Application of reverse transcriptase PCR for monitoring expression of the catabolic *dmpN* gene in a phenol-degrading sequencing batch reactor. Appl. Environ. Microbiol. 61: 3981-3985.

Shiaris, M.P. 1989. Seasonal biotransformation of naphthalene, phenanthrene and benzo[a]pyrene in estuarine sediments. Appl. Environ. Microbiol. 55: 1391-1399.

Sims, R.C. and Bass, J. 1984. Review of In Place Treatment Techniques For Petroleum Contaminated Surface Soils. Vol 1. EPA Report No. EPA-540/2-84-003a.

Sims, R.C. and Overcash, M.R. 1983. Indigenous Microorganisms in Biodegradation. In: Bioremediation of Petroleum Contaminated Sites. Riser-Roberts, E. (Ed.). 1992. CRC Press, Inc. FL, U.S.A. pp. 35-57.

Singer, M.E. and Finnert, W.R. 1984. Microbial Metabolism of Straight Chain and Branched Alkanes. In: Petroleum Microbiology. MacMillan Publishing Co., NY., U.S.A. pp. 99-128.

Skinner, F.A. 1976. Cited by Davis, J.S. and Westlake, D.W.S. 1978. Crude oil utilization by fungi. Can. J. Microbiol. 25: 146-156.

Smith, J.E. 1968. Cited by Worne, H.E. and Fortune, I. 1992. Bioremediation of hazardous and toxic wastes (presentation to HMC/SUPERFUND). Epicore Networks Inc. Publication, Canada..

Snellman, E.A., Collins, R.P. and Cooke, J.C. 1988. Utilization of fuel oil by fungi isolated from oceanic tar balls. Lett. Appl. Microbiol. 6: 105-107.

Song, H.G., Pedersen, T.A. and Bartha, R. 1986. Hydrocarbon mineralisation in soil: Relative bacterial and fungal contribution. Soil Biol. Biochem. 18: 109-111.

Spadaro, J.T., Gold, M.H. and Renganathan, G. 1992. Degradation of azo dyes by the lignin degrading fungus *P. chrysosporium*. Appl. Environ. Microbiol. 58: 2397-2401.

Spain, J.C., Milligan, J.D., Downey, D.C. and Slaughter, J.K. 1989. Excessive bacterial decomposition of H_2O_2 during enhanced bioremediation biodegradation. Ground Water 27: 163-167.

Spain, J.C., Pritchard, P.H. and Bourquin, A.W. 1980. Effects of adaptation on biodegradation rates in sediment/water cores from estuarine and freshwater environments. Appl. Environ. Microbiol. 40: 726-734.

Steitz, L.M., Sauer, D.B., Burroughs, R., Mohr, H.E. and Hubbard, J.D. 1979. Ergosterol as a measure of fungal growth. Phytopath.69: 1202-1203.

Stevenson, F.J. 1982. Humus Chemistry. Wiley and Sons Publications, NY., U.S.A. pp 123-142.

Stubberfield, L.C.F. and Shaw, P.J.A. 1990. A comparison of tetrazolium reduction and FDA hydrolysis with other measures of microbial activity. J. Microbiol. Meth. 12: 151-162.

Suflita, J.M., Howortiz, A., Shelton, D.R. and Tiedge, J.M. 1982. Dehalogenation: a novel pathway for the anaerobic biodegradation of haloaromatic compounds. Sci. 218: 1115-1117.

Suler, D.J. and Finstein, M.S. 1977. Effect of temperature, aeration and moisture content on CO_2 formation in bench-scale continuosly thermophilic composting of solid waste. Appl. Environ. Microbiol. 33: 345-349.

Swisher, R. and Carroll, G.C. 1980. Fluorescein diacetate hydrolysis as an estimator of microbial biomass on coniferous needle surfaces. Microb. Ecol. 6: 217-226.

Tansey, M.R. and Brock, T.D. 1978. Microbial Life in Extreme Environments. Academic Press, NY., U.S.A. pp. 159-216.

Thomas, J.M., Yordy, J.R., Amador, J.A. and Alexander, M. 1986. Rates of dissolution and biodegradation of water-insoluble organic compounds. Appl. Environ. Microbiol. 52: 290-296.

Tong, H.Y. and Karasek, F.W. 1984. Flame ionisation detector response factors for compound classes in quantitative analysis of complex organic mixtures. Anal. Chem. 56: 2124-2128.

Torrey, S. 1979. Sludge Disposal by Landfarming Techniques. Noyes Data Corp, U.S.A. p.372.

Tothill, I.E., Harris, D. and Magan, N. 1992. The relationship between fungal growth and ergosterol content of wheat grain. Mycol. Res. 96: 965-970.

Tucker, B., Radtke, C., Cook, W.S. and Anderson, A. 1995. Suppression of bioremediation by *Phanerochaete chrysosporium* by soil factors. J. Haz. Mat. 41: 251-265.

U.S. EPA. 1983. Methods for Chemical Analysis of Water and Wastes. Washington DC.

U.S. EPA. 1989. Innovative Technology :Slurry-Phase Biodegradation. Fact Sheet 9200.5-252FS, Solid Waste and Emergency Response (OS-220), Washington, D.C.

Van Wedel, R.J., Mostquera, J.F., Goldsmith, C.D., Hater, G.R., Wong, A., Fox, T.A., Hunt, W.T., Paules, M.S., Quiros, J.M. and Wiegand, J.W. 1988. Bacterial biodegradation of petroleum hydrocarbons in groundwater: *In situ* augmented bioreclamation with enrichment isolates in California. Water Sci. Technol. 20: 501-503.

Vyas, B.R.M., Bakowski, S., Sasek, V. and Matucha, M. 1994. Degradation of anthracene by selected white rot fungi. FEMS Microbial Ecol. 14: 65-70.

Wainwright, M. 1992. Fungi in Environmental Biotechnology. In: An Introduction to Fungal Biotechnology. John Wiley and Sons. NY., U.S.A. pp. 81-101.

Waksmann, S.A. and Nissen, W. 1931. Cited by Buswell, J.A. 1995. Potential of spent mushroom substrate for bioremediation purposes. Mushroom News 43: 28-34.

Walker, J.D. and Colwell, R.R. 1974. Cited by Davis, J.S. and Westlake, D.W.S. 1978. Crude oil utilization by fungi. Can. J. Microbiol. 25: 146-156.

Warcup , J.H. 1955, 1957. Cited by Davis, J.S. and Westlake, D.W.S. 1978. Crude oil utilization by fungi. Can. J. Microbiol. 25: 146-156.

Ward, C.H., Thomas, J.M., Fiorenza, S., Rifai, H.S. Bedient, P.B., Wilson, J.T. and Raymond, R.L. 1989. Cited by Dineen, D., Slater, P.H., Holland, J. and Clendening, D.L. 1990. *In Situ* Biological Remediation of Petroleum Hydrocarbons in Unsaturated Soils. In: Petroleum Contaminated Soils. Vol. 3. Kostecki, P.T. and Calabresse, E.J. (Eds.). Lewis Publishers Inc., MI, U.S.A. pp. 177-187.

Ward, D.M. and Brock, T.D. 1978. Anaerobic metabolism of hexadecane in marine sediments. Geomicrobiol. 1: 1-9.

Weissenfels, W.D., Klewer, H.-J. and Langhoff, J. 1992. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: influence on biodegradability and biotoxicity. Appl. Microbiol Biotechnol. 36: 689-690.

West, A.W., Grant, D.W. and Sparling, G.P. 1987. Use of ergosterol, diaminopimelic acid and glucosamine contents of soil to monitor changes in microbial populations. Soil

Biol. Biochem. 19: 607-612.

Westlake, D.W.S., Jobson, A., Phillippe, R. and Cook, F.D. 1974. Biodegradability and crude oil composition. Can J. Microbiol. 20: 915-928.

Williams, R.T. and Keehan, K.R. 1993. Hazardous and Industrial Waste Composting. In: Science and Engineering of Composting: Design, Environmental and Utilisation Aspects. Hoitink, H.A. and Keener, H.M. (Eds.). Renaissance Publications, OH., U.S.A. pp. 363-382.

Williams, R.T. and Myler, C.A. 1990. Bioremediation using composting. BioCycle 31: 78-80, 82.

Wilson, G.B., Parr, J.F., Epstein, E., Marsh, P.B., Chaney, R.L., Colacicco, D., Burge, W.D., Sikora, L.J., Tester, C.F. and Hornik, S. 1980. Manual for Composting Sewage Sludge by the Beltsville Aerated-Pile Method. U.S. Department of Agric./U.S. EPA, EPA-600/8-80-022, Cincinnati, OH., U.S.A.

Wilson, G.B., Sikora, L.J. and Parr, J.F. 1983. Composting of Chemical Industrial Wastes Prior to Land Application. In: Land Treatment of Hazardous Wastes. Parr, J.F., Marsh, P.B. and Kla, J.M. (Eds.). Noyes Data Corporation, NJ., U.S.A. pp. 263- 273.

Webb, C., Fukuda, H. and Atkinson, B. 1986. The production of cellulase in a spouted bed fermentor using cells immobilised in biomass support particles. Biotechnol. Bioeng. 28: 41-50.

Wodzinski, R.S. and Bertolini, D. 1979. Physical state in which naphthalene and bibenzyl are utilised by bacteria. Appl. Microbiol. 23: 1077-1081.

Woodhull, P.M. and Jurger, D.E. 1994. Bioremediation using a commercial slurry-phase

biological treatment system: Site-specific applications and costs. Remediation 3: 353-362.

Worne, H.E. and Fortune, I. 1992. Bioremediation of hazardous and toxic wastes (presentation to HMC/SUPERFUND). Epicore Networks Inc. Publication, Canada. pp. 1-28.

Wu, L. and Stahmann, M.A. 1975. Chromatographic estimation of fungal mass in plant materials. Phytopath. 65: 1032-1043.

Zeddel, A., Majcherczyk, A. and Huttermann, A. 1993. Degradation and mineralization of polychlorinated biphenols by white rot fungi in solid phase soil incubation experiments. Pre-print of Proceedings of The Second International Symposium for *In Situ* and On-Site Bioreclamation, San Diego, CA., U.S.A.

ZoBell, C.E. 1946. Cited by Atlas, R.M. 1981. Microbial degradation of petroleum hydrocarbons: An environmental perspective. Microbiol. Rev. 45: 180-209.

APPENDICES

APPENDIX A: Soil Fertility Test

(Conducted by Cedara Agricultural Development Institute, Department of Agriculture, KwaZulu-Natal, P.Bag X9059, Pietermaritzburg, 3201)

Sample parameter	Value
Density	1.19
P	4.6 mg l^{-1}
K	18.3 mg l^{-1}
Ca	25 mg l^{-1}
Mg	4.3 mg l^{-1}
Acidity (Al+H)	0.40
Total cations	0.20 %
Acid saturation	49.3 %
pH (KCl)	3.81
Zn	8.86 mg l^{-1}
NIRS Organic carbon	>5.0 mg kg^{-1}
Total nitrogen	0.069 mg kg^{-1}

APPENDIX B: Complex Mineral Salts Medium (Coutts *et al.*, 1987)

Basal Salts:

K_2HPO_4	1.50 g
$MgCl_2.6H_2O$	0.20 g
$NaH_2PO_4.2H_2O$	0.85 g
Na_2SO_4	1.40 g
NH_4Cl	0.90 g

Sterilise (121°C, 15 min) in 900 ml glass-distilled water

$NaHCO_3$*	0.50 g
Na_2CO_3 *	0.20 g

*Sterilise (121°C, 15 min) separately in 96 ml glass-distilled water.

Trace element solution A (g l^{-1} glass-distilled water):

$FeCl_2H_2O$	1.500
NaCl	1.500
$MnCl_2.4H_2O$	0.197
$CaCl_2.6H_2O$	0.238
$CuCl_2.H_2O$	0.017
$ZnSO_4$	0.287
$AlCl_3$	0.050
H_3BO_3	0.062
$NiCl_2.6H_2O$	0.024
conc HCl	10ml

Trace element solution B (g l^{-1}glass-distilled water):

Na_2MoO_4	0.0484
$Na_2Se0_3.5H_20$	0.0025
$NaNO_3$	0.0033

Vitamins and amino acids (g l^{-1} glass-distilled water):

Biotin	0.010
p-aminobenzoic acid	0.010
Pyridoxine HCl	0.020
Thiamine	0.020
Riboflavin	0.030
Nicotinic acid	0.050

One litre of the complex medium is made by adding: 1ml $NiCl_2.6H_20$ (1 mM);* 1ml Trace element solution A*; 1ml, Trace element solution B*; and 1ml, vitamin/amino acid solution to 996 ml basal salts.

* filter sterilised (0.2 μm, nitrocellulose, Whatman).

APPENDIX C: Soxhlet Oil-Extraction.

This method was used for the solvent extraction of residual hydrocarbons from soil samples for GC analysis.

Materials and Methods:

(1). The soil was sieved and mixed with anhydrous sodium sulphate (Na_2SO_4, 20% w/w);

(2). 100 g samples were placed into cellulose extraction thimbles (Whatman, 40x 120 mm). Six thimbles could be processed in the apparatus (Büchi 810 Soxhlet fat extractor) at once;

(3). 170 ml of dichloromethane were placed in each solvent flasks;

(4). The heating mantle was set at 120°C;

(5).Coil condensers were cooled by a continuous flow of tap water (2 l min^{-1}).This resulted in a solvent recycling time of approximately 12 minutes;

(6).The apparatus was run continuously for six hours. During this time levels in the solvent flasks were monitored and adjusted for solvent losses; and

(7). The volume of each extract was reduced under a stream of nitrogen gas and then diluted with dichloromethane to 10 ml in volumetric flasks.

All the processes above were conducted under a fume extraction hood.

APPENDIX D: Extraction and Analysis of Residual Hydrocarbons From Batch Cultures

This is a modification of Method 506 (Anon.,1983) for determining the hydrocarbon content of groundwater samples by gas chromatography.

Materials and Methods:

(1). The contents of each culture flask (basal salts solution, glass beads and fungal mycelium) were diluted to 150 ml with glass-distilled water;

(2). Six grams of NaCl (reagent grade) and 0.3 ml of concentrated HCl were added to each flask;

(3). 15 ml dichloromethane were added to each flask and the flasks were then placed on an orbital shaker (5°C, 100 r.p.m., 1 h);

(4). The flasks were transferred to an ultra-sonic water bath (Whaledent biosonic) for 15 minutes. This process was included to increase the extraction of hydrocarbons adsorbed to the fungal mycelium;

(5). The contents of the flasks were transferred to 500 ml separating funnels, through conical funnels, which contained a Whatman GF/C glass fibre filter. The GF/C filters were used to retain solid material such as the glass beads and mycelial fragments;

(6). The glass beads and fungal mycelia were rinsed with 10 ml dichloromethane in their original culture flasks. These solvent volumes were then poured back into the separating funnels through the GF/C filters;

(7). The contents of the flasks were shaken vigorously for two minutes. The solvent phases were allowed to separate and were decanted into 150 ml conical flasks;

(8). All glassware was finally rinsed with 10 ml dichloromethane which was poured through the top of the funnels to remove residual hydrocarbons;

(9). Step (7) was repeated;

(10). 5 g of analar grade Na_2SO_4 (anhydrous) were added to the filtrates which were then shaken for 30 seconds;

(11). The solutions were transferred to Eppendorf vials which each contained 1 g of activated Florosil™; and

(13). After 24 hours the vials were centrifuged (10 000 r.p.m.x g, 5 min) and the supernatants were then analysed by GC.

All the processes above were conducted under a fume extraction hood.

The GC (Varian 3600) conditions were as follows:

Split injector at 240°C ;
FID detector at 300°C;
Carrier gas, helium, at 30 ml min^{-1};
Oven conditions: 50°C to 320°C at a ramp rate of 10°C min^{-1} which was held at 320°C for 15 min, total run time of 43 minutes;
Column: J&W DB5, length 30 m, i.d. 0.326 mm; and film thickness 1.0 μm.

A solvent blank was run after every two samples to ensure "ghost" peaks from preceding injections were not causing any interference.

APPENDIX E: Statistical (ANOVA) Analysis of TPH Reduction in Microcosm Trial 3

Table E(i): Anova of TPH reduction

Source of variation	d.f	s.s	m.s.	V.r.	F.pr.
REPS	2	3.011E+05	1.505E+05	0.34	
TREAT	6	4.465E+07	7.441E+06	16.98	<0.001
Contrasts [1] NSC vs Rest	1	2.638E+07	2.638E+07	60.20	<0.001
[2] Tillage/Water vs other supplements	1	1.654E+07	1.654E+07	37.75	<0.001
[3] Straw vs other supplements	1	8.311E+05	8.311E+05	1.90	0.172
[4] Manure vs NPK	1	7.180E+05	7.180E+05	1.64	0.204
Deviations	2	1.792E+05	8.962E+04	0.20	0.815
TIME	6	2.085E+08	3.474E+07	79.29	<0.001
TREAT.TIME	36	4.640E+07	1.289E+06	2.94	<0.001
NSC vs Rest.TIME	6	1.204E+07	2.006E+06	4.58	<0.001
Tillage/Water vs other supplements.TIME	6	1.133E+07	1.889E+06	4.31	<0.001
[5] NPK/Manure vs Manure/NPK/straw. TIME	6	1.038E+07	1.731E+06	3.95	0.001
Deviations	18	1.265E+07	7.028E+05	1.60	0.074
Residual	96	4.207E+07	4.382E+05		
Total	143	6.419E+08			

Table E(ii): Standard errors and coefficients of variation

Stratum	d.f.	s.e.	cv%
REPS	2	55.4	1.2
REPS. Units	96	662.0	14.8

Table E(iii): Comparison of means within contrast groupings

Contrast group [A]	Contrast group [B]	Group [A] mean (mg kg^{-1} soil)	Group [B] mean (mg kg^{-1} soil)
NSC	Rest	5507	4296
Tillage and water	Supplements	5107	4134
Straw	Other supplements	4312	4090
Manure	NPK	3997	4182
Manure/NPK only	Manure/NPK with straw	4069	4111

APPENDIX F: Statistical (ANOVA) Analysis of Fluorescein Absorbance Data in Microbial Activity (FDA) Determinations for Microcosm Trial 3.

Table F(i): Anova of microbial activity (FDA) determinations (Data skew).

Source of variation	d.f	s.s	m.s.	V.r.	F.pr.
REPS	2	1.19	0.0020834	0.0010417	
TREAT	6	0.7601046	0.1266841	144.37	<0.001
Contrasts [1] NSC vs Rest	1	0.1557351	0.1557351	177.47	<0.001
[2] Tillage/Water vs other supplements	1	0.0856567	0.0856567	97.61	<0.001
[3] Straw vs other supplements	1	0.0918489	0.0918489	104.67	<0.001
[4] Manure vs NPK	1	0.3737335	0.3737335	425.90	<0.001
Deviations	2	0.0531304	0.0265652	30.27	<0.001
TIME	6	0.5943483	0.0990580	112.88	<0.001
TREAT.TIME	36	0.7511680	0.0208658	23.78	<0.001
NSC vs Rest.TIME	6	0.0820201	0.0136700	15.58	<0.001
Tillage/Water vs other supplements.TIME	6	0.1114731	0.0185789	21.17	<0.001
[5] NPK/Manure vs Manure/NPK/straw. TIME	6	0.0732551	0.0122092	13.91	<0.001
Deviations	18	0.4844196	0.0269122	30.67	<0.001
Residual	96	0.0842420	0.0008775		
Total	146	2.1919460			

Table F(ii): Standard errors and coefficients of variation

Stratum	d.f.	s.e.	cv%
REPS	2	0.00461	3.9
REPS.Units	96	0.02962	25.1

Table F(iii): Comparison of means within contrast groupings

Contrast group [A]	Contrast group [B]	Group [A] mean (absorbance)	Group [B] mean (absorbance)
NSC	Rest	0.0385	0.1315
Tillage and water	Supplements	0.0732	0.1432
Straw	Other supplements	0.0840	0.1579
Manure	NPK	0.2246	0.0912
Manure/NPK only	Manure/NPK with straw	0.1343	0.1816

APPENDIX G: Statistical (ANOVA) analysis of TPH Reduction in Microcosm Trial 4

Table G(i): Anova of TPH reduction

Source of variation	d.f	s.s	m.s.	V.r.	F.pr.
REPS	2	1.328E+05	6.640E+04	0.85	
TREAT	4	3.635E+06	9.087E+05	11.70	<0.001
Contrasts [1] NSC vs Rest	1	2.331E+06	2.331E+06	30.00	<0.001
[2] Wood-chips vs supplements	1	1.197E+06	1.197E+06	15.41	<0.001
[3] SMS vs Mushroom compost	1	4.158E+04	4.158E+04	0.54	0.467
[4] Wood-chips vs Manure/wood-chips mixture	1	6.517E+04	6.517E+04	0.84	0.363
Deviations TIME	6	2.629E+07	4.382E+06	56.41	<.001
TREAT.TIME	24	2.980E+06	1.242E+05	1.60	0.068
NSC vs Rest.TIME	6	7.013E+05	1.169E+05	1.50	0.190
Wood-chips vs supplements.TIME	6	1.862E+06	3.103E+05	3.99	0.002
SMS vs Mushroom compost.TIME	6	1.575E+05	2.625E+04	0.34	0.914
Deviations	6	2.594E+05	4.324E+04	0.56	0.763
Residual	68	5.282E+06	7.768E+04		
Total	104	3.832E+07			

Table G(ii): Standard errors and coefficients of variation

Stratum	d.f.	s.e.	cv%
REPS	2	43.6	4.3
REPS.Units	68	278.7	27.8

Table G(iii): Comparison of means within contrast groupings

Contrast group [A]	Contrast group [B]	Group [A] mean (mg kg^{-1} soil)	Group [B] mean (mg kg^{-1} soil)
NSC	Rest	1300	928
Wood-chips	Supplements	1135	859
SMS	Mushroom compost	425	456
Wood-chips	Wood-chips and manure	567	407

APPENDIX H: Statistical (ANOVA) Analysis of Fluorescein Absorbance Data in Microbial Activity (FDA) Determinations for Microcosm Trial 4.

Table H(i): Anova of microbial activity (FDA) determinations

Source of variation	d.f	s.s	m.s.	V.r.	F.pr.
REPS	2	0.007676	0.003838	0.90	
TREAT	4	17.105371	4.276343	1007.54	<0.001
Contrasts [1] NSC vs Rest	1	6.012532	6.012532	416.60	<0.001
[2] Wood-chips vs supplements	1	9.098720	9.098720	2143.74	<0.001
[3] SMS vs Mushroom compost	1	1.988168	1.988168	468.43	<0.001
[4] Wood-chip vs Manure/wood-chips mixture	1	0.005952	0.005952	1.40	0.240
Deviations TIME	6	5.728408	0.954735	224.94	<0.001
TREAT.TIME	24	4.491823	0.187159	44.10	<0.001
NSC vs Rest.TIME	6	1.478358	0.246393	58.05	<0.001
Wood-chips vs supplements.TIME	6	2.142822	0.357137	84.14	<0.001
SMS vs Mushroom compost.TIME	6	0.703240	0.117207	27.61	<0.001
Deviations	6	0.167403	0.027901	6.57	<0.001
Residual	68	0.288614	0.004244		
Total	104	27.621893			

Table H(ii): Standard errors and coefficients of variation

Stratum	d.f.	s.e.	cv%
REPS	2	0.01047	1.7
REPS.Units	68	0.06515	10.3

Table H(iii): Comparison of means within contrast groupings

Contrast group [A]	Contrast group [B]	Group [A] mean (absorbance)	Group [B] mean (absorbance)
NSC	Rest	0.1521	0.7503
Wood-chips	Supplements	0.1803	0.9403
SMS	Mushroom compost	0.5755	0.3580
Wood-chips	Wood-chips and manure	0.0901	0.4770

APPENDIX I: Estimation of Total Nitrogen Concentration in Soils and Sediments

This method was based on the method described by Nelson and Sommers (1972).

Materials and Methods:

(1). The soil sample (5 g) was ground with a mortar and pestle and sieved (500 μm mesh);

(2).Three sub-samples (0.20 g) of the sieved soil were weighed into Folin-Wu digestion tubes;

(3). 1.1 g of a catalyst (K_2SO_4, $CuSO_4.5H_2O$ and Se, 100:10:1 w/w) and 3 ml of concentrated sulphuric acidwere added to each digestion tube;

(4). The digestion tubes were placed into a pre-heated (>350°C) digestion block for three hours;

(5). The tubes were removed from the digestion block and allowed to cool;

(6). The residues in each digestion tube were diluted to 50 ml with glass-distilled water in volumetric flasks; and

(7). The volumetric flasks were then sealed and stored at 5°C until ammonium determinations were made (<48 h).

Calibration standards and measurement of soil samples

(1). A series of ammonia standards (viz. 10^{-1}M, 10^{-2}M, 10^{-3}M ,10^{-4}M, 10^{-5}M)

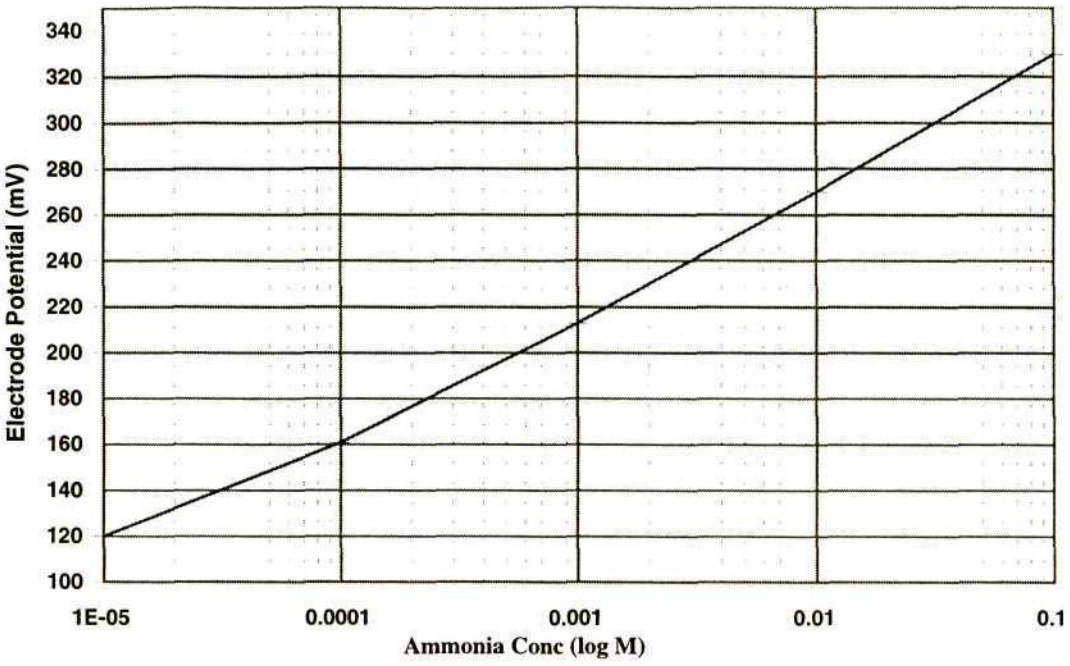

Fig. I.1. Ammonia standard curve.

were made with an appropriate dilution of a NH_4Cl (0.1 M) solution. Dilutions of the ammonia standard corresponded to the nitrogen concentrations in Table I.1 below:

Table I.1: Ammonia to nitrogen concentration conversion factors

Moles l^{-1}	**ppm N**
10^{-5}	0.14
10^{-4}	1.4
10^{-3}	14
10^{-2}	140
10^{-1}	400

(2). 5 ml of NaOH (1 N) were added a 50 ml sample so that the pH of the solution was >12.5. This was checked with pH test strips. Highly acidic samples required more than 5 ml NaOH; and

(3). After approximately 45 seconds of continuous stirring (magnetic stirrer) the electrode potential of each sample solution was recorded with an ammonia probe (Orion 95-12 Ammonia Electrode and 701A/Digital Ionalyser).

The standard curve, from which unknown sample values were determined, is presented in Figure I.1: