

**THE MICROBIOLOGY OF *EX SITU* BIOREMEDIATION
OF PETROLEUM HYDROCARBON - CONTAMINATED SOIL**

by

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ABSTRACT

Bioremediation is the process whereby the degradation of organic polluting compounds occurs as a result of biochemical activity of macro- and microorganisms. Bioremediation of hydrocarbon contaminated soils can be practised *in situ* or *ex situ* by either stimulating the indigenous microorganisms (biostimulation) or introducing adapted microorganisms which specifically degrade a contaminant (bioaugmentation).

This investigation focused on *ex situ* remediation processes with special attention to the processes and microbiology of landfarming and thermal bioventing. Landfarming was investigated at pilot-scale and full-scale, and thermal bioventing at laboratory and pilot-scale.

This study indicated that pilot-scale bioremediation by landfarming was capable of effecting a total petroleum hydrocarbon concentration (TPHC) reduction of 94% (m/m) from an initial concentration of 320 gkg⁻¹ soil to 18 gkg⁻¹ soil over a period of 10 weeks. Reactors receiving biosupplements showed greater rates of bioremediation than those receiving nutrients. Promotion of TPHC catabolism by addition of a commercial or a site-specific microbial biosupplement was similar. Seedling experiments proved that bioremediation did not necessarily leave the soil in an optimal condition for plant growth.

The full-scale landfarming operation reduced the TPHC concentrations from 5 260 - 23 000 mgkg⁻¹ to 820 - 2 335 mgkg⁻¹ soil over a period of 169 days. At full-scale, the larger

fraction of more recalcitrant and weathered petroleums, and the less intensive treatment resulted in a slower rate of TPHC reduction than was found in the pilot-scale study. Three distinct decreases in the TPHC were observed during the full-scale treatment. These presented an ideal opportunity to investigate the microbiology of the soil undergoing treatment. The dominant culturable microorganisms were isolated and identified. The bioremediation process was dominated by *Bacillus* and *Pseudomonas* species. The method used to study the population was, however, biased to culturable, fast growing microorganisms which represent a small portion of the total microbial population. For this reason, a method to study the total eubacterial population *in situ* with rRNA targeted oligonucleotide probes was adapted and found to be a valuable technique.

Soil microorganisms respiratory activity was investigated at different times in the full-scale treatment. A clear correlation between activity and degradation was recorded. The effect of a supplement, anaerobically digested sludge, was also assessed by this method.

Thermal bioventing was investigated as an *ex situ* in-vessel treatment technology for small volumes of highly contaminated soils. This proved to be a viable technique for the bioremediation of petroleum hydrocarbons at laboratory-scale. Volatilisation contributed to at least 40% of the reduction. Of the two supplements evaluated, dried sludge promoted degradation to a greater extent than chicken manure.

The pilot-scale study proved that a chemical contaminant reduction of at least 50% could be achieved in 13 weeks by thermal bioventing. Of the supplemented reactors, the presence of

dried sludge and commercial biosupplement effected the largest contaminant decrease. As a possible supplement to increase the rate of bioremediation, dried anaerobically digested sludge was more effective than chicken manure. A parallel laboratory-scale experiment gave similar results. Gravimetric analyses were found to be conservative indications of the remediation process.

The results of this study shed some light on our, still, limited understanding of bioremediation. The gap between the technology in the laboratory and field was narrowed and a better understanding of the soil microbiology was achieved. Due to the limited control of environmental parameters in the case of landfarming, thermal bioventing was investigated and proved to be an effective alternative. The latter technology is novel in Southern Africa.

There are no mistakes, only lessons:

Growth is a process of trial and error: Experimentation.


The “failed” experiments are as much part of the process
as the experiment that ultimately “works”.

Chérie Carter-Scott

Thank you for the opportunity to make mistakes, to learn and grow.

DECLARATION

I hereby certify that this research, unless specifically indicated to the contrary in the text, is the result of my own investigation.


Heidi G. Snyman

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And lastly,

I thank my Creator.

ABBREVIATIONS

Chemical symbols for the elements are not included in the list of abbreviations below.

ARC	Agricultural Research Council
ATP	Adenosine Triphosphate
a_w	water activity
BD	Below Detection
BOD	Biochemical Oxygen Demand
BTEX	Benzene, Toluene, Ethyl benzene and Xylene
°C	Celsius degrees
cfu	colony forming units
cm d ⁻¹	centimetre per day
C/N	Carbon to Nitrogen ratio
C:N:P	Carbon to Nitrogen to Phosphorus ratio
COD	Chemical Oxygen Demand
CSIR	Council for Industrial and Scientific Research
d	Day(s)
2,4-D	2,4-dichlorophenoxyacetic acid
DCM	Dichloromethane
DDT	Dichlorodiphenyl-trichloroethane
DOC	Dissolved Organic Carbon
DNA	Deoxyribonucleic acid
DWAF	Department of Water Affairs and Forestry
E_h	Redox potential
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency (USA)
°F	° Fahrenheit
FID	Flame Ionization Detector
GC	Gas Chromatograph
HC	Hydrocarbon

HP	Hewlett Packard
HPLC	High Performance Liquid Chromatography
IR	Infra Red
K	Biokinetic constant
kg	kilogram
kl	kilolitre
kPa	kilo Pascal
kV	kilovolts
ℓ	litre
LAAP	Louisiana Army Ammunition Plant
lbsi	pounds per square inch
m	metre
MAP	Mono-ammonium Phosphate
mg	milligram
min	minutes
mℓ	millilitre
MLSS	Mixed Liquor Suspended Solids
m/m	mass per mass
mm	millimetre
mo	month
MS	Mass Spectrometer
m/v	mass per volume
NA	Nutrient Agar
no.	number
OECD	Organisation for Economic Cooperation & Development
PAH	Polycyclic Aromatic Hydrocarbon
PBS	Phosphate Buffered Saline
PCB	Polychlorinated Biphenyls
PDA	Potato Dextrose Agar
PHC	Petroleum Hydrocarbon Concentration
ppm	parts per million

Pt	Point
PV	Pore Volume
PVC	Polyvinyl chloride
PWV	Pretoria, Witwatersrand & Vereniging
RNA	Ribonucleic acid
RSA	Republic of South Africa
rRNA	ribosomal RNA
SEA	Soil Extract Agar
sec	second
SDS	Sodiumdodecylsulphate
SEM	Scanning Electron Microscopy
SIM	Selected Ion Mode
TPHC	Total Petroleum Hydrocarbon Concentration
TRIS	N-Tris(hydroxymethyl)aminomethane
$\mu\ell$	microlitre
μm	micrometre
USA	United States of America
VOC	Volatile Organic Carbons
v/v	volume per volume
wk(s)	week(s)
WRF	White-rot Fungus
w/w	weight per weight
XRF	X-ray Fluorescence

CHAPTER 1

INTRODUCTION

Bioremediation is the process whereby the degradation of polluting compounds occur as a result of biochemical activity of macro- and microorganisms (Mason, Sticher & Hamer, 1992; Swett, 1992. Pearce; Snyman, Van Heerden, Greben & Oellermann, 1996).

Hazardous organic chemicals are mineralized aerobically to carbon dioxide and water, and anaerobically to methane and carbon dioxide.

The petrochemical industry is a well developed economic sector in South Africa.

Notwithstanding the accidental spills and leaks that can happen on site, petrochemical products often have to be transported long distances from the refineries and harbours, inland to industrial areas, particularly in the province of Gauteng (formally known as the Pretoria, Witwatersrand & Vereniging (PWV) region). Thus, a risk factor exists for accidental spills or pipeline/tank leaks. In addition to accidents and spills, hazardous waste is increasingly posing health and environmental problems in South Africa since technologies for cleaner production and waste minimization results in more concentrated wastes. In the past, landfilling and incineration seemed to be an acceptable expedient. However, since the available landfill sites in South Africa are being depleted rapidly, the need for other and more effective cleanup technologies has led to increased interest in bioremediation technologies. Throughout the world, governments, international organisations and major corporations, as well as ordinary citizens, are insisting that planning and decision making

must take cognizance of the impacts of human actions on the environment (Fuggle & Rabie, 1992). The advantages of biological treatment strategies, compared with physical and chemical methods such as incineration and solvent extraction, lie in reduced cost and likelihood that either toxic compounds or derivatives are produced (Mason *et al.*, 1992; Rubin, Buckner-Powers & Setzer, 1992). The rising popularity of biological treatment technologies is substantiated by the projected growth of 15.8% in the US bioremediation market, from \$228 million in 1995 to \$ 475 million by the year 2000 (Business Trends, 1996). A survey investigating the nature and extent of contaminated sites in South Africa revealed a total of 78 contaminated sites, much of which were contaminated with petroleum products (Pearce, Snyman, Van Heerden, Greben & Oellermann, 1995). This dissertation focuses on the bioremediation of such petroleum-contaminated sites.

1.1 PURPOSE OF THE STUDY

The main purpose of the study was to investigate the feasibility of two *ex situ* technologies, landfarming and thermal bioventing, for South African conditions. Since these technologies are underpinned by microbiological processes, several techniques were used to investigate the microbial population in the soil. The understanding of the bioremediation process and the adaptation of laboratory techniques facilitated the development of a short duration assessment of a contaminant's biodegradability in which the feasibility of bioremediation for a specific case could be assessed.

1.2 SCOPE AND STRUCTURING OF THIS THESIS

Innovative technologies, particularly biotechnologies, appear to be a solution to many of the environmental problems facing us today. Bioremediation is one of the new technologies which holds great potential (Nichols, 1992). Bioremediation was identified as a novel technology for South Africa (Lees & Senior, 1992). A survey made in 1994 identified 28 sites in South Africa where bioremediation had been used in recent years (Pearce *et al.*, 1995). The feasibility of *in situ* bioremediation is under investigation at the University of Natal, Pietermaritzburg and the CSIR and is not included in the present study. This investigation focuses on *ex situ* remediation processes with special attention to landfarming and thermal bioventing (Figure 1.1). Landfarming was investigated at pilot-scale and full-scale, and thermal bioventing at laboratory and pilot-scale. Results from a pilot-scale study were extrapolated and compared to the results obtained from a full-scale site investigation which resulted in the successful treatment of the petroleum hydrocarbon contaminated soil. The culturable and total microbial populations present in the soil during the full-scale landfarming operation were investigated and respiration studies made to establish the correlation between degradation rate, respiration and culturable microbial numbers.

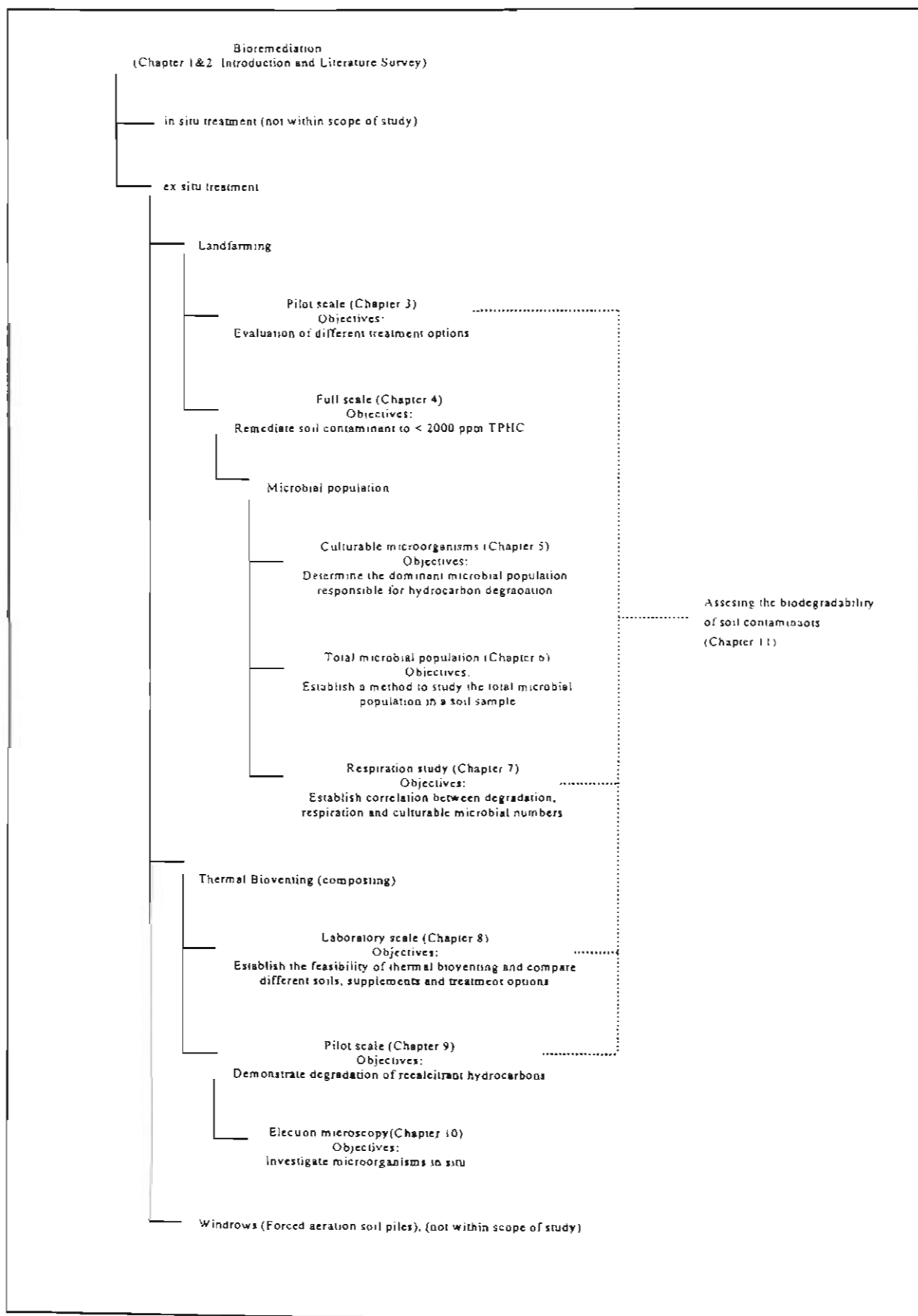


FIG. 1.1 Scope and structure of this thesis.

Due to the limited control of environmental parameters in the case of landfarming, thermal bioventing was investigated as an alternative technology. This technology is novel in Southern Africa. The feasibility of thermal bioventing was investigated at laboratory and pilot-scale (Figure 1.1). In the laboratory study, different soil types, treatment options and amendments were compared. Based on these results, a pilot-scale experiment was made and the microorganisms studied *in situ* by scanning electron microscopy.

In the field of bioremediation, as seen on numerous occasions in this study, remedial technologists need to know the extent of biodegradability or treatability of the contaminant. This can be approached by laboratory simulations and treatment optimisations such as those described in this thesis, or by assessing the biodegradability of the contaminant by standard or adapted laboratory screening tests. In this thesis, a biodegradability assessment test is suggested and the limitations discussed.

CHAPTER 2

BIOREMEDIATION OF PETROLEUM-CONTAMINATED SOILS: LITERATURE SURVEY

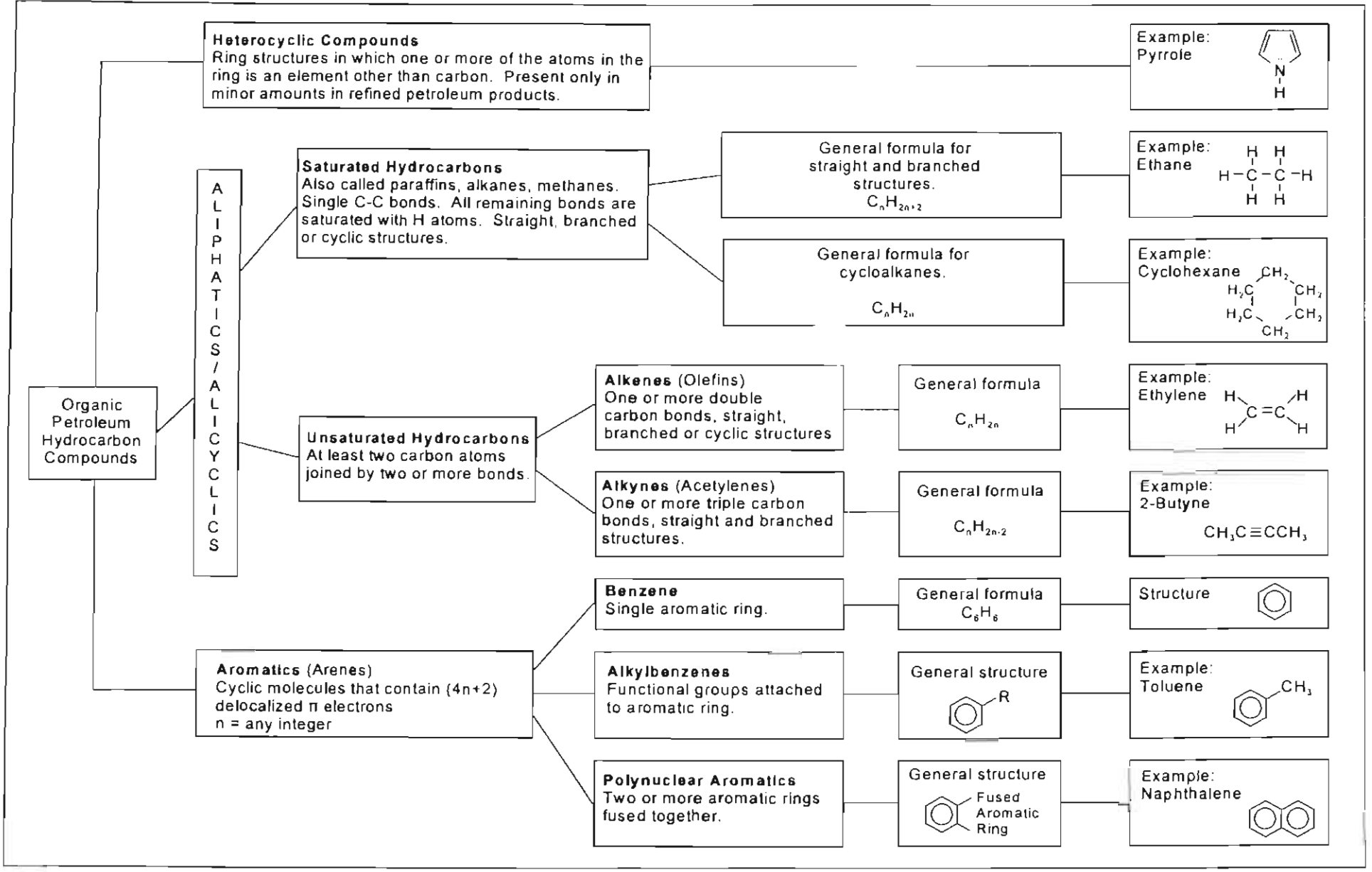
2.1 INTRODUCTION

Hydrocarbon contaminants, typically, include gasoline, diesel, jet fuel and petroleum based oils, but can also include wood processing chemicals such as creosote, pesticides, herbicides and organic solvents.

Figure 2.1 illustrates the many different compounds found in petroleum hydrocarbons and the broad classes in which they can be grouped. In a typical gasoline sample, over 100 individual hydrocarbon compounds can be found. A measurement of these hydrocarbons is expressed as the Total Petroleum Hydrocarbon (TPH). TPH can broadly be divided into aromatic and aliphatic compounds. Aromatic compounds contain one or more benzene rings and can have a functional group attached to the ring. Aromatic compounds of particular interest include benzene, toluene, ethyl benzene, and xylene which are collectively referred to as BTEX. High soil concentrations of BTEX are a good indication of fresh (unweathered) gasoline contamination. Also of interest are polynuclear aromatic hydrocarbons (containing two or more fused benzene rings), referred to as PNAs or PAHs, such as naphthalene, anthracene and benzopyrenes. High soil concentrations of PAHs are a good indication of diesel or heavier petroleum contamination (Schwerko, 1993).

FIG. 2.1

A breakdown of the typical composition of Total Petroleum Hydrocarbons



Aliphatics and alicyclics are straight chain (aliphatic) or closed ring (alicyclic) non-aromatic hydrocarbons. Both aliphatics and alicyclics can contain branched structures and single, double, or triple carbon-carbon bonds. Included in this group are alkanes (or paraffins), cycloalkanes, alkenes (or olefins), cycloalkenes and alkynes (or acetylenes) (Schwerko, 1993).

Microorganisms may attack hazardous organic molecules in one of three ways (Bennett & Olmstead, 1992):

- Mineralize the compound directly, which means that the compound is converted to harmless inorganic molecules such as carbon dioxide, water and salts;
- Degrade the compound as a co-metabolite, which means that the microorganisms require some other organic compound(s) for growth or to induce formation of the enzymes required for degradation of the target compound; and
- Convert the compound to an intermediate, which may also be toxic and may be recalcitrant to further degradation.

The first scenario is clearly the most desirable.

The degradation of hydrocarbons may be considered as a multi-step process. Hydrocarbon uptake is an essential first step for the microorganism to catabolise both straight chain (labile) and ring (semi-recalcitrant) compounds. Extracellular surfactants may assist in this process (Jackson, 1993). The initial steps in the catabolism of aliphatic, cyclic and aromatic

hydrocarbons by bacteria and fungi involve the oxidation of the substrate by oxygenases, for which molecular oxygen is required. Aerobic conditions are, therefore, essential (Leahy & Colwell, 1990). Once the hydrocarbon constituents have been catabolized to smaller molecules they are utilized in metabolism to obtain the energy required for microbial growth and division. If the process is permitted to go to completion, the hydrocarbons are completely degraded (mineralized). This is followed by a rapid decline in the viable population density in response to the removal of the most prevalent carbon sources (Jackson, 1993).

For the biological treatment to be effective, optimal conditions for microbial growth must be created. Major factors which are considered include pH, oxygen availability, concentration and availability of inorganic nutrients (primarily nitrogen in the form of ammonia and phosphorus as orthophosphate), E_h (redox potential), water activity (a_w) and temperature. For any biological treatment system to function effectively it is important that the only limiting factor for biological growth is the availability of labile organic substrates. Thus, all nutrients and oxygen should be available in concentrations in excess of those required for metabolism of the target molecule(s) (Galaska, Skladany & Nyer, 1990).

2.2 BIOREMEDIATION TECHNOLOGIES

Bioremediation can be practised by a number of technologies. The contaminated soil can be treated *in situ* or can be excavated and treated elsewhere (*ex situ*). Because excavation and earthmoving is omitted when using an *in situ* bioremediation technology, it is generally

more cost effective. However, before the technical approach is selected, the following issues need to be evaluated:

- Types and concentrations of contaminants:
- The contaminated site, especially physical and chemical parameters including seasonal factors such as temperature; geological and geohydrological factors;
- Microbiological and biochemical characteristics, especially consideration of whether or not known mechanisms for biodegradation can be applied; and
- Other factors, such as legal and regulatory matters, public, social and political concerns, process economics and overall business objectives (Slater, 1992; Leahy & Brown, 1994).

2.2.1 *In situ* remediation technologies

In situ strategies imply that the contaminated soil is treated in place and essentially remains undisturbed. The most common form of *in situ* treatment is the biodegradation of contaminants within the saturated zone of the soil (Wilson & Jones, 1993). *In situ* technologies which have been used to remediate contaminated sites include soil washing, low temperature thermal treatment, soil venting, bioventing, enhanced bioreclamation, bioslurping and passive remediation.

i. Soil washing

This method involves the injection of a synthetic surfactant or solvent into the contaminated zone to promote release of hydrophobic contaminants to the aqueous phase (Hoeppel, Hinchee & Arthur, 1991). Arthur, O'Brien, Marsh & Zwick (1989) claimed that this technology had been implemented with limited success. A total of 53 synthetic surfactants were screened for their ability to enhance natural biodegradation rates in jet fuel contaminated soils and neither promotion nor inhibition were recorded. Tiehm (1994), however, proved that synthetic surfactants could indeed enhance the degradation of polycyclic aromatic hydrocarbons (PAHs) as long as the surfactants were non-toxic and non-ionic. Sodium dodecyl sulphate (SDS), an ionic surfactant, hampered degradation of the PAHs since it was preferred as a growth substrate.

ii. Low temperature thermal methods

These techniques include heated gas or steam injection into and radio frequency heating of soils. These methods are costly, due to the energy and equipment needed, and are therefore not used routinely to remediate hydrocarbon spillages (Hoeppel *et al.*, 1991).

iii. Soil venting

This process, which is also known as soil vapour extraction or *in situ* soil stripping, involves the controlled flow of air saturated with the volatile and some semi-volatile contaminants

from the soil under the influence of a vacuum applied to the vadose zone. The gas leaving the soil may be treated to recover or destroy the contaminants, e.g. by condensation, activated carbon adsorption, biofiltration, or thermal treatment (Hoeppel *et al.*, 1991; Goldfarb & Vogel, 1994). The technology is attractive since it has potential to treat contaminated soil and remove volatile organic carbons (VOCs) with less capital and operational costs than conventional decontamination methods such as excavation and disposal, or *in situ* solvent washing (Cho, Kampbell, Wilson & DiGuilio, 1990). This technique is widely used for removing volatile hydrocarbons from the subsurface, especially from the vadose zone.

iv. *Bioventing*

Soil vapour extraction and bioventing are often described as the same technology. However, these processes differ considerably. Bioventing actually improves on vacuum extraction by increasing the flow of air through the soil to speed the removal of organic compounds. Degradation is, therefore, enhanced by the indigenous microorganisms *in situ* (Hoeppel *et al.*, 1991; Torma, 1994). Thus, unlike soil vacuum extraction technologies, bioventing attempts to stimulate the biodegradative activity while minimizing stripping of volatile organics (Sayles, Brenner, Hincbee, Leeson, Vogel & Miller, 1994). The major advantage of this process is that the unsaturated zone of the contaminated soil is used as a bioreactor (Cho *et al.*, 1990) thus eliminating the additional costs of an above ground treatment facility for the off-gasses. Despite this, the remediation rates are slow, typically 2 to 6 mgkg⁻¹day⁻¹, and should be considered when exploring bioventing as a treatment option

(Sayles *et al.*, 1994). The specific degradation rate and clean up time can be estimated by determining the oxygen utilization rate of the indigenous microorganisms in the contaminated plume, by an *in situ* feasibility test (Pearce, Van Heerden, Snyman & Whyte, 1996).

v. *Enhanced bioreclamation*

This technique involves the stimulation of the metabolic capabilities of soil microorganisms which degrade or detoxify contaminants residing within the soil or groundwater (Hoepfel *et al.*, 1991). Two methods are commonly used, landfarming and bioaugmentation. These techniques can also be employed *ex situ*. *In situ* biodegradation (landfarming) attempts to enhance the activity of naturally present microorganisms, by either the addition of suitable additional nutrients which were otherwise limiting, and/or increasing their numbers (Bradford & Krishnamoorthy, 1991; Mason *et al.*, 1992). Bioaugmentation involves the introduction of specially adapted or engineered microorganisms known for their ability to degrade a particular compound to the contaminated soil (Mason *et al.*, 1992). Most USA remediation experts avoid the use of engineered microorganisms, in response to the strict federal rules on their use and the fact that indigenous microorganisms catabolize most contaminants (Rubin *et al.* 1992). Landfarming has the virtue of simplicity and consequent modest capital and operational costs. Unfortunately it is encumbered by the problems of land availability (in the case of off-site treatment) and a serious potential for contaminating water, air and soil (Savage, Diaz & Golueke, 1985b).

vi. *Bioslurping*

Bioslurping is a new approach to remediation at petroleum-release sites which have free phase light, nonaqueous-phase, liquid contamination. Bioslurper systems are designed to recover the free product via vacuum-enhanced pumping, while simultaneously initiating the remediation of the vadose zone soils via bioventing (Kittel, Hinchee, Hoepfel & Miller, 1995).

vii. *Passive remediation*

Passive remediation, also called intrinsic bioremediation, developed from the recognition that biological, physical and chemical processes are constantly operating in nature. Under specific conditions, untouched contaminants undergo natural degradation which reduces contaminant concentrations to acceptable levels. Passive remediation should not be confused with "no-action" alternatives since it entails substantial site reviewing and assessment, analytical investigations and monitoring (Nelson, 1994).

2.2.2 *Ex situ* remediation technologies

Ex situ techniques are applied to overcome the limitations of on-site or *in situ* techniques. These limitations may arise from the difficulty of establishing and maintaining the association of microorganisms needed due to problems with supplemental nutrient delivery and mixing. For example, bacteria applied to oil deposited on coastal, intertidal regions

would be rapidly washed away from the site of pollution. In some cases, it may be necessary to remove the contaminated soil/sediment from the site, because of a human health threat or when the water table is such that there is a possibility of contamination through seepage. Most of the *in situ* treatments can also be made *ex situ* by excavating the soil and treating it elsewhere. There are cases where vessels or reactors are used such as slurry reactors and soil columns. Slurry-phase bioremediation is a process whereby contaminated soils are treated as aqueous slurries in large bioreactor systems usually situated close to the contaminated sites. The technology allows for intimate mixing and contact of microorganisms with the contaminants and provides the best environmental conditions for microbial biodegradation of target contaminants (Stegmann, Goede & Ginster, 1994). Britto, Sherrard & Truax (1992) developed an effective continuous flow bioreactor treatment for petroleum-contaminated soils.

Some of the more recent popular approaches to site remediation are solidification or stabilization. In solidification, hazardous wastes are encapsulated in a solid matrix to prevent the waste constituents from leaching out and polluting surrounding soil and groundwater. In stabilization, the waste is modified in such a way that it no longer poses a leaching threat to the environment. These technological alternatives are used on inorganic metals rather than complex organic compounds (Olfenbuttel, 1991). However, these technologies do not provide a guarantee that, due to corrosion of the solid matrix or destabilization with time, leaching of the hazardous wastes can not take place.

Environmental contamination could, therefore, reoccur later.

In the next section a promising new technique is discussed which is also an *ex situ* treatment for contaminated soils. Although the technique is often called *composting* in the literature, this term causes confusion and will be referred to as *thermal bioventing* in this thesis.

2.3 THERMAL BIOVENTING

Thermal bioventing is an aerobic solid-phase bioremediation technique in which the contaminated soil is mixed with a bulking agent such as wood chips or straw to provide porosity for air flow. Oxygen can be provided by forced-air fans or by mechanically turning the pile (Bradford & Krishnamoorthy, 1991).

Composting is widely used to treat wastewater sludges, processing wastes and municipal refuse. Applications of composting to hazardous wastes are few. According to Savage *et al.* (1985b) the first report was made by Rose & Mercer (1968) who investigated the use of composting to degrade insecticides in agricultural wastes.

The three basic technologies used for composting solid wastes include windrows, aerated static piles and in-vessel composting (Davis & Russell, 1993). The windrows and aerated static pile processes are used most frequently for municipal sludge composting. The steps to follow in both processes are similar. In the windrow method, oxygen is drawn into the pile by natural convection and mechanical turning of the compost. In a static pile, aeration is induced by forced air circulation. In the windrows composting process, the composting mixture is placed in long parallel rows called windrows. As with other composting systems,

heat is generated through aerobic microbial metabolism. Since temperature control is affected primarily by mechanical turning of the compost, closely regulated temperature regimes in such a system may be difficult to achieve. In aerated static pile composting, the material to be composted is mixed with a bulking agent, commonly wood chips, and formed into a pile. The pile is placed over an aeration system comprised of blowers and piping, and air is forced or drawn through the pile to provide oxygen and to regulate the temperature. Depending on the design and operating parameters of the aeration system, more precise temperature control may be possible with this approach compared to windrows composting.

Mechanical composting is accomplished inside an enclosed vessel. The system provides a higher degree of process control and better odour control compared to open windrow or aerated static pile systems. The primary differences among mechanical composting systems are the methods of process control. Some provide aeration by tumbling or dropping the materials from one level to another. Other systems use devices such as augers, to stir the composting mass, or rotating drums, to enhance mixing and aeration (Davis & Russell, 1993).

Each method has the advantages and disadvantages usually characteristic of their application with conventional wastes. In the selection of a particular system for composting a hazardous waste, these advantages must be considered in terms of their effects on the control of emissions from the composting operation. One of the more important departures from the composting of conventional wastes is the need for careful control of all emissions and discharges from the operation. The three broad types of composting systems are

equally amenable to the effective control of solid and liquid emissions and discharges.

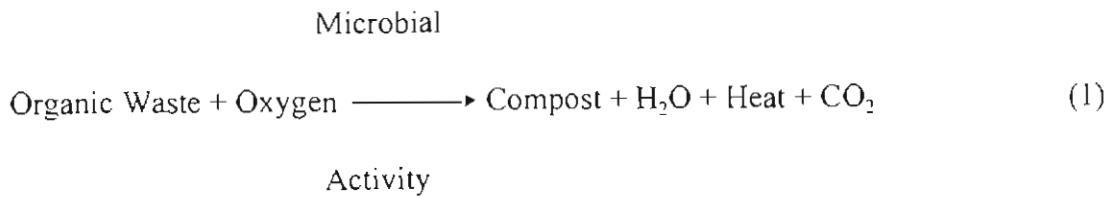
Obviously, the degree of importance of emission control depends upon the severity of the hazard posed by the discharges and emissions on health and the environment. If the hazard is deemed serious, the selected technology should involve an in-vessel system. Forced aeration should thus include a suction phase where all air is passed through a single duct and treated to remove the hazard (Savage *et al.*, 1985b).

The primary benefits gained by traditional composting are reductions in the volume and moisture content of the waste, destruction of pathogens and odour producing nitrogen- and sulphur-containing compounds, and stabilization of the waste for ultimate disposal or use as a marketable product.

In contrast, the objective of hazardous material composting is solely to convert hazardous substances into innocuous end products. This shift in objective has several important consequences. For example, operating parameters such as treatment time may need to be modified and more carefully controlled to ensure acceptable contaminant destruction.

The potential for bioremediation of contaminated environmental matrices by thermal bioventing is promising primarily because of the intensity of the microbial activity within a composting matrix. This activity is facilitated by the, generally, warm, moist, aerobic, and nutrient and carbon rich environment. The production of metabolic heat together with the insulative properties of the physical matrix create a self-heating environment that serves to further stimulate microbial activity (Williams & Myler, 1990). The overall composting

process can be represented as follows:



According to Williams & Myler (1990), the overall transformation potential for contaminants within a composting mass is worthy of consideration for several reasons. First, elevated (thermophilic) temperatures facilitate a higher reaction rate than that generally achievable with ambient conditions. Second, the opportunity for co-oxidation (degradation of a semi-recalcitrant compound while a microorganism obtains carbon and energy from more labile compounds) is enhanced due to the range of alternative substrates present and the high level of metabolic activity. Third, the changing physical/chemical microenvironment within a composting mass results in a diversity of microbial communities and metabolic activity thereby increasing the number and type of microorganisms to which a contaminant is exposed. Finally, elevated temperatures, typically, result in increased contaminant solubility and higher mass transfer rates, making hazardous chemicals available for metabolism at increased concentrations (Williams & Myler, 1990). The pressure also plays an important role in the solubility of gas-liquid systems which exist in the soil/liquid/gas system. At a given temperature, a rise in pressure increases the solubility of the hydrocarbon gasses. At these low pressures, the gas solubility is directly proportional to pressure (Henry's law) (Masterton, Slowinski & Staninski, 1985). The primary advantage of composting compared with traditional bioremediation is that the toxic affects of the contaminated soil can be minimized or reduced by adding a soil supplement or bulking

agent under controlled conditions (Davis & Russell, 1993). Savage *et al.* (1985b) also emphasized the advantages and rationale of this method of bioremediation. The authors concluded that the successful result attained in the landfarming of hazardous wastes is indicative of the potential of composting in the destruction of hazardous wastes. This success can be extrapolated to composting because the microbiology and the biological processes involved in both systems are comparable. For example, pseudomonads, which are most active in land treatment are among the prominent microorganisms in composting. This method also combines many of the good points of incineration and landfarming and minimizes their disadvantages (Savage *et al.*, 1985b).

2.3.1 Feasibility of the technology

The application of composting to the treatment of hazardous wastes is not an untried approach. In the late 1960's, researchers explored the potential of composting for decomposing diazinon, parathion, dichlorodiphenyl-trichloroethane (DDT) and dieldrin (Rose & Mercer, 1968). Through aerobic mesophilic-thermophilic composting they lowered the diazinon concentration by 50% in 10 days, and by > 98% in 42 days. The concentration of parathion was reduced by approximately 50% in 12 days but the effects on DDT and dieldrin were minimal. Furthermore, work done under the auspices of the Boston Metropolitan District Commission showed that, apparently, most of the polynuclear aromatic hydrocarbons can be decomposed by composting (Savage *et al.*, 1985b).

The feasibility of bioremediation by thermal bioventing has also been demonstrated by

extensive work with *Phanerochaete chrysosporium*. Current research is bringing commercialization of white-rot fungus (WRF)-based bioremediation of contaminated soils closer to reality. White-rot fungi degrade an extremely wide range of toxic organic molecules, including polychlorinated aromatic hydrocarbons, carbon tetrachloride, chloroform, methyl chloride, trichloroethane and DDT. The full-scale application entails excavation of the soil, addition of nutrients and a bulking agent and the WRF inoculum. The mixture is placed in an aerated closed vessel or pile for the desired time (IB Market Forecast, 1994).

McMullen & Regan (1991) made a laboratory study on a simulated jet fuel-contaminated soil mixture in an active compost and monitored the fate of the fuel mixture. This preliminary study showed considerable promise. In general, they found that the components of the synthetic fuel mixture were effectively removed from the soil/compost matrix within 6 wks. However, the authors stated that fuel-contaminated soil is geographically widespread and included most soil types. As such, it is not possible to use any one soil as a representative of all contaminated soils. McMullen & Regan (1991) stated that a major incentive for pursuing this work was that composting is believed to be comparatively inexpensive compared to other approaches and can be accomplished with commonly available materials.

The primary objective of the Louisiana Army Ammunition Plant (LAAP) study made by Williams & Myler (1990) was to validate the concept of thermal bioventing as a technology for remediating soils and sediments contaminated with explosives. Concrete test pads were

constructed adjacent to the contaminated lagoons at the LAAP. Drainage channels in the pads were connected to a sump and water from the sump was reapplied to the compost piles as necessary. The mixture to be composted was prepared from horse manure and soiled bedding (straw), alfalfa, horse feed and contaminated sediment. Sawdust, wood chips and baled straw were used to construct the compost pile base and insulating cover. A mechanical feed system was used to homogenize the sediment and to mix the material. The temperature and oxygen content in the compost piles were controlled with a system of perforated and non-perforated polyethylene drainage tubes placed in a wood chip base and connected to a radial blade blower. An induced draught fan was used to pull air through the compost pile. Blower cycling was controlled by both timer and temperature feedback systems. Both mesophilic and thermophilic temperatures were investigated to determine if the higher microbial diversity (and metabolic diversity) present with mesophilic temperatures would result in greater overall contaminant destruction than that observed with thermophilic temperatures. They found that thermophilic temperatures resulted in higher rates of transformation but they were mediated by a narrower range of microorganisms. The appearance of the compost also changed considerably over the test period. When the compost was initially mixed, it had a highly fibrous appearance, a rough texture and it smelled of manure and feed. After 100 days, the compost had become more soil-like and less fibrous in appearance. At the end of the test period, the compost had both the appearance and smell of loamy soil (Williams & Myler, 1990). The results of these field demonstrations indicated that thermal bioventing is a feasible technology for reducing the concentrations of contaminants in soils and sediments. A cost analysis indicated that if the cost of supplements was kept low and a soil fraction of > 20% was used, the soil could be

treated for 2.5 times less than incineration costs.

Taddeo, Findley, Dooley-Danna & Fogel (1989) made experiments with soil contaminated with 20 000 ppm of coal tar. Laboratory experiments were made to determine if higher concentrations of coal tar-contaminated soil (than those treated by landfarming) were biodegradable by thermal bioventing. Compost test containers, or "microcosms", were set up with wood chips as a bulking agent. Tests were then made in a bench-scale composting vessel (15 l) to determine optimal moisture, fertilizer amounts and effects of high molecular mass polycyclic aromatic hydrocarbon (PAH) concentrations on biodegradability. Here, full-size wood chips, high concentrations of coal tar and continuous forced aeration were used. The glass vessel was double jacketed and completely airtight. The effluent gas from the vessel was passed through activated carbon to trap volatile organics (Taddeo *et al.*, 1989). Data from the 15 l composter indicated that in 15 days, monoaromatics, consisting mainly of xylenes, trimethylbenzene and indan, were degraded by approximately 90% and volatilized <6%, while 2-ring PAHs were reduced 95% and 3-ring PAHs were reduced 50% by biodegradation. Therefore, these results showed that the technology was feasible, even when high concentrations of contaminants required treatment (Taddeo *et al.*, 1989).

2.3.2 Optimal operating requirements

Environmental conditions such as pH, moisture content, oxygen supply and C/N ratio can influence the extent of PAH removal. Optimum operating conditions according to Adenuga, Johnson, Cannon & Wan (1992) are listed in Table 2.1. Although these

conditions are listed as optimal for the process of thermal bioventing, they could be extrapolated to a broad spectrum of remedial technologies and apply in most biological soil treatment systems.

TABLE 2.1. Optimal composting parameters (Adenuga *et al.*, 1992)

Parameters	Range
pH	6.0-8.0
C/N (w/w)	20:1-30:1
Moisture (% w/w)	50-60
Soil porosity (%)	30-35

i. Nutrients

If the waste has the nutrient (C,N,P,K) concentrations and the physical characteristics required for the growth and activity of the microorganisms involved in its decomposition, no additional materials are required. Generally, for a particular hazardous waste to be degraded, it must serve as a source of carbon/energy for one or more of the microbial populations involved in the process. If the waste is lacking in one or more nutrients, supplemental nutrients can be added. Key determining factors for the selection of a suitable nutrient mixture include the rate of biodegradation as well as the cost (Taddeo *et al.*, 1989). In the composting of refuse, the chemical nature of the substrate is a key factor for determining the rate of the process. The quantity and the balance of nutrients, as well as their availability to various microorganisms are important. One of the principal aspects of the total nutrient balance is usually expressed by the C/N ratio. The effects of the C/N ratio

on composting of refuse have been investigated by many researchers and have shown that a ratio of 25 or 30:1 is the optimum and that higher values slow the rate of organic material decomposition. In contrast, the only disadvantage for having a C/N ratio lower than 20:1 is loss of nitrogen (Nakasaki, Yaguchi, Sasaki & Kubota, 1992). Although this information was applied to the composting of garbage the same considerations should apply to the composting of hazardous wastes.

ii. *pH*

The optimal pH range for the composting process is between 5.5 and 8.0 (Internal Report, University of Natal, 1994). It is well known that bacteria favour neutral pH and fungi function optimally at acidic pH values. Therefore, when adjusting the pH of the compost heap, it has to be known which microorganism(s) to favour under the particular conditions.

iii. *Temperature*

Since thermal bioventing is a biological process and biological activity provides heat, heat generation gives an indication of the success of the treatment conditions. In the study of Taddeo *et al.* (1989), temperatures of a compost were kept between 65 and 85°F (18 and 29°C) with an aeration blower. High temperature is the most salient feature of the composting environment. Due to the elevated temperatures, the selection pressure on microflora involved in the process is intensified (Crawford, Johnson & Goetz, 1993). Kaplan & Kaplan (1982) made laboratory-scale composting systems where temperatures of

55°C were used. During this study several thermophilic microorganisms were isolated and identified and included actinomycetes, fungi and bacteria (*Bacillus stearothermophilus*, *B. subtilis* and *B. coagulans*).

iv. Aeration

According to Crawford *et al.* (1993) composting requires oxygen concentrations > 5% (v/v). The amount and thoroughness of aeration determine the rate and extent of the destruction of the waste because the breakdown of biodegradable hazardous wastes is, essentially, an aerobic process. Aeration also determines the limit to which the temperature will rise in the composting mass, inasmuch as the temperature rise is a result of exothermic bacterial activity. The amounts and rates required to ensure adequate aeration are determined by the chemical and physical makeup of the waste being composted. Insufficient aeration leads to anaerobiosis and an accompanying generation of objectional odours (Savage *et al.*, 1985b). It must be noted, however, that in some cases it is necessary to include an anaerobic pretreatment process, e.g., haloaromatic treatment. Improved methodology has meant that anaerobic studies have progressed and anaerobes have been shown to metabolise a wide range of organic compounds including chlorinated phenols, PAHs and polychlorinated biphenyls (PCBs) (Singleton, 1994) and should, therefore, not be excluded without consideration when choosing treatment options for a specific case.

Nakasaka, Watanabe & Kubato (1992) compared the rates of organic matter decomposition under aerobic and anaerobic conditions and showed the difference in the patterns of

microbial succession. They found that the rate of organic matter degradation could be accelerated under oxic conditions.

v. *Moisture content*

The optimum moisture content is a combined function of two factors. The first is the moisture required for optimal bacterial activity. According to Savage *et al.* (1985b) bacterial activity becomes severely inhibited when the moisture content drops below about 40% (w/w), and fungi and actinomycetes are more tolerant of lower moisture contents than bacteria. For example, the soil moisture content of compost reactors has been reported to be between 40 and 60% (w/w) of the field capacity (moisture content at -1/3 bar [-33kPa]) (McFarland, Qui, Sims, Randolph & Sims, 1992). The second factor is ultimately related to the availability of oxygen as determined by the volume of air in the interstices of the composting mass. The volume of air, in turn, is a function of the porosity of the composted mass and the ratio of air to water in the interstices. Consequently, the maximum moisture content is a function of the physical structure of the wastes (Savage *et al.*, 1985b).

vi. *Bulking agent*

The high moisture content and amorphous structural characteristics of wastes are two serious difficulties encountered in the composting of hazardous wastes but they can be overcome through the use of a bulking agent.

According to Savage *et al.* (1985b) an ideal bulking agent has the following properties:

- Provides ample porosity under all moisture conditions;
- Is absorbent;
- Resists compaction;
- Degrades very slowly, if at all, and;
- Can easily be recovered from the composted wastes and subsequently recycled.

With the possible exception of inadequate absorbency, wood chips could serve as a satisfactory bulking agent in the composting of hazardous wastes. However, it would be preferable to use the compost product. An external bulking agent would have to be used for the first composting pass but the product from this could serve as the bulking agent in the second composting - and so on throughout the life of the operation. Recycling the compost eliminates the need to import new bulking agent after each round and it also reduces the amount of residue to be disposed of (Savage *et al.*, 1985b).

The bulking agents used differ considerably from researcher to researcher. Kamnikar (1992) claimed that a manure/wood chip (in a 1:1 (v/v) ratio) combination effected an increase in temperature generation as the manure/wood chip concentration increased. The ratio of the compost mixture used by Crawford *et al.* (1993) consisted of one part manure, one part wood chips, and four parts soil whereas Kaplan & Kaplan (1982) used horse manure, alfalfa hay, grass clippings, dead hardwood leaves and garden soil. Taddeo *et al.* (1989) made studies on different bulking agents. Materials, including wood chips, wood

shavings, peat moss, sand, vermiculite, sawdust and cacao shells were tested. They found that none of the bulking agents significantly inhibited the extent of degradation. Treatment of hazardous waste in this manner should be low technology and low cost in order to be competitive. Therefore, to meet these objectives, it is necessary to use materials that are readily available and of consistent quality. In addition to the bulking agent supplementation, researchers have also added manure.

Manure provides three components needed for successful composting of hydrocarbon contaminated soils:

- Microorganisms which catabolise petroleum hydrocarbons:
- An additional energy source for the microorganisms since the hydrocarbons alone are insufficient to sustain the microorganisms, and;
- Moisture (Kamnikar, 1992).

vii. Time

A treatment time can never be standardised, since several factors determine the treatment time. These include legislative cleanup standards, the nature and extent of the contaminant and environmental factors such as the soil characteristics and the microbial population.

2.4 HYDROCARBON ANALYSES

Although the application and science underpinning the technology of bioremediation and composting are relatively low technology, the chemical analyses are considered very difficult. When monitoring the degradation of a single contaminant, the analyses are relatively simple. However, in hydrocarbon contamination there are many compounds present which include the whole aliphatic range and, normally, one to five ring aromatics and even chlorinated compounds (Figure 2.1). What complicates matters even more, is that the contaminant and the soil matrix are also dynamic systems, which change with time. For example, oxidation and reduction reactions may take place and the bioremediation process, albeit slow, is initiated. This exemplifies the difficulty involved in the analysis of the hydrocarbons. If an incorrect or inadequate analytical procedure is followed, it can have profound consequences. For example, an undetected breakdown product can be more toxic to the environment than the more inert primary compound (Alexander, 1981). Therefore, the analytical procedure used to monitor the contamination and treatment efficiency must be carefully chosen. The selected method must be specific as well as sensitive to be able to measure hydrocarbons in environmentally realistic concentrations.

2.4.1 Infra-red spectroscopy

Methods such as Environmental Protection Agency (EPA) Method 418.1, *Total recoverable petroleum hydrocarbons by infra-red spectroscopy*, measure the total recoverable petroleum hydrocarbons. This screening tool is often accepted and is taken as an accurate analytical

method without recognising its limitations.

The analytical bias associated with Method 418.1 has been documented (Douglas, McCarthy, Dahlen, Seavey, Steinhauer, Prince & Elmendorf, 1992) and is listed below:

- Poor extraction efficiency of freon for high molecular weight hydrocarbons;
- Loss of volatile hydrocarbons during extract concentration;
- Differences in molar absorptivity between the calibration standard and product type;
- Fractionation of soluble low IR absorbing aromatic hydrocarbons during water washout;
- Removal of 5- to 6-ring alkylated aromatics during the silica cleanup procedure;
- Preferential biodegradation of n-alkanes;
- Product differences in molar absorptivity;
- Partitioning of soluble aromatics from the bulk product because of oil washout;
- Measurement of naturally occurring saturated hydrocarbons; and
- IR dispersion of clay particles.

It must be noted that all these factors are not exclusive to this method since other methods, still to be discussed, could also be affected by one or more of these factors.

2.4.2 Gas chromatography

Most researchers seem to use a GC equipped with a Flame Ionization Detector (FID) and,

typically, helium as the carrier gas to identify contaminants. The analytical sensitivity for most modified GC/FID methods for total recoverable petroleum hydrocarbon (PHC) ranges from 1 to 10 mgkg⁻¹ dry weight of soil. The limits for PHCs in soil are, generally, within the range of environmental background concentrations (Douglas *et al.*, 1992). The GC/FID chromatogram can be used to identify product type, based on n-alkane pattern distributions, pristane and phytane ratios, the width of unresolved complex mixtures, and unidentified compound peaks matching in the "grass" region of the chromatogram. Light to moderate product degradation resulting from weathering and bacterial degradation can be measured. The GC may be linked to a Mass Spectrometer (GC/MS) to improve the accuracy of product identification. Further quantification of the isolated target analytes can be made by GC/MS in the selected ion mode (SIM). When an extract is analyzed by GC/MS-SIM, a clear picture of the concentration and distribution of the analytes are observed in the one part per trillion range (Douglas *et al.*, 1992). However, these analyses are expensive and time consuming and, therefore, GC alone is normally used in routine laboratory analyses.

2.4.3 Other analyses

The above two methods are the most common but some researchers have used ash determinations which involves a two-phase heating process in tared crucibles (McMullen & Regan, 1991). High Performance Liquid Chromatography (HPLC) is sometimes used for the determination of the heavier aromatic fractions. Respirometry is used in cases where the mineralization of a radioactive indicator compound is monitored.

2.5 SUMMARY

Bioremediation offers a cost effective and safe cleanup method for several different organic contaminants, with the added advantage of being relatively low technology. In this chapter, several of these bioremediation technologies were described. A new technology called thermal bioventing, which shares the principles of composting, was discussed. This technology addresses highly contaminated soils where hazardous emissions are present.

CHAPTER 3

BIOREMEDIATION OF PETROLEUM-CONTAMINATED SOIL BY LANDFARMING: A PILOT-SCALE STUDY

3.1 INTRODUCTION

Landfarming is, in most cases, an *ex situ* solid-phase bioremediation technique where contaminated soil is treated above ground, using conventional soil management practices, i.e. tilling, irrigation and fertilization, to enhance the microbial degradation of contaminants (Brunsbach & Reineke, 1993). This type of equipment and skills are readily available in South Africa, making landfarming a cost effective and viable option to treat hydrocarbon-contaminated soils.

In this study, the development and implementation of a treatment programme for pilot-scale bioremediation of petroleum hydrocarbon-contaminated soil is described. Soil, for this study, was sampled from a site where contamination had occurred accidentally when a storage tank valve had been left open and petroleum oil had spilled into a small stream. The aim was to assess the extent of the contamination and evaluate selected treatment options by manipulating various key parameters to achieve the fastest rate of degradation. Both approaches to bioremediation were investigated, viz. bioaugmentation and biostimulation. A commercial biosupplement was also compared with a biosupplement prepared with indigenous microorganisms.

This study stresses the necessity of a preliminary investigation, where biological parameters are assessed to enhance the remedial effectivity and efficiency, before capital is invested in the full-scale treatment.

3.2 HISTORICAL BACKGROUND

The spill occurred during the rainy, summer time (January 1994) at a site in Gauteng (Figure 3.1), RSA. The history of the spill was as follows. Instructions were given that the company's main lubrication oil product¹ should be circulated between storage tanks during the night, as is the norm. However, a valve on a connecting jet line was left open. The receiving tank had a smaller capacity than required and started to overflow. The spill should have been contained within the bunded area of the tankfarm but, unfortunately, the outlet valve had been left open. The mixture of oils thus spilled into the stormwater drain which discharges into the adjacent stream. The oil passed directly through the separator, which was already at its capacity with oil and water, and into the stream and nearby veld. It was estimated that a total of 120 000 ℓ entered the stormwater system. A retaining wall at the stormwater drain outlet retained some of the oil, and a further fraction was recovered from an old building foundation further downstream which intercepted the oil. Pumps were used to recover any free product and an absorbent was used to recover some oil from the soil. It was estimated that 10 000 ℓ of oil were not recovered and remained in the stream and surrounding soil.

¹ The nature of the lubrication oil cannot be revealed, because of client confidentiality

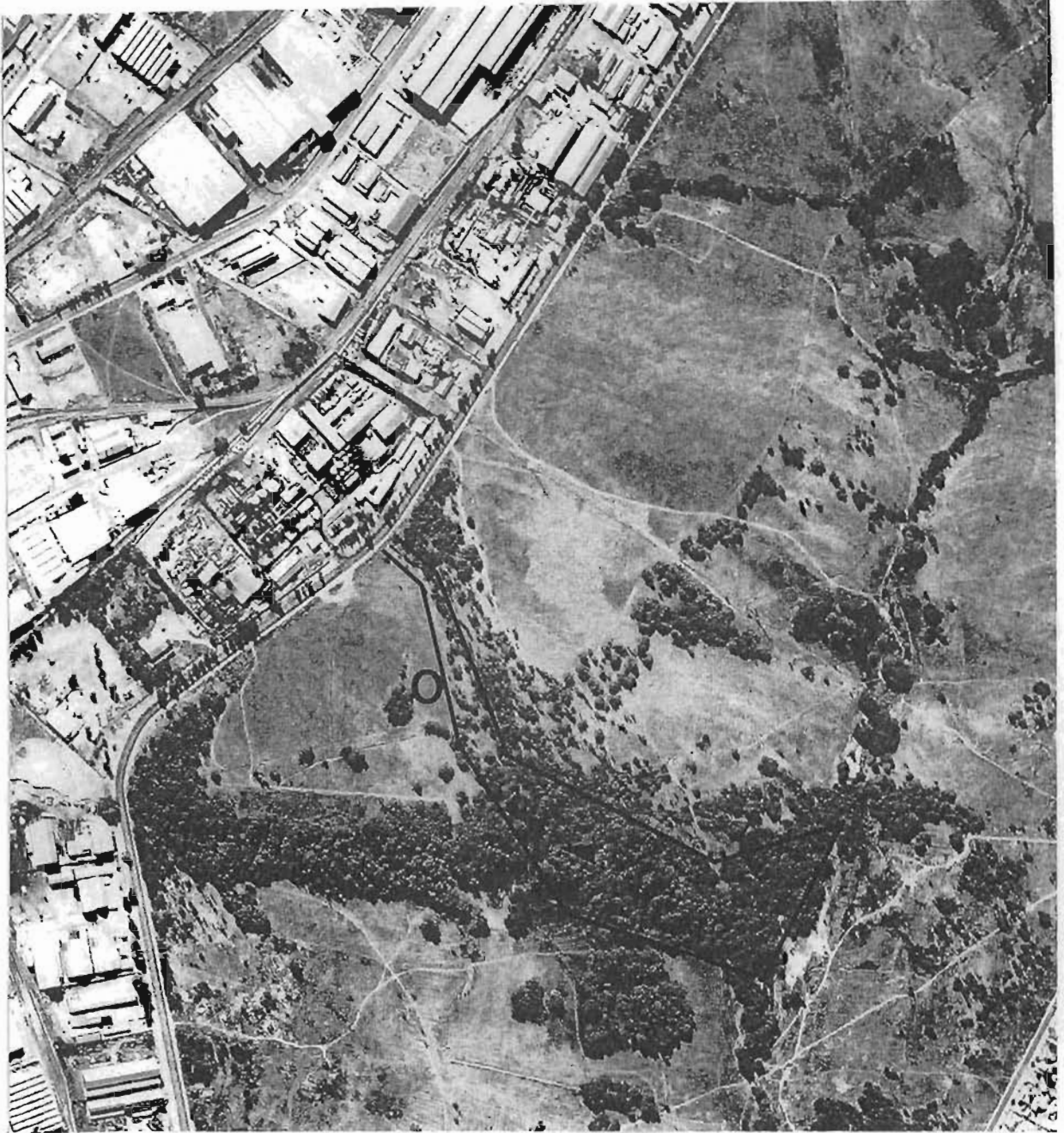


FIG. 3.1 Aerial photograph (1: 8300) showing area of oil spill. The box indicates the extent of the spill. The arrow indicates where the material used in the pilot-scale evaluation was collected. The open circle indicates where the uncontaminated soil was collected.

3.3 NATURE AND EXTENT OF CONTAMINATION

A visual inspection of the spillage site showed substantial soil surface contamination from petroleum hydrocarbons for a distance of about 500 m down the stream. The sides of the stream were blackened, as too was the soil surface area just above the level of the water. The immediate vegetation was black with oil and some dead vegetation was evident, possibly due to the layer of oil inhibiting photosynthesis. A considerable area was affected downstream where the stream broadens into a larger clearing. However, it appeared that a recent fire also contributed to vegetation damage in this area. The small stream joins a larger stream which is heavily contaminated with domestic effluent and litter. Oil, visible in the small stream, was absent in the combined streams where the water was clear.

3.3.1 Groundwater

The threat of possible groundwater contamination was investigated. Information on numerous boreholes in the area was obtained from the Department of Water Affairs and Forestry (DWAF) and is shown in Table 3.1. The location of the boreholes could not be disclosed in this thesis because of client confidentiality.

Although variation in the depth of the water table does occur with time, the depths between the soil surface and water table (Table 3.1) show that contamination of the groundwater due to the oil spill was highly unlikely. At boreholes 5 and 6 contamination could be expected if continuous contamination occurred thus saturating the soil to the extent that migration to

the groundwater occurred.

TABLE 3.1. Depths of the water table in the area of the spill from borehole data

Borehole no.	Depth of water table (m)
1	27.5
2	45.2
3	41.6
4	23.8
5	14.8
6	13.0

3.4 MATERIALS AND METHODS

3.4.1 Soil

A total of ca. 250 kg of contaminated soil was collected from the site, mixed thoroughly with a shovel and stored at 4°C (in the dark) until analysed or used in the reactor vessels. The soil was removed from the area of the stream (indicated on Figure 3.1 by an arrow) at the clearing over a distance of ca. 2 m² and to a depth of approx 0.05 m. Uncontaminated soil (10 kg) was collected from the site from an area marked with an open circle (O) on the map (Figure 3.1). Two samples of 1 kg each were taken from this soil and were submitted for analyses of total petroleum hydrocarbons using the methodology recommended in EPA 418/1.

3.4.2 Nutrients available in the soil

Nitrogen and phosphorus concentrations were determined in both the contaminated and uncontaminated soils. Phosphorus analyses were made using the Bray 1 method (Standard Soil Testing, 1990) rather than by digestion. The total nitrogen, using a digestion and colorimetric method, and the available NH_4 , NO_3 and NO_2 , using a saturated water paste extraction, were determined (Standard Soil Testing, 1990).

3.4.3 Isolation and characterization of indigenous soil microorganisms

Soil samples (1 g) were taken and dilution series prepared with sterile aqueous physiological saline (0.85% m/v). The dilutions were plated out on soil extract agar (SEA) (Parkinson, Gray, & Williams, 1971) and nutrient agar (NA) to estimate total colony forming units. Soil extract agar was prepared by autoclaving (15 psi (103 kPa), 121 °C, 20 min) 500 g of soil in one litre of distilled water. The suspension was suction filtered through a Büchner funnel lined with no. 5 Whatman paper and 15 g of agar were added to the filtrate. The pH was adjusted to 6.8 with NaOH and 1.0 g glucose, 5.0 g yeast extract and 0.2 g of K_2HPO_4 were added before reautoclaving (Parkinson *et al.*, 1971). The dilutions were also inoculated onto potato dextrose agar (PDA) which contained 500 mg of chloramphenicol per litre PDA, and Czapek-Dox agar (Harrigan & McCance, 1966) to estimate the total numbers of yeasts and other fungi present in the soil. These plates were incubated at 25 °C for 24 h for bacteria and for 4 days for fungi. Subsequently, monocultures of bacteria and fungi were isolated from the plates. Gram stains and light

microscopy studies were made on all bacterial cultures to assess culture purity and to study cell morphology. Preliminary identifications were made on fungal cultures using spore morphology (Baxter, Rong, Roux, Schutte & Van der Linde, 1994).

3.4.4 Plate counts of total- and petroleum-degrading species

Samples (1 g) were collected weekly from each soil reactor and analyzed for the total number of indigenous microorganisms present by dilution series plating on NA (Wollum, 1982). The soil was also analyzed for the total number of petroleum hydrocarbon degrading bacteria by inoculating on an OECD (Organisation for Economic Cooperation & Development) described minimal medium which contained 30 ml commercially available lubrication oil as sole carbon source. The oil was of the same chemical composition as the contaminant. The OECD/lubrication oil medium was prepared by adding 17 g agar, 4 ml FeCl_3 (0.25 g l^{-1}), 1 ml each of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (22.5 g l^{-1}), CaCl_2 (27.5 g l^{-1}) and $(\text{NH}_4)_2\text{SO}_4$ (40 g l^{-1}) to 2 ml of the following mixture: KH_2PO_4 (8.5 g l^{-1}), K_2HPO_4 (21.75 g l^{-1}), $\text{NaH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$ (33.4 g l^{-1}) and NH_4Cl (1.7 g l^{-1}) and diluting to one litre with distilled water. The mixture was autoclaved (15 psi (103 kPa), 121 °C, 20 min) and, after cooling, the lubrication oil was added and the agar sonicated before pouring the plates.

3.4.5 Total petroleum hydrocarbons concentrations

Total petroleum hydrocarbons were determined using the EPA 418.1 method (US Environmental Protection Agency, 1979).

3.4.6 Experimental design

Seven oval shaped polyvinyl chloride (PVC) soil pans (30 ℓ capacity with a major and minor axis of 270 mm and 208 mm, respectively, and a height of 201 mm) were each filled with 25 kg of contaminated soil. Agricultural lime (CaCO₃, 175 g) was added to all reactors to raise the soil pH to approx 6.5 - 7.5. The soil of one reactor (Reactor 0) was treated twice with 57 g (92 ml) sodium azide to establish a metabolically inhibited control hereafter referred to as “sterile control”. The 57g of sodium azide was dissolved in distilled water and diluted to 2 ℓ and mixed into the soil. Table 3.2 shows the experimental design for each soil reactor.

TABLE 3.2. Experimental design of the soil reactors

Reactor	Addition of water	Addition of MAP	Turned daily	Addition of H ₂ O ₂	Addition of commercial supplement	Addition of indigenous microbial culture
0	no	no	no	no	no	no
1	no	no	no	no	no	no
2	yes	no	yes	no	no	no
3	yes	yes	yes	no	no	no
4	yes	yes	yes	yes	no	no
5	yes	yes	yes	no	yes	no
6	yes	yes	yes	no	no	yes

The pH values of the soil reactors were determined weekly. Agricultural lime (Leo), 7 gkg⁻¹, was added to maintain the pH between 5.0 and 7.5. The room temperature was measured daily. The reactor soil temperatures were also taken daily to establish whether an

increase in microbial activity raised the soil temperature.

Water (250 ml to 500 ml) was added to each reactor every second day as determined by visual inspection. Turning the soil daily with a spade introduced oxygen to the soil.

Hydrogen peroxide was also investigated as an alternative source of oxygen and was added at a concentration of 500 mg l^{-1} (600 μl of a 39% solution) once a week (Thomas & Ward, 1989).

Ammonia and phosphate were added in the form of mono-ammonium phosphate (MAP)(Kynoch Fertilizers) at a dosage of 1 g kg^{-1} soil. The nutrients were dosed four times during the study to all relevant reactors (Reactor 3,4,5 and 6, Table 3.2).

An indigenous microorganism-biosupplement was prepared which contained enriched indigenous soil microorganisms from the contaminated soil. A contaminated soil sample (2 g) was inoculated into a broth (pH 4) which contained (g l^{-1} distilled water): NaNO_3 , 2; KCl , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; Na_2HPO_4 , 1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; sucrose, 3; 30 ml lubricating oil (adapted from Harrigan & McCance, 1966). The mixture was homogenized using an Ultra Turrax (Janke & Kunkel) at $20\,000 \text{ r min}^{-1}$ for at least 20 sec. A second soil sample (2 g) was inoculated into soil extract broth (Parkinson *et al.*, 1971) which contained 30 ml lubricating oil as additional carbon source. This broth was also sonicated before inoculation. The microbial suspensions were incubated and aerated via a sintered glass diffuser at 25°C for 4 days. This facilitated enrichment of both fungi and bacteria. The enrichment reactors were manufactured from glass and were fitted with a glass tap at the

bottom. This made it possible to decant the microbial suspensions without oil contamination. The populations of bacteria were estimated by plate counts on nutrient agar.

The commercial biosupplements, formulated specifically for treatment of hydrocarbon oils (Bi-Chem 1738 CN and 1008 SF), were obtained from Sybron Chemicals (SA) (Pty) Ltd and resuscitated according to manufacturer's specifications. The concentrations of bacteria in both commercial and indigenous biosupplements were adjusted to 10^8 cfu ml⁻¹.

The commercial biosupplement and enriched microbial solution containing indigenous microorganisms were added weekly at a dosage of 50 ml each per reactor.

Reactor 1 was used as a "natural" control and contained contaminated soil. With the exception of the initial pH, no parameters were regulated.

3.4.7 Rehabilitation of remediated soil

After the termination of the pilot-scale experiment, the soil was tested for vegetation rehabilitation. Wheat seed, *Triticum aestivum* cv. L. Betta, with a germination efficiency of 96%, was used in the experiment. The wheat seed, which was harvested in Reitz during 1991, was sown in soil from Reactor 6 and Reactor 1. Seeds were also planted, one seed per cell, in 24-cell seedling trays which contained soil samples (pH adjusted to 6) from these reactors. The control and experiment seedling trays, and the reactors, were watered frequently. The seedlings were harvested after 32 days and shoot and root lengths were

determined. The soil pH and the percentage emergence in the seedling trays were established.

3.5 RESULTS AND DISCUSSION

3.5.1 Characterization of soil contamination

Table 3.3 summarizes the characteristics of the contaminated soil.

Total petroleum hydrocarbons (TPHC) for the two soil samples were determined to be 236 gkg⁻¹ and 322 gkg⁻¹. The discrepancy between the soil samples could be ascribed to either the heterogeneity of the soil taken from the site, despite mixing, or to the method of extraction and/or analysis.

As, typically, concentrations of phosphorus in soil vary between 12 and 18 mgkg⁻¹, the phosphorus content of the soil had to be increased. According to Bremner & Mulvaney (1982), the total nitrogen content of most soils ranges from < 0.02% (m/m) in subsoils to > 2.5% (m/m) in peats. The total N value of the soil was therefore, within the norm. The inorganic combined N in soils is predominantly NH₄ and NO₃, with NO₂ (nitrite) seldomly present in detectable concentrations. Inorganic N normally represents about 2% of the total N in soils (Keeney & Nelson, 1982). In the case discussed in this chapter, the inorganic nitrogen forms represented 0.2% of the total N. This indicated that additional inorganic nitrogen was required.

TABLE 3.3. Characteristics of contaminated soil

Parameter	Concentration
TPHC (two samples)	32 gkg ⁻¹ and 24 gkg ⁻¹
pH	5.5
Loss on drying (105°C): water and volatile organic matter	41.5% (m/m)
Non-volatile fraction and water of crystallization	15.8% (m/m)
Major element	Silicon
Minor elements	Ca, K, P, Al, Fe, Ti
Nitrogen (as % total N)	0.14 %
Available NH ₄ (as N)	2.36 mgkg ⁻¹
Available NO ₃ (as N)	0.62 mgkg ⁻¹
Available NO ₂ (as N)	BD ^a
Phosphorus (as o-PO ₄)	6.8 mgkg ⁻¹
Bacteria	1.6 x 10 ⁶ cfug ⁻¹
Fungi	3.7 x 10 ⁵ cfug ⁻¹
Yeasts	1.2 x 10 ⁶ cfug ⁻¹

a Below detection (BD)

The initial loss of mass on drying at 105°C was 41.5%. This represented mainly water and volatile organic matter. A further 15.8% loss on ignition at 800°C represented non-volatile organic matter.

The residue after ashing consisted of a yellow-brown, free flowing material which was submitted for X-ray fluorescence (XRF) analysis. The only major element determined by XRF was silicon. Minor elements, each accounting for < 5% (m/m) of the residue were

calcium, potassium, phosphorus, aluminium, titanium and iron.

As there were no elements present in sufficient quantity to combine stoichiometrically with silicon to give a compound of known composition, it was assumed that the base material consisted of silica of siliceous rock forming materials which had been heavily contaminated with organic material.

3.5.2 Degradation of contaminant oil

Figures 3.2 to 3.4 show the decreases in TPHC with time. The efficacy of each reactor was compared with the sterile control (Reactor 0). Reactor 0 was treated with sodium azide which acts as an inhibitor of electron transport by blocking electron flow in cytochrome oxidase (Stryer, 1988). This results in a shortage of ATP and cell death follows which establishes a biologically sterile control. The decrease in TPHC in the sterile control, from 322 to 117 gkg⁻¹, was used as a measure of the petroleum hydrocarbons lost through volatilization as no microbiological activity was possible. Decreases in TPHC concentrations in Reactors 1 to 6 beyond 117 gkg⁻¹ could, therefore, be attributed mainly to bioremediation. Volatilization, thus, initially contributed largely to the reduction of petroleum hydrocarbons. However, after the volatile fractions had been displaced, the bacteria then degraded the heavier, less volatile fractions of the lubrication oil.

Reactor 6, supplemented with enriched indigenous microorganisms, showed the largest decrease of 85% TPHC during the first four weeks, followed by Reactor 4, supplemented

with H₂O₂, and Reactor 5, supplemented with the commercial biosupplement, with decreases of 79.6% and 78.4%, respectively.

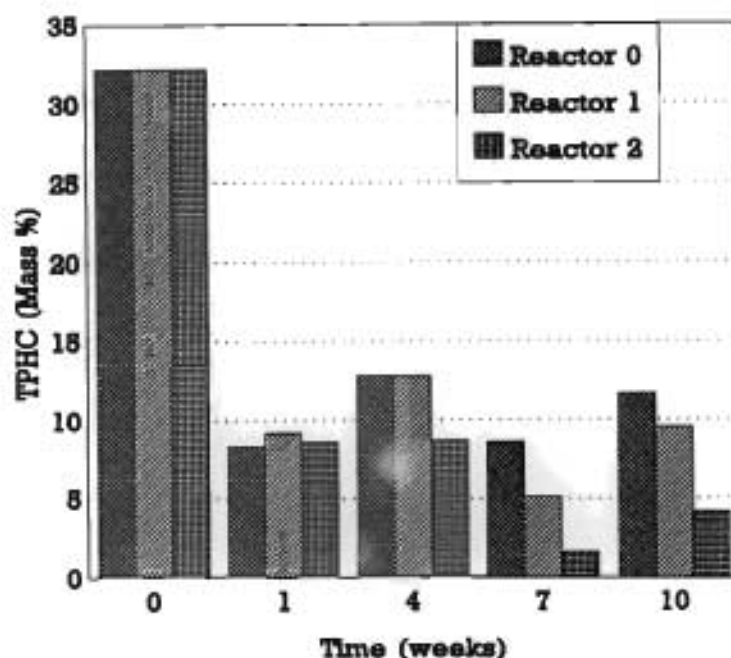


FIG. 3.2 Changes in TPHC concentrations in Reactor 1 (natural control) and Reactor 2 (moisture and oxygen) in comparison with the Sterile Control (Reactor 0).

In week 10, Reactors 5 and 6 had similar residual TPHC concentrations of 18 gkg⁻¹. As non-indigenous microorganisms may initially have had difficulty in adapting to new environments, the initial lag time in Reactor 5 was expected. An overall reduction of petroleum hydrocarbons of 94% was recorded in Reactors 5 and 6. Reactor 4 followed closely with residual concentrations of 224 gkg⁻¹ TPHC.

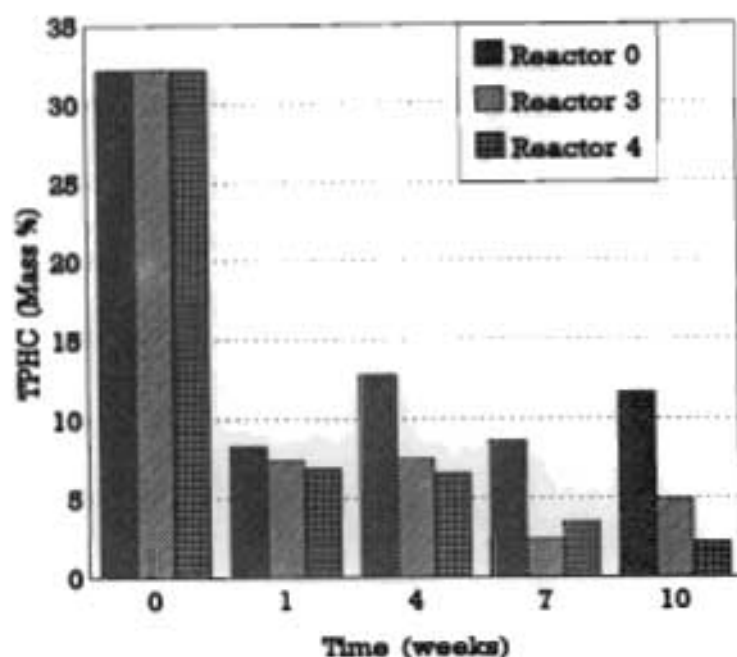


FIG. 3.3 Changes in TPHC concentrations in Reactor 3 (moisture, MAP & oxygen) and Reactor 4 (moisture, MAP, oxygen & H₂O₂) in comparison with the Sterile Control (Reactor 0).

The natural control, as simulated by Reactor 1, showed a residual TPHC concentration of 96 gkg⁻¹. This indicates the extent to which regulation of the parameters, to produce a favourable environment for the microorganisms, can increase the rate at which bioremediation proceeds.

The similarities in the residual TPHC concentrations of Reactors 2 and 3 show that in this particular soil, the nutrients were not limiting. Table 3.3 shows the concentrations of available nutrients. The addition of MAP increased the phosphorus concentration from 6.8 to 43.9 mgkg⁻¹ and the total nitrogen concentration from 0.14 to 0.16% (m/m).

Anid, Ravest-Webster & Vogel (1993) found that aerobic conditions could be enhanced by the addition of H_2O_2 . In this study, a comparison of the residual TPHC concentrations in Reactors 3 and 4 verifies that the additional oxygen supplied to Reactor 4, via H_2O_2 , significantly enhanced bioremediation rates.

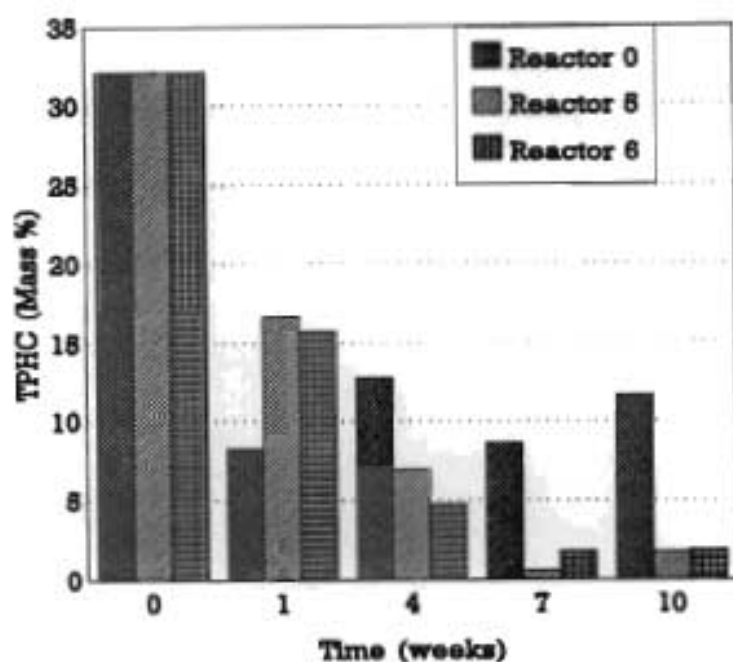


FIG. 3.4 Changes in TPHC concentrations in Reactor 5 (moisture, MAP, oxygen & commercial biosupplement) and Reactor 6 (moisture, MAP, oxygen & indigenous microbial biosupplement) in comparison with the Sterile Control (Reactor 0).

3.5.3 Isolation and characterization of indigenous soil microorganisms

Morphological and cultural information on the microorganisms isolated from the contaminated soil are detailed in Appendix 1. The isolation method revealed only culturable

microorganisms, which accounts only for a fraction of the total microbial population (Amann, Ludwig & Schleifer, 1995). Preliminary identification indicated the presence of eight different indigenous microorganisms in the soil, including *Pseudomonas* spp. Fungal species isolated from the contaminated soil included *Aureobasidium pullulans*, an *Eurotium* sp. and several species of the *Penicillium* and *Aspergillus* genera. Typically, bacterial and fungal counts should be of the order of 10^8 to 10^9 and 10^6 to 10^7 cfug⁻¹ soil, respectively (Wollum, 1982). The low counts (Table 3.3) of the contaminated soil, although not statistically proven, could indicate a stress situation. The absence of the fungal genera, *Pythium* and *Fusarium*, in the soil further indicated the toxic nature of the soil to natural indigenous species. It is also possible that the microbial population had shifted to an unculturable state.

3.5.4 Plate counts of total- and petroleum-degrading species

Figures 3.5 and 3.6 show the weekly total plate counts and counts of hydrocarbon-degrading species for each soil reactor.

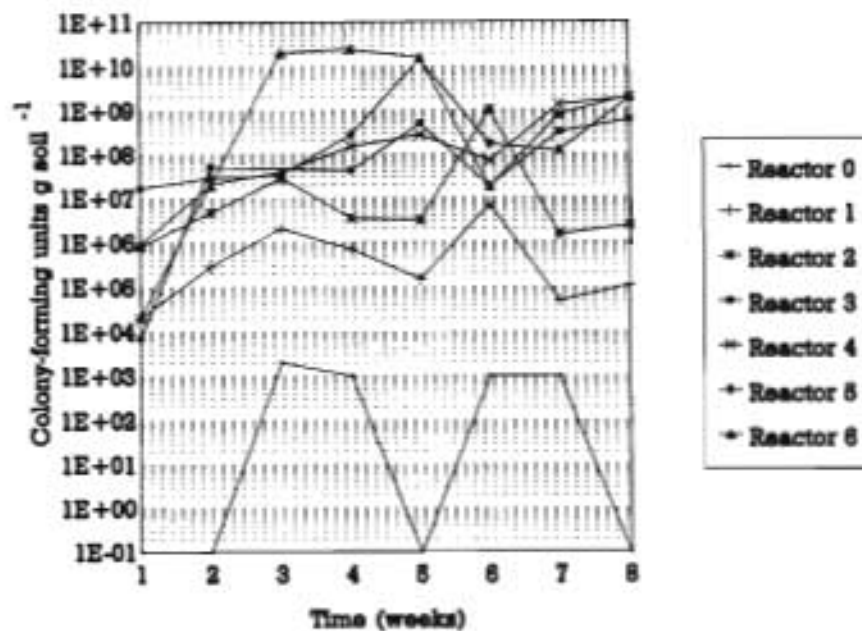


FIG. 3.5 Changes in the numbers of bacteria (as demonstrated by total plate counts) in the soil bioreactors. Reactors 0 and 1 represent the sterile and natural control, respectively. Reactors 2 to 6 were all aerated and received water. Reactors 3 to 6 were treated with MAP. In addition, Reactor 4 received H_2O_2 , Reactor 5, a commercial biosupplement and 6 an indigenous microbial mixture.

The number of hydrocarbon-degrading microorganisms was similar to the total cfu concentration in the first week (before the effect of the treatment became apparent). It can therefore be assumed that only hydrocarbon tolerant microorganisms could tolerate the environment. Therefore, it is possible that the microorganisms capable of degrading the oil were dominant in the soil, thus indicating the potential toxicity of the chemical contaminant to other soil microorganisms.

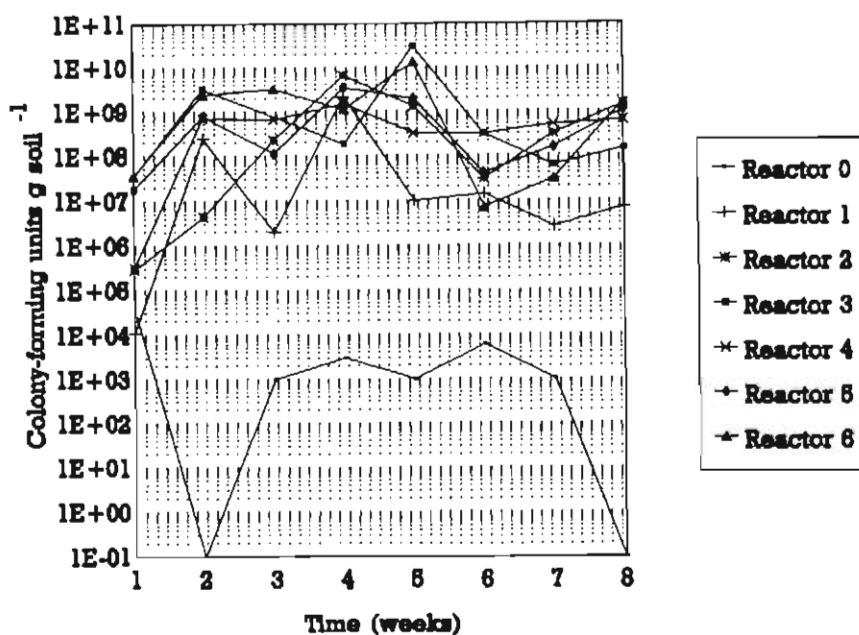


FIG. 3.6 Changes in the total counts of hydrocarbon-degrading microorganisms (as determined by total plate counts on OECD minimal medium). Reactors 0 and 1 represent the sterile and natural control, respectively. Reactors 2 to 6 were all aerated and received water. Reactors 3 to 6 were treated with MAP. In addition, Reactor 4 received H_2O_2 , Reactor 5, a commercial biosupplement and 6 an indigenous microbial mixture.

It is not yet known what percentage of the hydrocarbon breakdown can be attributed to fungal species although it is suspected that they do play a substantial role in conjunction with bacteria (Cerniglia, 1984). The total fungal counts increased, while the bacterial counts decreased, in the reactors which tended towards an acidic pH (Table 3.4). Fungi prefer acidic environments (Parkinson *et al.*, 1971) and have been known to create acidic environments. The decreases in pH in some reactors may have been due to either the fungi or the formation of phosphoric acid from the addition of the MAP. It may be speculated that the latter may be a more feasible explanation since the pH decreases were significantly

greater in the reactors supplemented with MAP (Tables 3.2 and 3.4).

The pH values of the soil reactors varied between 5 and 6.5 (Table 3.4) and the room temperature varied from 21 to 27 °C. The soil temperature did not increase above the room temperature at any time. However, this does not indicate an absence in microbial activity since the open, shallow reactors facilitated efficient heat exchange.

Table 3.4. The pH of soil reactors during treatment

Reactor	Week 1 (pH)	Week 2 (pH)	Week 3 (pH)	Week 4 (pH)	Week 5 (pH)	Week 6 (pH)	Week 7 (pH)	Week 8 (pH)	Week 9 (pH)
0	6.5	6.5	6.2	5.5	5.5	5.5	6.0	6.0	5.5
1	5.5	6.0	6.5	6.5	6.0	5.5	6.5	6.5	6.0
2	6.0	6.0	5.5	5.0	5.5	5.0	5.0	5.5	5.5
3	5.5	5.5	5.5	5.8	5.5	5.0	5.5	5.5	5.5
4	5.5	5.5	5.5	5.5	5.5	4.5	5.5	5.5	5.5
5	5.5	5.5	5.5	5.5	5.5	4.5	5.5	5.0	5.0
6	5.5	6.0	6.5	7.0	6.0	6.5	6.8	6.2	7.5

3.5.5 Rehabilitation of remediated soil

The effect of the bioremediation treatment on the rehabilitation of the soil was investigated by performing seedling germination tests. The pH of the soil in the reactors were adjusted to between 6 and 6.5, using agricultural lime, after termination of the experiment.

i Germination tests using seedling trays

The percentage germination of the wheat seeds in the seedling trays was found to be 95.8% in the treated contaminated soil (Reactor 6) and 87.5% in the untreated contaminated soil (Reactor 1). However, in the former case 8% of the resulting seedlings exhibited stunted growth. When this value is subtracted from the percentage germination in the treated soil it indicates that there was no significant difference in the percentage germination between the treated and untreated soil. Figure 3.7 shows the stunted root growth among the seedlings grown in the remediated soil as compared to those grown in the untreated soil. Statistical ratings of shoot and root lengths substantiated these observations (Table 3.5).

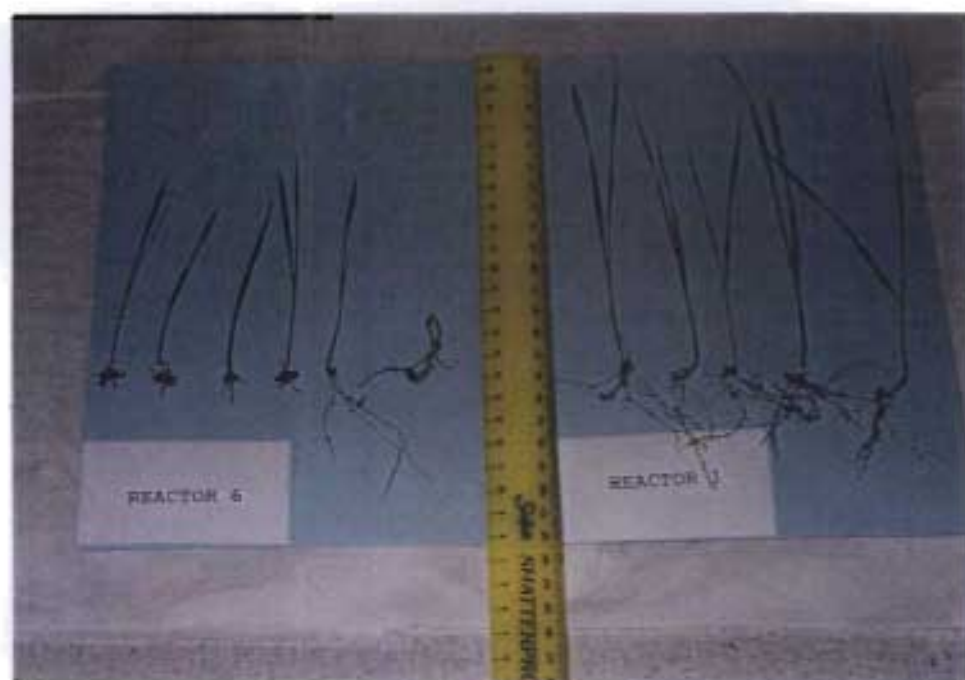


FIG. 3.7 Photograph showing that seedlings grown in the remediated soil (Reactor 6) were clearly stunted and had stunted roots compared to seedlings grown in the untreated soil (Reactor 1).

TABLE 3.5. Statistical ratings of wheat seedlings grown in the remediated soil (Reactor 6) and untreated soil (Reactor 1) in seedling trays

Reactor no.	Shoot length (mm)		Root length (mm)	
	6	1	6	1
Number of seedlings	19	22	19	22
Mean length	108.2	127.2	23	52.6
Standard deviation	36	34.2	12.2	36

According to the Student's t test there was a respective 95% and 99.5% certainty that the shoot and root lengths differed significantly between the treated and untreated soils. This could indicate a dilemma which should receive attention in future experiments. These results may at first seem contradictory, since it was expected that the remediated soil, which is less contaminated would be more conducive to plant growth than the untreated soil.

These results could be explained as follows. Firstly, the soil that had been bioremediated had received extensive supplements. This, in turn, could have altered the soil structure by chemical precipitation making it difficult for small primary roots to penetrate. Secondly, although the nutrient conditions were favourable for remediating bacteria, they were not necessarily optimal for plant growth.

ii. Germination tests directly in reactors

In the case of the untreated soil (Reactor 1), yellow seedling tips were observed which were absent in the case of Reactor 6. Although it is possible that these could have been due to a nutrient deficiency, toxic effects cannot be ruled out. Percentage germinations were not

determined in the seedlings grown in the reactors. The reactor walls caused shadows in some places, which would cause this data to be statistically incorrect. It was for this reason that percentage germination was determined in the seedling trays (i).

TABLE 3.6. Statistical ratings of wheat seedlings grown in the remediated soil of Reactor 6 and untreated soil of Reactor 1

Reactor no	Shoot length (mm)		Root length (mm)	
	6	1	6	1
Number of seedlings	83	73	83	73
Mean length	106	152.5	13.4	57.1
Standard deviation	3.06	33.8	7.5	23.6

The trends observed in the seedling trays (Table 3.6) also occurred in the reactors. These results indicate that even highly petroleum-contaminated soils are tolerated by *Triticum aestivum*. Plants have previously been used in remediation of petroleum-contaminated soils i.e. phytoremediation. With the help of plants, microbial degradation of organic waste constituents in the rhizosphere was shown to accelerate (Anderson, Guthrie & Walton, 1993; Lee & Banks, 1993). Although phytoremediation seems an inviting option, the nature of the site in this study militated against this biotechnology.

3.6 CONCLUSIONS

The following conclusions were made from this study:

- Pilot-scale bioremediation by landfarming proved capable of effecting a TPHC reduction of 94% (m/m) from an initial concentration of 320 gkg⁻¹ soil to 18 gkg⁻¹ soil over a period of 10 wks;
- Biosupplements improved the degradation and overall TPHC reduction compared to the case in which nutrients only were used. Final concentrations of 1.8% (m/m) TPHC were reached. Promotion of TPHC catabolism by addition of a commercial biosupplement compared to a site-specific microbial biosupplement was insignificant. Also, the commercial biosupplement showed a lag phase during which it is possible that the microorganisms adapted to the soil conditions;
- The addition of oxygen, either by turning the soil regularly or by H₂O₂ supplementation, enhanced degradation rates. Oxygen concentration in the soil may be an important limiting factor and can significantly affect bioremediation rates;
- Bioremediation can be enhanced by both manipulation of nutrients, oxygen and moisture, to create a favourable environment for increased degradation rates, and bioaugmentation; and
- Bioremediation does not necessarily leave the soil in an optimal condition for plant growth.

CHAPTER 4

BIOREMEDIATION OF A PETROLEUM-CONTAMINATED SITE

BY LANDFARMING:

A FULL-SCALE CASE STUDY

4.1 INTRODUCTION

Recent events in South Africa have impacted on social, economic and political structures placing renewed emphasis on the preservation of land and water resources. South Africa has a low, variable rainfall averaging 502 mm per annum (Department of Water Affairs, 1986) which makes protection of surface and groundwater resources imperative.

Environmentally conscious petroleum industries are recognising their responsibilities in terms of cleaning contaminated sites. This chapter summarises the bioremediation of highly weathered petroleum products in soil and sediment by landfarming techniques. The main objective was to remediate the contaminated soil to < 2 000 ppm TPHC. The effects of various critical parameters were investigated at pilot-scale (Chapter 3). Based on results of the pilot-scale test, a treatment programme for full-scale landfarming operation was implemented and assessed.

Landfarming has been used successfully in many countries although this biotechnology has the reputation of being time-consuming. Genouw, Naeyer, Van Meenen, Van de Werf, De

Nijs & Verstraete (1994) documented that oil sludge can be effectively treated by landfarming, reaching catabolic rates of 4 to 15 gHCkg⁻¹ dry soil per year. However, the study documented in this chapter may have proved that if the environmental contaminants are controlled effectively, the degradation rate can be enhanced substantially.

4.2 MATERIAL AND METHODS

4.2.1 Determining regulatory cleanup standards

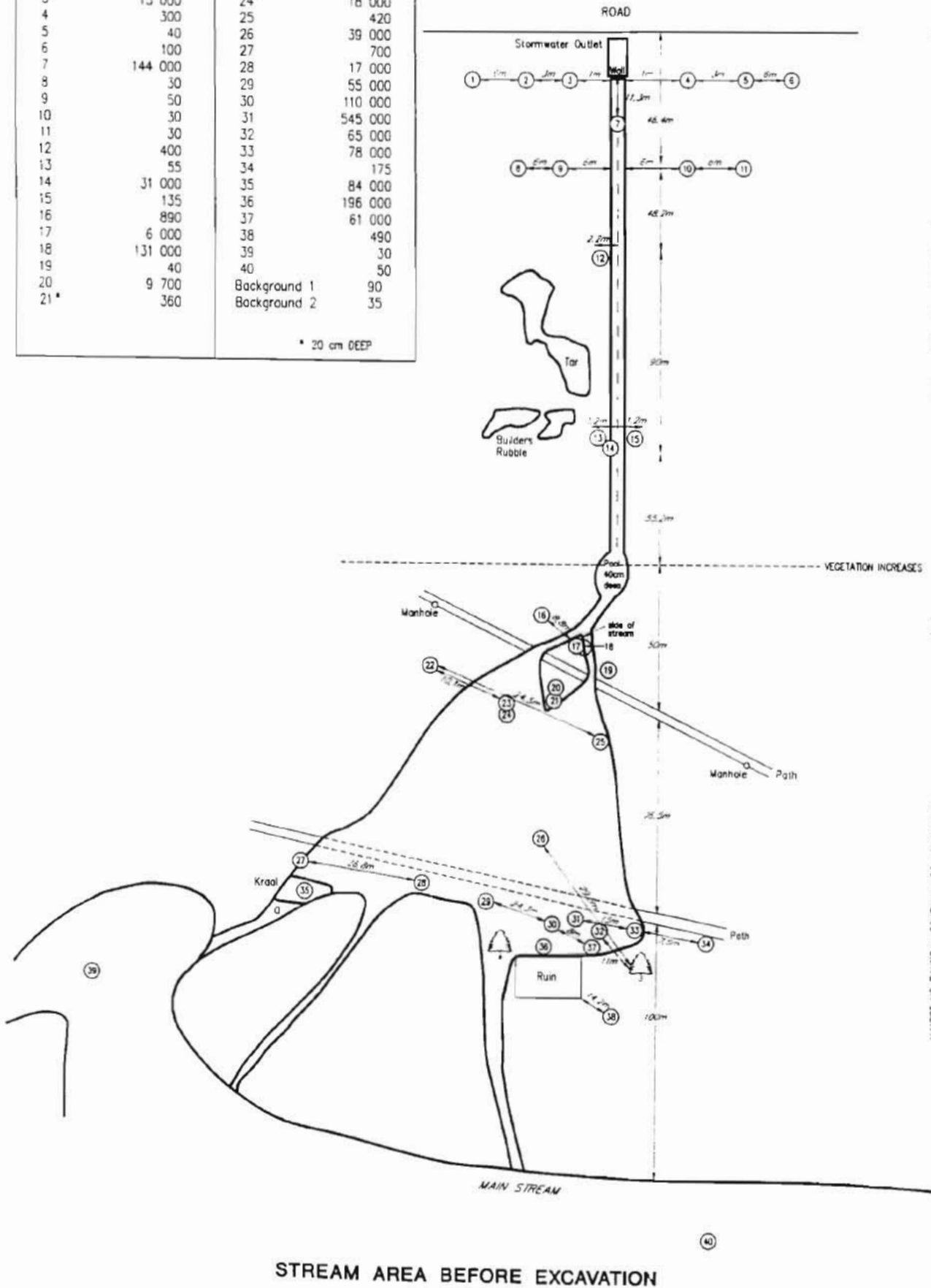
Remediation target concentrations were negotiated with the local town council and the DWAF, using the results of background samples and the pilot-scale study. The nature of the contaminant, current and possible future use of the land and risks were considered.

4.2.2 Extent of contamination

Following the visual inspection of the extent of the contamination, the preliminary boundaries of the contamination were determined. A sampling grid was defined in this area and extensive sampling was undertaken to determine the extent of the contamination by chemical analyses. The sampling points were marked on site and mapped (Figure 4.1). Soil samples were immediately transported to an independent analytical laboratory where they analyzed for TPHC by the EPA 418.1 method.

LEGEND:			
Sampling Pt	TPHC mg/kg	Sampling Pt	TPHC mg/kg
1	130	22	180
2	210	23	250
3	15 000	24*	18 000
4	300	25	420
5	40	26	39 000
6	100	27	700
7	144 000	28	17 000
8	30	29	55 000
9	50	30	110 000
10	30	31	545 000
11	30	32	65 000
12	400	33	78 000
13	55	34	175
14	31 000	35	84 000
15	135	36	196 000
16	890	37	61 000
17	6 000	38	490
18	131 000	39	30
19	40	40	50
20	9 700	Background 1	90
21*	360	Background 2	35

* 20 cm DEEP



B685-1 SCALE 1=1

STREAM AREA BEFORE EXCAVATION

FIG 4.1 Extent of the contamination determined by 11...

4.2.3 Surface cleaning

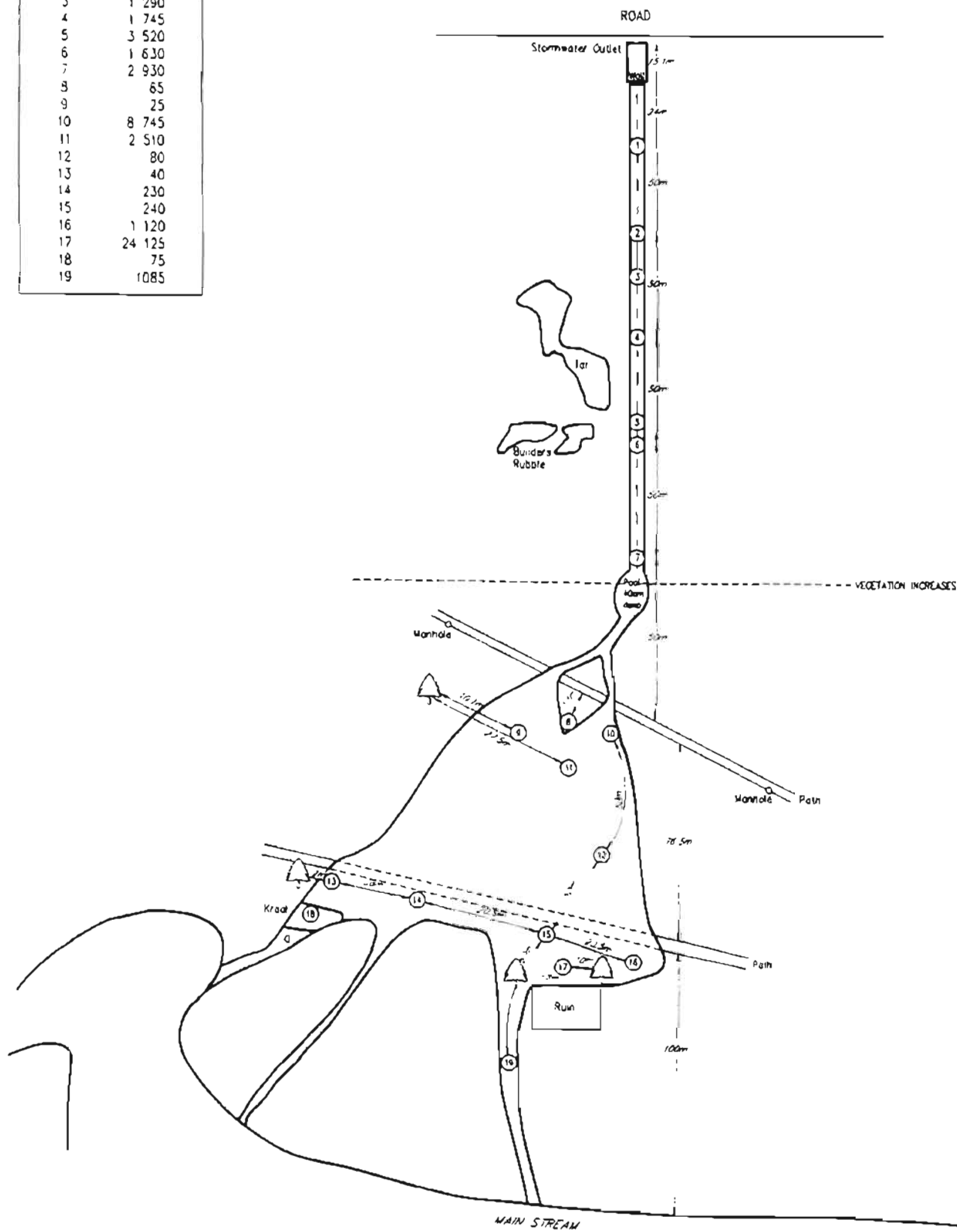
The stormwater drain feeding the stream was closed for the duration of the operation. The concrete and rock surfaces were cleaned by means of a high pressure water jet or steam. The downstream contamination caused by the cleaning procedure was restricted through use of absorbent fibres and booms (Drizit, S.A.).

4.2.4 *Ex situ* remediation site preparation and excavation

A site (50 x 70 m), 50 m from the stream, was cleared of vegetation using a grader. Bund walls (500 mm high) were constructed to contain run-off. The depth of the ground water table (13 m) was considered to be susceptible to ground water contamination, thus the decision to excavate and bioremediate *ex situ*. The contaminated soil was excavated with a top load and back actor in the narrow stream area and a grader and front end loader in the areas where the contamination had spread. Samples were taken at the locations shown in Figure 4.1 and analyzed for TPHC (Figure 4.2). The soil was excavated at the locations where the analyses indicated values higher than the target concentrations. The excavated soil and sediment were heaped to a depth of 0.4 m using a front end loader. An irrigation system was then installed.¹

¹ The irrigation system was stolen before it could be used.

LEGEND:	
Sampling p:	TPHC mg/kg
1	3 359
2	2 340
3	1 290
4	1 745
5	3 520
6	1 630
7	2 930
8	65
9	25
10	8 745
11	2 510
12	80
13	40
14	230
15	240
16	1 120
17	24 125
18	75
19	1085



CONTAMINATION WITHIN RIVER BANKS

ALMOST NO BANKS - CONTAMINATION SPREADING

Reids 2 SCALE 1:1

STREAM AREA AFTER EXCAVATION

FIG. 4.2 TPHC values of soil determined after initial excavation

4.2.5 Bioremediation

i. Aeration

The site was ploughed weekly, using available agricultural tilling equipment, to ensure mixing and aeration.

ii. Moisture

Water (30 kℓ) was added twice weekly using a water tanker. Watering was terminated during the rainy season when natural precipitation was sufficient.

iii. pH

Twenty tons of Lytton dolomite lime (44% (m/m) CaCO_3 , 31% MgCO_3 (m/m)) was added with the onset of remediation. This was repeated after two months. The soil pH was tested monthly with the Holte's soil test.

iv. Nutrients

Monoammonium phosphate (MAP), containing 11% (m/m) nitrogen and 22% (m/m) phosphate (Kynoch Fertilizer Ltd.), 550 kg was added at the beginning of the study and again after three months (350 kg). The fertilizers were ploughed in to the full depth of the

site (0.4 m). On the 155 day of treatment, anaerobically digested sludge was added to the site.

v. *Biosupplement*

A biosupplement consisting of microorganisms indigenous to the contaminated soil was prepared every fortnight and added through a mobile sprinkler unit fixed to a tractor. The biosupplement was prepared by adding NaNO_3 (1 kg), KCl (250 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (250 g), K_2HPO_4 (500 g), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (5 g), sucrose (1.5 kg), yeast extract (50 g) and commercial motor oil (5 l) to 750 l of potable water. With the exception of the oil, all chemicals were obtained from Unilab. Saarchem (Pty) Ltd. Samples (2 kg) of heavily contaminated soil were selected from the bioremediation site, mixed with potable water (3 l) for 5 min and allowed to settle. The soil slurry was agitated again and strained through a sieve (1 mm mesh). The resulting liquid was used as inoculum, and was added to the oily growth medium. The mixture was aerated for seven days with two industrial diffusers and was then added to the site without any additional treatment.

vi. *Sampling*

Samples were taken every month with a manual auger. The site was divided into a predetermined grid system and permanent markers were installed to ensure that samples were taken at the same location and to rule out bias sampling. The site was divided into six horizontal lines, 10 m apart, designated A, B, C, D, E and F. Three orthogonal lines, 20 m

apart in the horizontal plane, were used to form a grid. A sample, for example the A sample, was taken on the A line at each of the three intersections. At each of these points, samples were taken at two depths, 50 to 250 mm and 250 to 400 mm. All the samples from the A line were then pooled and mixed thoroughly to prepare a composite sample. Three Consol Glass canning jars were filled, leaving no headspace. The process was repeated for all the lines to give six composite samples from the 36 sample points. The soil samples were stored at 4°C prior to moisture, pH, nutrient, microbiological and TPHC analyses.

4.2.6 Analytical methods

Total and hydrocarbon-degrading species plate counts were made (2.4.4). TPHC concentrations were determined by the EPA 418.1 method (Environmental Protection Agency (USA), 1979). Nitrogen and phosphorus concentrations were determined according to standard methods (Standard Soil Testing, 1990).

4.3 RESULTS AND DISCUSSION

4.3.1 Determination of regulatory cleanup standards

After negotiations with the local town council and DWAF, a clean-up target of 2000 ppm was considered acceptable. This decision was based on the fact that the spill had occurred in an industrial area where the future use of the land would probably be industrial.

4.3.2 Extent of contamination

Figure 4.1 (not drawn to scale) illustrates the extent of the contamination based on a visual and analytical assessment. Based on the results obtained from the TPHC analyses, the extent of the contamination and hence excavation was determined. The results indicated that the spill was contained within the banks of the stream for the first 230 m from the stormwater outlet. For example, Sample 7, which was taken in the middle of the stream bed, had a TPHC of 144 000 ppm, compared to Sample 8 or 10 which had TPHC values of the same magnitude as the background samples.

After the first 230 m, the contamination had spread as the banks of the stream disappeared. Thick vegetation made sampling and mapping very difficult. A "hot spot" was identified around Sample 31. Visually, the area did not seem to be particularly contaminated since the samples were loose and had little petroleum odour to them. Yet, Sample 31 had a TPHC value of 545 000 ppm. There were two contributing factors to this finding. Firstly, this spot was compromised by a previous spill, hence the visual change in the oil and the fact that the lighter fractions which are normally detected by smell had already been degraded or volatilized. Secondly, it should be noted that the EPA 418.1 method correlates the contaminant concentration according to the mass of the whole sample (matrix + contaminant). In this case, the matrix was the leaf debris on the forest bed which is much lighter than soil and has a higher capacity to absorb oil than soil has. In addition to the mass, organic material can also contribute to the TPHC (Douglas *et al.*, 1992). For example although Sample 38 was taken outside the contaminated area it had a TPHC of 490 ppm.

This value can only be ascribed to the organic matter content.

Sample 35 was taken in an old animal pen where the oil had dammed up and settled in a 30 cm thick soil/oil slurry. The area marked "a" south east of the pen was not excavated. The bulk of the oil was contained by the walls of the pen and little oil seeped through. The plants in this area, unlike the rest of the contaminated area, were indigenous. As a consequence, it was concluded that excavation of the oil contaminated soil in this area would be more harmful to the ecology than the presence of a small amount of oil.

The main stream was also investigated for evidence of contamination. Samples 39 and 40 were taken up-stream and down-stream of the contaminated inlets. The TPHC determinations showed no petroleum contamination.

4.3.3 Excavation

After excavation, samples were collected again (Figure 4.2) and the concentrations of several samples were still > 2 000 ppm. Where possible, the soil was excavated again to meet the requirements of the DWAF. The excavated area was measured to be 8 494 m² and accounted for 1 500 m³ of petroleum-contaminated soil.

4.3.4 Landfarming

i. Moisture

The minimum moisture content during the remediation programme was 2.4% (m/m) and the maximum was 12.6% depending on the time of day and the weather. During the first 50 days of remediation, the soil dried out within a day of watering. For example, 2 h after the water tanker watered the site, the moisture content was 6.8% (m/m) while 48 h later the moisture had dropped down to 3.6%. After this period the annual summer rains started which kept the site moist on a more regular basis. The average site moisture stabilised at 8.4% (m/m) \pm 2.7.

ii. pH

The soil pH was between 5.5 and 6 at the start of the project. After the addition of lime, the pH was 6.5.

iii. Nutrients

Nutrients were added on two occasions. Table 4.1 documents the effect of each nutrient addition.

TABLE 4.1. Nutrient concentrations before and after the addition of monoammonium phosphate during the remediation

Time	Carbon (mgkg ⁻¹)	Nitrogen (mgkg ⁻¹)	Phosphorus (mgkg ⁻¹)
Before nutrient addition	51 000	1 000	12.4
After first nutrient addition	51 100	2 500	158.6
Before second nutrient addition	31 433	1 100	40.6
After nutrient addition	32 100	3 000	103.6

The results show that the addition of the MAP was adequate to enhance the nutrient concentration. Note that between the first and second additions, the nutrients were depleted, thus necessitating the second supplement.

iv. Biosupplement

The biosupplement was cultivated for one week before addition. The microorganisms in the liquid biosupplement were enriched from $<10^6$ cfum ℓ^{-1} to $>10^{12}$ cfum ℓ^{-1} in the first three days and the carbon source (commercial motor oil) decreased from 2 946 to 1 573 mg ℓ^{-1} , indicating microbial catabolism. Enrichment values of $>10^{12}$ cfum ℓ^{-1} were unusually high. However, the two control series, where the agar and the saline were tested, ruled out contamination. An open nutrient agar plate was placed in the environment where the plate counts were performed which ruled out environmental contamination as well. Concern over residual oil introduced into an already contaminated site, should be addressed. If, for example, the biosupplement was introduced to the soil after only 50% (m/m) of the oil was catabolized (three days of incubation) the residual oil would contaminate the soil with

0.5 ppm. If this is compared to the average concentration of contamination at the start of the study, the effect of adding the biosupplement with residual oil, would be insignificant (0.004% (m/m)). Separating the biomass from the residual oil would have been costly, both financially and in terms of the biomass that would be lost in the separation process. However, the effect of different sucrose and oil concentrations is currently under investigation.

This method of cultivating biomass from a fraction of the contaminated soil for subsequent use as an inoculum for bioaugmentation (soil activation) of the same soil was investigated at laboratory scale by Otte, Gagnon, Comeau, Matte, Greer & Samson (1994) with success. In the study investigated by the author, soil activation was applied at full-scale. The biosupplement was prepared and added every 14 d. A new inoculum was prepared from the soil on each occasion. The intention was, therefore, to enrich the microbial population that was dominating at that specific time of the remedial process. Theoretically, the petroleum degrading population should change with time. This population shift is currently under investigation.

v. *Contaminant reduction and microbiology*

The results of the TPHC analyses and microbiological plate counts of the full-scale treatment are shown in Table 4.2. After the first month of treatment, the TPHC was higher than at the beginning. This was thought to be due to the effects of mixing. Initially, the soil was heterogeneous but became more homogenous during the first month. Decrease in

TPHC was initially slow, with the limiting factors considered to be low levels of moisture in the soil due to the lack of natural precipitation, the low moisture retaining capacity of the stream sediment, and the high evaporation rates. The lower moisture levels were probably responsible for lower soil microbial numbers and, consequently, slower degradation rates. Increased water dosages, as well as the start of the rainy season, increased the soil moisture and is likely to be the factor which promoted the degradation to an average of 2 000 mgkg⁻¹ mo⁻¹. A final TPHC concentration of 1 390 mgkg⁻¹ was obtained, which is below the target level of 2 000 mgkg⁻¹ required by the DWAF.

TABLE 4.2. TPHC and plate counts of soil samples from the full-scale treatment

Grid Line	TPHC mgkg ⁻¹ soil							
	Time 0	26 days	56 days	78 days	96 days	124 days	155 days	169 days
A	7 425	9 270	7 555	2 280	1 560	1 845	1 050	
B	7 845	10 905	8 555	3 310	2 900	2 665	820	820
C	20 720	19 575	18 460	5 250	8 330	5 820	2 405	1 770
D	9 490	10 610	21 410	5 960	8 620	6 270	3 725	2 335
E	5 260	10 755	7 985	2 560	5 180	4 280	2 250	1 135
F	22 990	21 350	20 215	4 915	5 680	4 065	7 130	1 240
Average	12 288	11 945	12 554 (excl D)	4 046	5 378	4 157	2 897	1 392
Hydrocarbon-degrading microorganisms (CFUg ⁻¹ soil) ^a	6 x 10 ⁶	2 x 10 ⁶	8 x 10 ⁶	7 x 10 ⁷	8 x 10 ⁷	6 x 10 ⁷	9 x 10 ⁷	> 10 ⁷

a Average of three plate counts on OECD minimal medium which contained commercially available lubricating oil as sole carbon source.

CFU Colony forming unit

TPHC Total petroleum hydrocarbon concentration

Interestingly, the hydrocarbon degraders twice increased by an order of magnitude during the treatment. The first occurred between days 56 and 78 and the second between days 155

and 169. On both these occasions the TPHC showed enhanced decreases. This was especially evident the first time when the TPHC dropped from 12 554 to 4 046 mgkg⁻¹. This could have been due to the effect of mass transfer. In other words, during the first 56 days, the contaminant was only available to a small fraction of the microbial community, due to the high contaminant concentration and, therefore, the resulting toxicity. These microorganisms might have had the enzymatic capability of overcoming the first rate-limiting step in the catabolic cascade. The resulting molecules could then have been more labile to other members of the microbial association. At this point the resulting increased population degraded the hydrocarbons rapidly and this was promoted by the general increase in moisture. Once this labile carbon source was depleted, the same scenario repeated itself, but to a lesser extent. This cycle could, theoretically, continue until the carbon source was completely depleted. The microbial populations present and their respiration rates were investigated and the results are detailed in Chapters 5, 6 and 7.

4.3.5 Comparing the pilot and full-scale investigations

It is generally agreed that results obtained during a controlled laboratory study cannot be duplicated in the field, probably due to a failure of the application to fully account for the rate-limiting factors prevalent in soil systems (Autry & Ellis, 1992). However, it is also generally considered risky to attempt a full-scale cleanup without some kind of prior treatability work. In fact, Block, Kabrick, Stroo & Swett (1992), who have been involved with more than thirty full-scale treatments, reported that the only bioremediation failures were at sites in which the clients broke *the cardinal rule*: "Always conduct some level of

treatability work to be sure a unique condition does not exist that will make the selected remedy ineffective."

Researchers in this area should, therefore, strive towards obtaining results which are as close to the field situation as possible, or at least simulate the worst case scenario in the laboratory. In this study for instance, the pilot-scale study was made on a soil sample contaminated with 320 000 ppm of hydrocarbon compared to the highest contaminated sample on the bioremediation site which was 23 000 ppm. In other words, during the excavation the soil was diluted considerably, a factor which was deliberately omitted from the pilot-scale study.

However, even though the worse case scenario was investigated at pilot-scale, the degradation rate still could not be extrapolated to the full-scale results. The catabolic rate at pilot-scale was much higher than in the full-scale application. In this particular case, there are reasons, other than the shift between the laboratory and the field, which contributed to the results. The soil sample used in the pilot-scale study was collected only days after the spill occurred whereas the full-scale study was initiated a few months after the spill. In other words, the oil had been weathering and volatilizing in the open environment for several months, resulting in a different residual contaminant. A fire also contributed to the weathering of the free product which had not seeped into the subsurface soil. The most important factor was that a large proportion of the oil treated was not from the 1994 spill but was from a previous spill of which very little history was known. This product was extremely weathered and recalcitrant thus making biological treatment very difficult.

With all the factors taken into consideration, the contaminant was successfully degraded by the treatment which also showed the best results at pilot-scale. However, since no controls could be established at this site it is unknown how the other treatments that were investigated at pilot-scale would have performed at full-scale.

4.4 CONCLUSIONS

The following conclusions were made from this study:

- Bioremediation by landfarming reduced the TPHC concentrations from initial values in the range 5 260 to 23 000 mgkg⁻¹ to values in the range 820 to 2 335 mgkg⁻¹ soil over a period of 169 days;
- Initial degradation rates were slow, probably due to dry conditions. Once the moisture levels increased, the degradation rates and microbial numbers increased; and
- At full-scale, the larger fraction of more recalcitrant and weathered petroleums, and the less intensive treatment compared to the pilot-scale study, resulted in a slower rate of TPHC reduction than was found in the latter.

CHAPTER 5

THE CULTURABLE MICROBIAL POPULATION PRESENT IN HYDROCARBON-CONTAMINATED SOIL

5.1 INTRODUCTION

Microorganisms were isolated from 1500 m³ of petroleum-contaminated soil during the full-scale landfarming project (Chapter 4). These microorganisms were cultured to high concentrations and then re-administered to the contaminated soil (4.2.5 v) with the objective of enriching the hydrocarbon degrading microorganisms in the soil. The question arose whether the cultured bacteria and fungi actually survived in the soil after administration and whether the soil microorganisms were of a similar type throughout the bioremediation period.

The principal objectives of the study described in this chapter were to determine the dominant microbial population responsible for degrading the hydrocarbon contaminant and to determine whether a shift in population took place as the contaminant concentration decreased.

These questions had to be addressed to enable a better understanding of the bioremediation process. For example, if the microorganisms in the biosupplement were not contributing to the degradation of the contaminant or surviving in the process, this time consuming and

costly step could be discarded in the treatment or alternatives investigated.

5.2 MATERIALS AND METHODS

A composite sample was taken monthly from each of the six lines on the full-scale bioremediation site (4.2.5 vi). Using sterilized equipment, equal sized batches (10 g) were taken from these samples and mixed thoroughly, resulting in a single sample representing the bioremediation site for a particular time during the treatment. This was done for every set of monthly samples.

From the combined sample, samples (1 g soil) in triplicate were suspended in 10 ml saline, serially diluted in physiological saline solution and plated on nutrient agar and OECD (Organisation for Economic Cooperation and Development) minimal medium which contained 30 ml commercially available base oil as sole carbon source. The base oil was of the same chemical composition as the contaminant. The OECD/base oil medium was prepared by adding 17 g agar, 4 ml FeCl_3 (0.25 g l^{-1}), 1 ml each of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (22.5 g l^{-1}), CaCl_2 (27.5 g l^{-1}) and $(\text{NH}_4)_2\text{SO}_4$ (40 g l^{-1}) to 2 ml of the following mixture: KH_2PO_4 (8.5 g l^{-1}), K_2HPO_4 (21.75 g l^{-1}), $\text{NaHPO}_4 \cdot 7\text{H}_2\text{O}$ (33.4 g l^{-1}) and NH_4Cl (1.7 g l^{-1}) and diluting to 1 l with distilled water. The mixture was autoclaved (15 psi, 121°C , 20 min) and after cooling the base oil was added and the agar sonicated before pouring the plates.

The plates were incubated for seven days at 30°C . The plates with the highest dilution still bearing visual species diversity of more than four were selected for further identification.

All bacterial colonies were then subcultured to nutrient agar plates and incubated at 30°C for 48 h. The cultures were purified by restreaking. The fungal species were transferred to potato dextrose agar (PDA) containing chloramphenicol (500 mgℓ⁻¹).

Bacterial identification was made by the Vegetables and Ornamental Plant Research Institute (ARC), Pretoria, using Bergey's Manual of Systematic Bacteriology (Holt, 1984). Fungal identifications were made by the Department of Botany, University of Pretoria, Pretoria, using spore morphology (Baxter *et al.*, 1994).

5.3 RESULTS AND DISCUSSION

Table 5.1 details the dominant microbial species isolated from the landfarming treatment site (Chapter 4). The days on which the samples were taken correspond with the days detailed in Table 4.2. A single biosupplement sample collected after cultivation from contaminated soil 70 days after treatment started was also investigated to establish whether the biosupplement microorganisms had survived in the soil after administration. The microorganisms isolated from the biosupplement should, therefore, be compared to those isolated from the site on day 78 since the biosupplement was prepared from soil sample extracts throughout the treatment time and not from a single soil sample from the beginning of the treatment.

TABLE 5.1. Bacteria and yeasts isolated from contaminated soil and the biosupplement during a full-scale bioremediation

Day of treatment	0	26	56	78	96	124	155	156	169	Biosuppl
Bacterial species and Yeasts										
<i>Bacillus azotoformans</i>	●	●								
<i>Pseudomonas aeruginosa</i>	●						●	●		
<i>Bacillus insolitus</i>	●	●						●	●	
<i>Bacillus mallei</i>	●									
<i>Pseudomonas acidovorans</i>		●								
<i>Bacillus badius</i>		●								
<i>Pseudomonas pseudoalcaligenes</i>			●							
<i>Planococcus halophilus</i>			●	●		●			●	●
<i>Xanthomonas axonopodis</i>			●						●?	
<i>Pseudomonas pseudomallei</i>			●		●	●			●	
<i>Micrococcus luteus</i>				●					●	
<i>Pseudomonas caryophylli</i>				●	●			●	●	●
<i>Pseudomonas oleovorans</i>				●		●				
<i>Micrococcus sedentarius</i>				●						
<i>Frateuria aurantia</i>				●			●			
<i>Bacillus firmus</i>				●	●					
<i>Bacillus pasteurii</i>										●
<i>Bacillus pulvifaciens</i>										●
<i>Pseudomonas halophilus</i>					●					
<i>Pseudomonas mendocina</i>					●	●	●	●	●	●
<i>Pseudomonas mallei</i>							●			
<i>Pseudomonas putida</i>							●	●	●	
<i>Sporosarcina urea</i>							●			
Yeasts				●	●	●	●		●	●

Table 5.1 illustrates that the biosupplement consisted of six dominant species during that time. If these species are compared to the time it was administered (day 78) *Planococcus halophilus*, *Pseudomonas caryophylli*, *Pseudomonas mendocina* and some yeasts could be detected in both the soil samples and the biosupplement. Two *Bacillus* species, *B. pasteurii* and *B. pulvifaciens* which were cultured in the biosupplement were not detected as dominant species in the soil.

The method used in this experiment is biased in several ways which should be noted. The method can only detect the culturable fraction of the population which according to Prakasam & Dondero (1967) could be less than 10% of the cell counts determined by direct microscopic techniques. Furthermore, the method is biased to fast growing microorganisms. Microorganisms which are slow growers on solid media, but metabolise the contaminant, may have been missed. A full investigation into the population shift was beyond the scope and budget of this study and its objectives. Within the constraints of the method used some conclusions were drawn.

The dominant hydrocarbon degraders were found to be *Pseudomonas* and *Bacillus* species. These species are known to degrade hydrocarbons (Antai, 1990). During the first 56 days of treatment, only four dominant species could be isolated. However, between days 56 and 78 the total number of colony forming units increased from 8×10^6 to 7×10^7 CFUg⁻¹soil and the TPHC decreased from 12 554 to 4046 mgkg⁻¹soil (Table 4.2). During this time the dominant species isolated also increased from four to seven (Table 5.1), illustrating that the microbial association changed to a more efficient hydrocarbon utilising consortium. During

the next 46 days (days 78 to 124) the TPHC did not change significantly (Table 4.2) and the microbial population appeared to vary between sample times while after day 124 the population seemed to be dominated by *Pseudomonas* spp. specifically *P. mendocina* and *P. caryophylli*. *P. mendocina* is known to degrade the BTEX group (Zhou & Tiedje, 1995). This might indicate that the microbial population had shifted to those members capable of degrading the aromatic compounds in the contaminant, which were left after the labile fraction of the contaminant such as the aliphatic compounds were degraded.

As the treatment of the contaminated soil was intended to be bacterial, the fungal population was only monitored during the first 78 days. The fungal population seemed to be dominated by *Penicillium* species (Table 5.2). This group are naturally occurring soil borne microorganisms which could contribute to the degradation of the contaminant.

Table 5.2. Fungal species isolated from the contaminated soil during a full-scale bioremediation

Fungal species	Day 0	Day 26	Day 56	Day 78
<i>Penicillium</i> sp.1	●	●	●	
<i>Penicillium</i> sp.4	●			
<i>Penicillium</i> sp.2		●		
<i>Cladosporium</i> sp.		●		
<i>Mortierella isabellina</i>		●		
<i>Aspergillus</i> sp.			●	
<i>Trichoderma</i> sp.			●	
<i>Penicillium</i> sp.3			●	
<i>Penicillium</i> sp.5				●
<i>Penicillium</i> sp.6				●

5.5 CONCLUSIONS

The following conclusions could be drawn from this study:

- A population dominated by *Bacillus* and *Pseudomonas* species, which are known to be degraders of complex hydrocarbons, was isolated throughout the bioremediation project;
- The dominating population in the biosupplement prepared from indigenous microorganisms from the contaminated site showed some overlap when compared to the dominating species isolated directly from the contaminated soil; and
- The method used to identify the populations at different time periods was identified to be biased toward fast growing culturable microorganisms.

CHAPTER 6

THE USE OF rRNA-TARGETED OLIGONUCLEOTIDES TO INVESTIGATE THE TOTAL MICROBIAL POPULATION PRESENT IN HYDROCARBON-CONTAMINATED SOIL

6.1 INTRODUCTION

The analysis of natural microbial communities is limited by reliance on growth dependent methods for identifying individual species. So far, two techniques for specific enumeration of bacteria have been established: selective plating; and immunofluorescence. Both techniques depend on the isolation of the target organisms. While selective plating techniques rely on strong phenotypic markers of the target organism, the immunofluorescence technique needs pure cultures of the target organism in order to raise specific antibodies. Even though selective isolation procedures do exist for many microorganisms, most members of natural bacterial communities seem to remain unculturable and their identity unknown (Hahn, Amann, Ludwig, Akkermans & Schleifer, 1992).

Molecular methods based on DNA or rRNA sequence analysis have reached a high level of acceptance in microbial ecology as new techniques for the specific enumeration of bacteria, unbiased by the limitations of culturability (Holben, Jansson, Chelm & Tiedje, 1989).

rRNA directed oligonucleotide probes have been designed and successfully used to detect

specific organisms without prior isolation (Hahn, Kester, Starrenburg & Akkermans, 1990). In recent years, this technology has developed to enable the detection and identification of individual cells *in situ*. Hahn *et al.* (1992) investigated the application of *in situ* hybridization with fluorescent labelled rRNA targeted oligonucleotides for the detection of specific target organisms in soil. Similar to the application of fluorescent antibodies for identification of bacteria in soil, protocols for *in situ* hybridization have to deal with non-specific binding of probe to soil particles, autofluorescence of soil components, recovery of introduced bacteria and specific detection of a target organism with large background of non-target microorganisms (Gray, 1990). In this study, these criteria were investigated using soil from a bioremediation site (Chapter 4). Since these microorganisms are subjected to a stress situation, the cells should have a much lower metabolic rate compared with microorganisms from natural, uncontaminated soil. Low metabolic activity implicates reduced amounts of RNA, and therefore less target molecules for the probes. The methods described by Hahn *et al.* (1992), therefore, needed to be adapted to detect the microorganisms in the petroleum-contaminated soil.

The objective of this programme was to establish a method to study the total microbial population in the contaminated soil undergoing bioremediation, using rRNA-targeted, fluorescently-labelled oligonucleotides.

6.2 MATERIALS AND METHODS

The experiment was repeated three times.

6.2.1 Sample preparation

Six soil samples (500 g) were collected from a full-scale land farming treatment operation after 26 and 78 days of treatment, and stored at 4°C. The samples were combined and mixed in equal proportions (m/m) resulting in a composite sample representing day 26 and one representing day 78. Sterile distilled water was added to two samples (30 g) of each of the composite mixtures and placed in a sealed glass container which contained an open petri dish filled with 30 ml of base oil contaminant. The soil was left to equilibrate for 72 h at room temperature.

Samples (1 g) were weighed and transferred to 25 ml glass bottles with Teflon coated screw caps, under sterile conditions. Sterile nutrient broth (100 µl and 1 ml) was added to the separate samples of the day 26 and 78 samples, vortexed for 20 sec and incubated at 30°C for 12 h. To ensure the same conditions, the control samples of soil were also placed in the incubator. After the 12 h incubation period, the samples containing the nutrient broth were placed on ice, while the other samples were “spiked” with 1 ml of an *Echerichia coli* log phase culture in nutrient broth and placed on ice for 1 h to allow some binding of the introduced cells to the soil particles.

6.2.2 Cell fixation and extraction from soil

Paraformaldehyde fixative (100 ml) was prepared by adding paraformaldehyde (4 g) to 65 ml double distilled water at 65°C. One drop of 2M NaOH solution was added and the solution was stirred rapidly until nearly clarified and then removed from the heat source. PBS x3 (33 ml) (phosphate buffered saline, 390 mM NaCl, 30 mM sodium phosphate buffer pH 7.2) was added and the pH adjusted to 7.2. The solution was rapidly cooled down to 4°C after sterile filtration. Paraformaldehyde (3 ml) was added to each of the samples and fixed overnight at 4°C. The samples were mixed on a vortex mixer for 10 seconds and kept on ice for 1 h to allow separation of heavy soil particles from the supernatants. The supernatants were removed and the soil pellets re-extracted with 2 ml fixation buffer. The combined supernatants were centrifuged at 5000 x g for 15 min, the supernatant discarded and the pellet washed twice in 1x PBS. One volume of ice-cold ethanol was added to all the samples. These fixed cells were stored in the freezer (-20°C) (adaptation of methods described by Hahn *et al.*, 1992; Amann, 1993).

6.2.3 Pretreatment of microscope slides

Hydrophobically coated slides with eight glass windows (Sterilab) were cleaned by soaking them in a warm detergent solution for one hour, rinsed with distilled water and air dried. The slides were then dipped in a warm (70°C) 0.1% (v/v) gelatin, 0.01% KCr(SO₄)₂ and allowed to dry in a vertical position (Amann, 1993).

6.2.4 *In situ* hybridization

A sample (3 μl) of each fixed cell suspension was spread on a gelatine coated slide over an area of 5 mm in diameter, and allowed to air dry. The cells were then successively passed through 50, 80 and 98% (v/v) ethanol washes (3 min each), to dehydrate the cells. Each of the preparations was hybridized in 8 μl hybridization buffer (20 mM Tris/HCl, pH 7.2; 0.01% SDS; 0.9 M NaCl) and 1 μl of probe (50 ng) at 46°C in an temperature equilibrated sealed moisture chamber containing a slip of Whatman 3MM paper soaked in hybridization buffer, for 90 min. After hybridization, the slides were removed from the moisture chamber and the hybridization was stopped by rinsing the probe from the slides with wash-buffer (20 mM Tris/HCl, pH 7.2; 5 mM EDTA; 0.9 M NaCl; 0.1 % (m/v) SDS) prewarmed to the hybridization temperature. The slides were then washed in wash-buffer for 20 min at 48°C and the salts removed by dipping the slides in double distilled water. The slides were allowed to air dry and mounted in a glycerol/PBS mountant with pH >8.5 (Citiflour Ltd, UK) and viewed with an epifluorescence microscope equipped with suitable filters.

6.2.5 Probes

An eubacterial probe designed by Amann *et al.* (1990) was used in this study. The probe (Eub338) was derived from *E. coli* 16S rRNA position (338 to 355), and has the sequence: 5'-GCTGCCTCCCGTAGGAGT-3' which is complementary to the 16S rRNA strand. The non-eubacterial probe (Non-Eub) has the same sequence as the *E. coli* 16S rRNA position (338 to 355) and is, therefore complementary to the eubacterial probe.

6.3 RESULTS AND DISCUSSION

6.3.1 The potential of *in situ* hybridization of soil microorganisms with r-RNA-targeted, fluorescently labelled oligonucleotides

Figure 6.1 (a and b) illustrates the potential of the method as a tool to study a biologically active bacterial population *in situ*. Whole cell *in situ* hybridization of the bacterial populations enabled the detection of the hydrocarbon degrading microorganisms, illustrated by an arrow on Figure 6.1a, compared to the results obtained from the traditional phase-contrast microscopy, of the same field, where no bacterial cells were observed (Figure 6.1b).

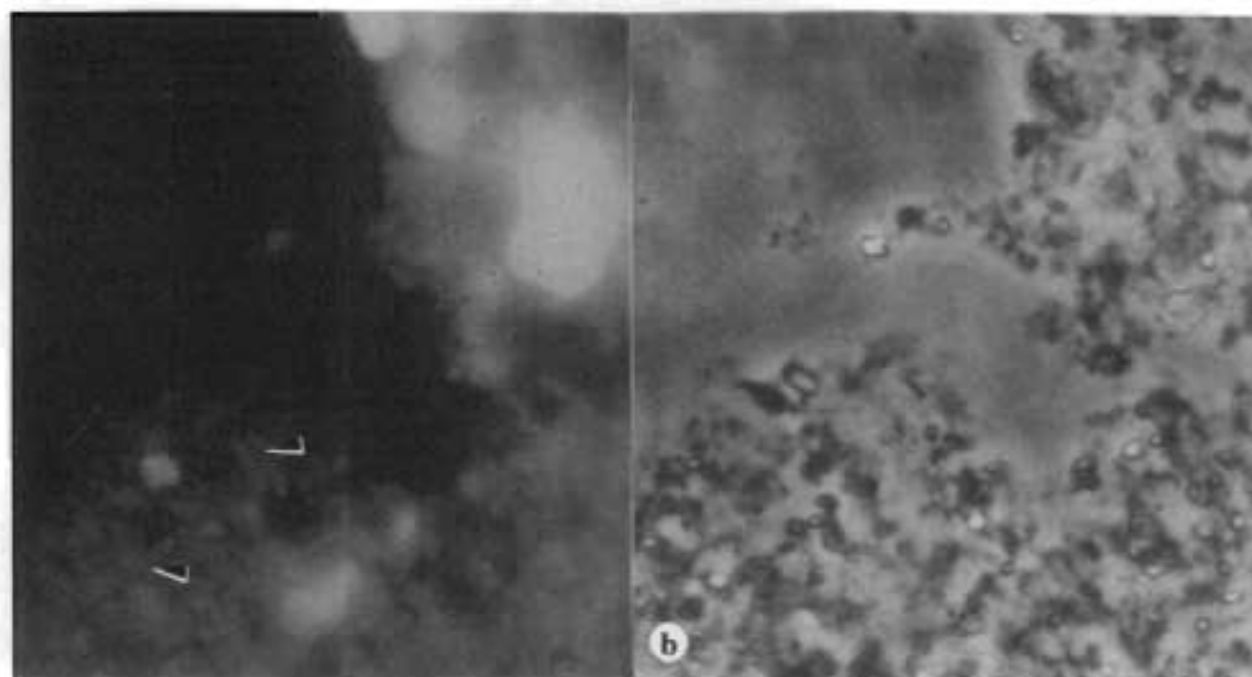


FIG. 6.1 The detection of bacterial populations in soil - (a) biologically active bacterial population after *in situ* hybridization with the universal probe Eub338 in soil which is undergoing bioremediation (the arrows show the bacterial cells), (b) the corresponding phase-contrast photograph.

6.3.2 Investigating population change during a full-scale bioremediation operation

Hahn *et al.* (1992) investigated the technique of *in situ* hybridization of fluorescently-labelled oligonucleotides to detect microorganisms directly in soils which have different contents of soil minerals and organic material. The authors succeeded in recovering introduced *Pseudomonas aeruginosa* cells. However, they were not successful in detecting natural bacterial populations in soil without activation of these microorganisms by adding nutrients. The authors explained that the amount of rRNA is correlated with the activity of the cells, and that activity is normally low in soil because of the lack of nutrients (Hahn *et al.*, 1992).

In the current study, the bacterial populations active in soil contaminated with hydrocarbons were investigated. These bacteria are known to be under stress and have low activity. However, the main objective of bioremediation is to enhance the biological activity in the contaminated soil by removing inhibiting factors (such as low nutrient values) as far as possible.

In this study, the natural populations in the contaminated soil were detected without the addition of nutrients. However, the fluorescence disappeared rapidly and photographs did not reveal any bacterial cells. When nutrients were added in the form of nutrient broth (100 μl), the cells could be detected more readily although the signals were not stable enough to photograph since the lens shutter had to be open for 10-20 sec and fluorescence disappeared during this time. Nutrient broth (1 $\text{m}\ell\text{g}^{-1}$ soil incubated for 12 h at 30°C)

resulted in clear cells that could be photographed readily (Figure 6.1a).

Once the method had been optimized, it was tested on soil samples originating from a full-scale bioremediation site and collected 26 and 78 days after treatment started. These samples were chosen since the rate of oxygen consumption increased ten fold in the soil samples collected after 78 days compared to the samples collected after 26 days, indicating an increase in biological activity and/or a population change (Chapters 4, 5, and 7). In addition to the oxygen consumption rate, the total petroleum hydrocarbon concentration decreased rapidly between these different times, also indicating an increase in biochemical activity.

Figure 6.2 illustrates the results obtained from the soil samples collected 26 and 78 days after the start of the full-scale bioremediation (Chapter 4). Figure 6.2a is the positive control, which represents a pure culture of *E. coli* cultured in nutrient broth probed with an eubacterial probe (Eub338). The corresponding negative control (an *E. coli* culture probed with Non-Eub) yielded no fluorescent cells (results not shown - black field). Figure 6.2c illustrates that *E. coli* cells introduced to the soil could be retrieved when probed with the Eub338 probe but not retrieved when probed with the Non-Eub probe (Figure 6.2b). Figure 6.2d illustrates the enriched natural population of the contaminated soil after 78 days of treatment. Interestingly, the enriched natural population from the soil samples taken after 26 days of treatment were not stable for long enough to photograph. This could indicate that the soil microorganisms did go through some kind of change, be it a population shift or becoming more biochemically active, between the 26th and 78th days of treatment.

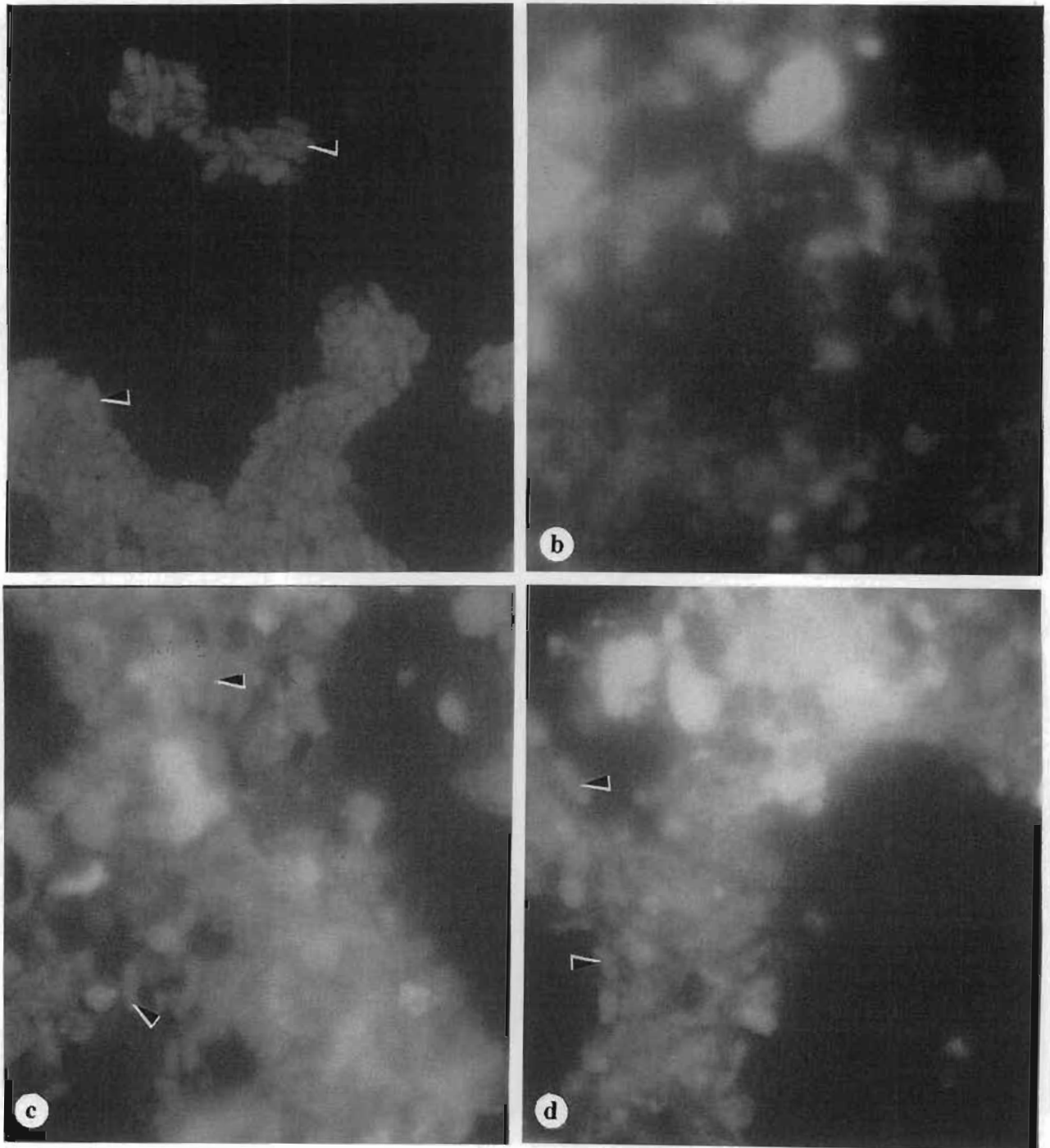


FIG. 6.2 Results obtained from the soil samples collected from a remediation site during treatment - (a) a positive control, representing a pure culture of *E. coli* probed with Eub338, (b) *E. coli* cells introduced to the soil probed with the Non-Eub, (c) *E. coli* cells introduced to the soil probed with the Eub338 probe, (d) enriched natural population of the contaminated soil after 78 days of treatment, probed with Eub338. The arrows show the bacteria.

6.4 CONCLUSIONS

- The study proved that *in situ* hybridization with rRNA-targeted, fluorescently-labelled oligonucleotides allows the total microbial population present in the contaminated soil to be investigated; and
- Standard methods needed adaptation and the soil samples needed to be activated by the addition of nutrients.

CHAPTER 7

RESPIRATION OF SOIL MICROORGANISMS DURING FULL-SCALE LANDFARMING

7.1 INTRODUCTION

Aerobic respirometry is the measurement of living cell oxygen consumption and carbon dioxide production measured as the uptake or generation rate per unit volume and unit time.

Respirometry has been used widely in the study of aerobic biodegradation processes. These studies include treatability tests, the measurement of biokinetic rate constants and the measurement of biological oxygen demand (BOD) (Barbeau, Ellis & Grady, 1995; Li & Zhang, 1996). For example, Lee & Suh (1995) were able to determine the first order biokinetic constant (K), ultimate BOD and adaptation period of microorganisms in wastewater. Based on these results the biodegradability and treatability of chemicals could be predicted.

Bioremediation is a slow process and the microbial activity in soil is not as high as in a water matrix. Respiration rates are, therefore, generally low. However, the primary objective of bioremediation is to enhance the microbial activity to such an extent that the contaminant is degraded in the shortest possible time. In this study, samples taken at different times during the bioremediation process (Chapter 4) were studied to investigate the

correlation between degradation and respiration. This correlation would enable the assessment of the effect of biosupplements, amendments and co-substrates in a short period of time without performing time consuming studies as the experiment detailed in Chapter 3.

7.2 MATERIALS AND METHODS

Three soil samples (800-1000g) per grid line were collected from a full-scale bioremediation site on a pre-determined grid of six lines (4.2.5 *vi*). The three soil samples were combined to form a composite sample, resulting in six soil samples. This was done on days 0, 26, 56, 78, 96, 124, 155, 156 and 169 after the start of the treatment process (Table 4.2). The samples were stored at 4°C. Samples (100 g) from each of the six samples from a specific day were combined to form a representative sample for that day in the treatment programme.

The soil samples were mixed thoroughly at 4°C and 50 g soil were added to each of the respirometer vessels. Due to the limited number of channels on the instrument, the soil samples were divided in two groups, day 0 to 78 and day 96 to 169. The samples were placed on the Micro-Oxymax respirometer and analysed every 2 h for at least 40 h at room temperature. The oxygen consumption and carbon dioxide production rates and cumulative values were measured and standardised for standard temperature and pressure, and difference in head space.

Duplicate samples were analysed and the experiments were repeated.

7.3 RESULTS

Figures 7.1 to 7.8 show the results of the respiration rates obtained. Figures 7.1 to 7.4 illustrate the respiration results from samples collected during the first 78 days of treatment and Figures 7.5 to 7.8 for the last 91 days of the treatment.

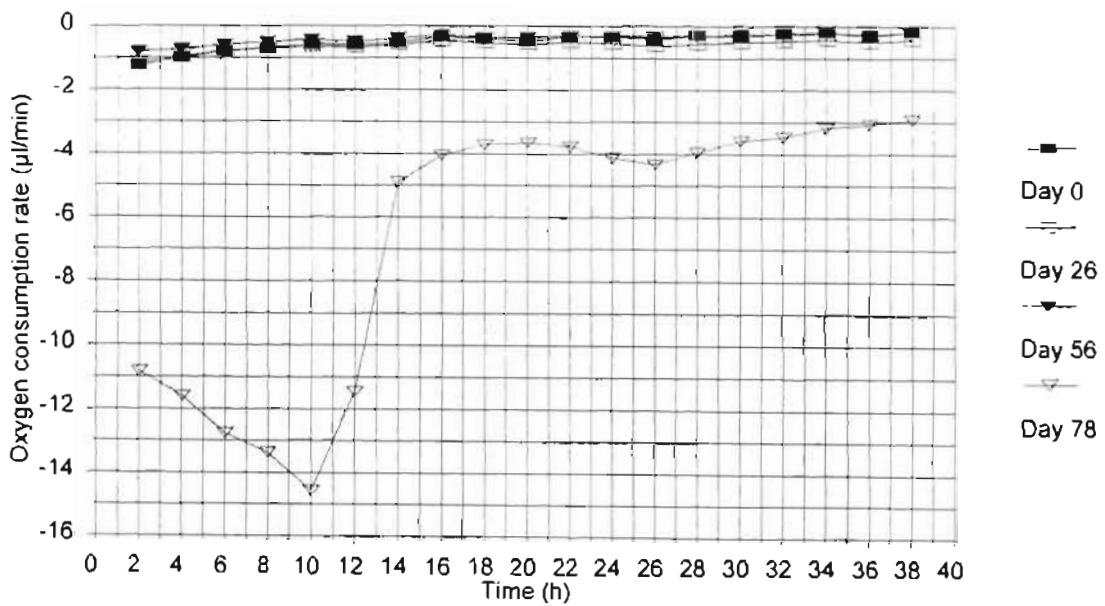


FIG. 7.1 Oxygen consumption rates (μlmin^{-1}) of soil samples collected from the full-scale remediation site on days 0, 26, 56 and 78.

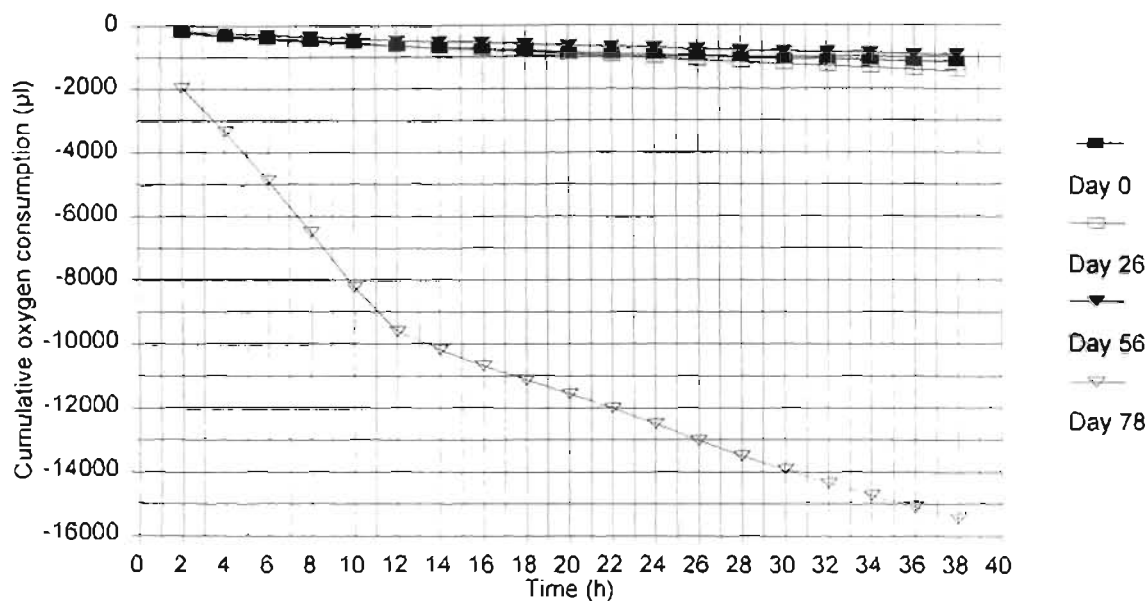


FIG. 7.2 Cumulative oxygen consumption (μl) of soil samples collected from the full-scale remediation site on days 0, 26, 56 and 78.

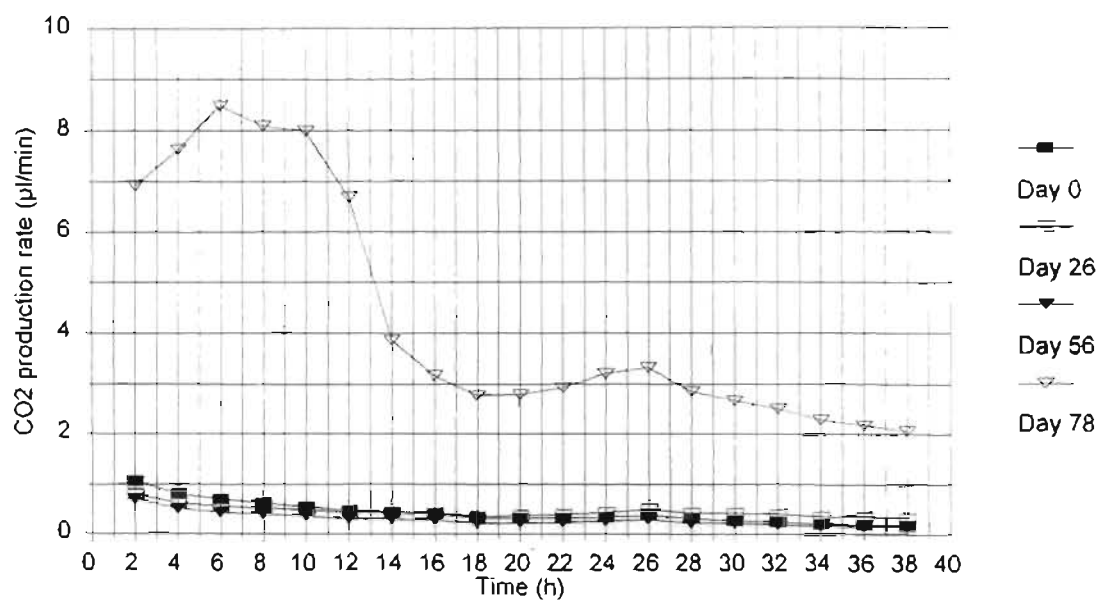


FIG. 7.3 Carbon dioxide production rates (μlmin^{-1}) of soil samples collected from the full-scale remediation site on days 0, 26, 56 and 78.

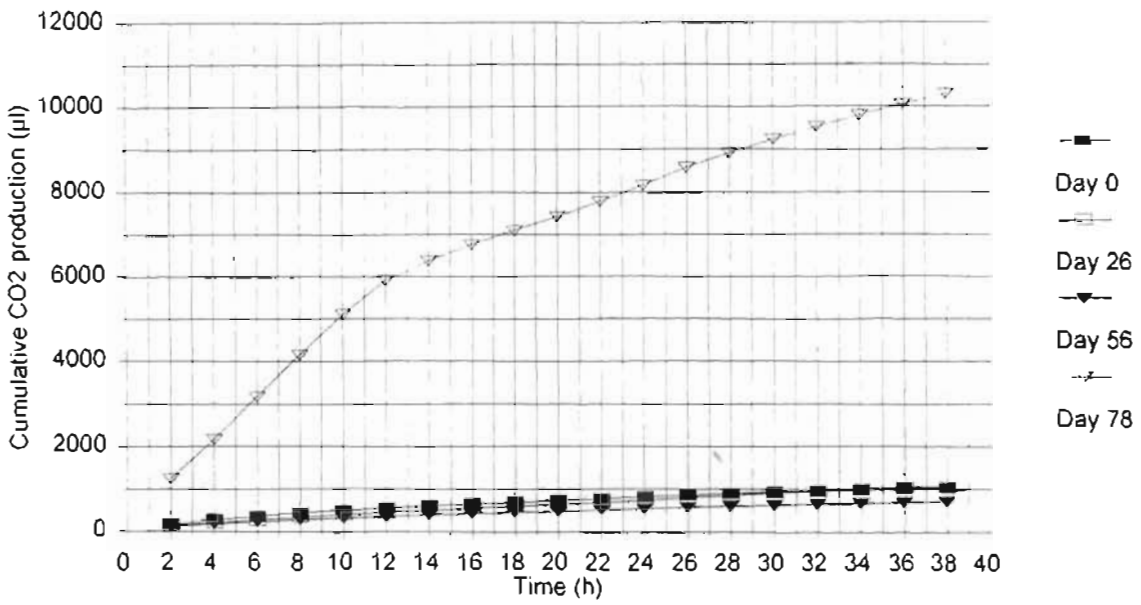


FIG. 7.4 Cumulative carbon dioxide production (μl) of soil samples collected from the full-scale remediation site on days 0, 26, 56 and 78.

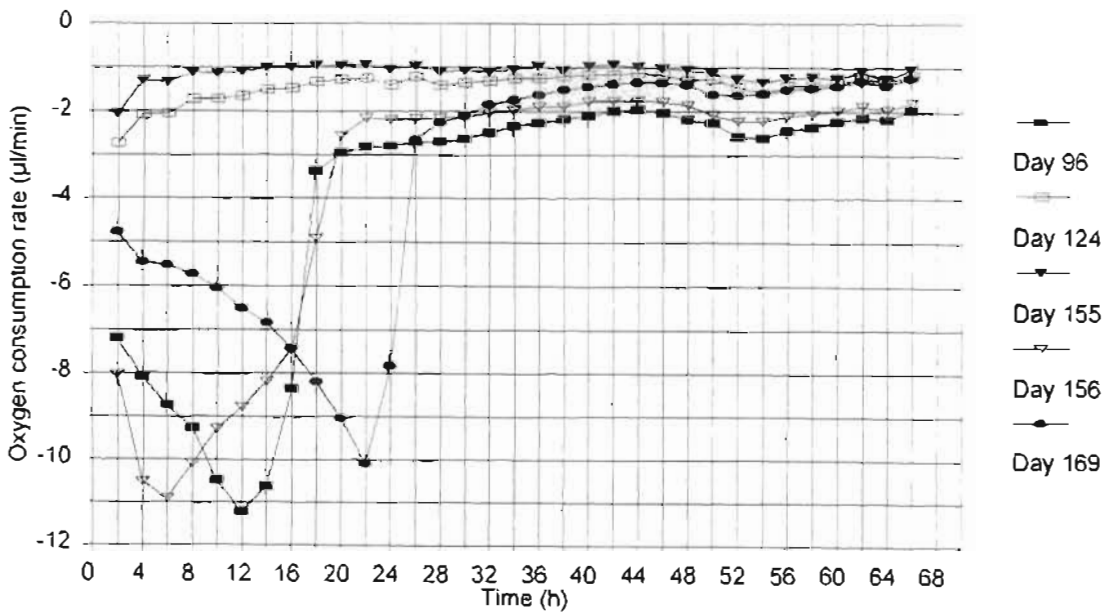


FIG. 7.5 Oxygen consumption rates ($\mu\text{l}/\text{min}$) of soil samples collected from the full-scale remediation site on days 96, 124, 155, 156 and 169.

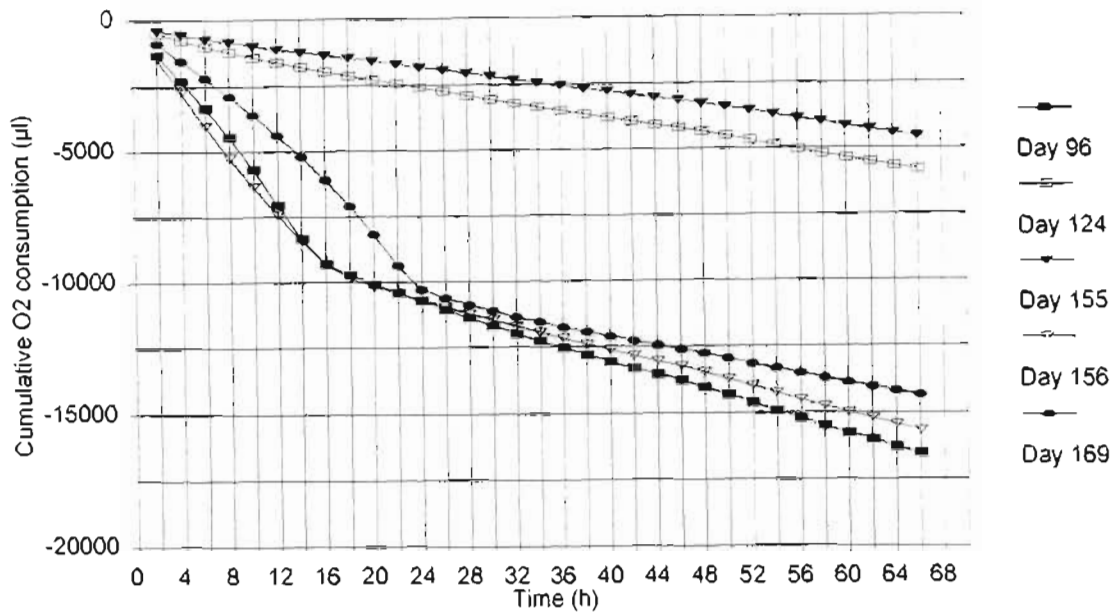


FIG. 7.6 Cumulative oxygen consumption (μl) of soil samples collected from the full-scale remediation site on days 96, 124, 155, 156 and 169.

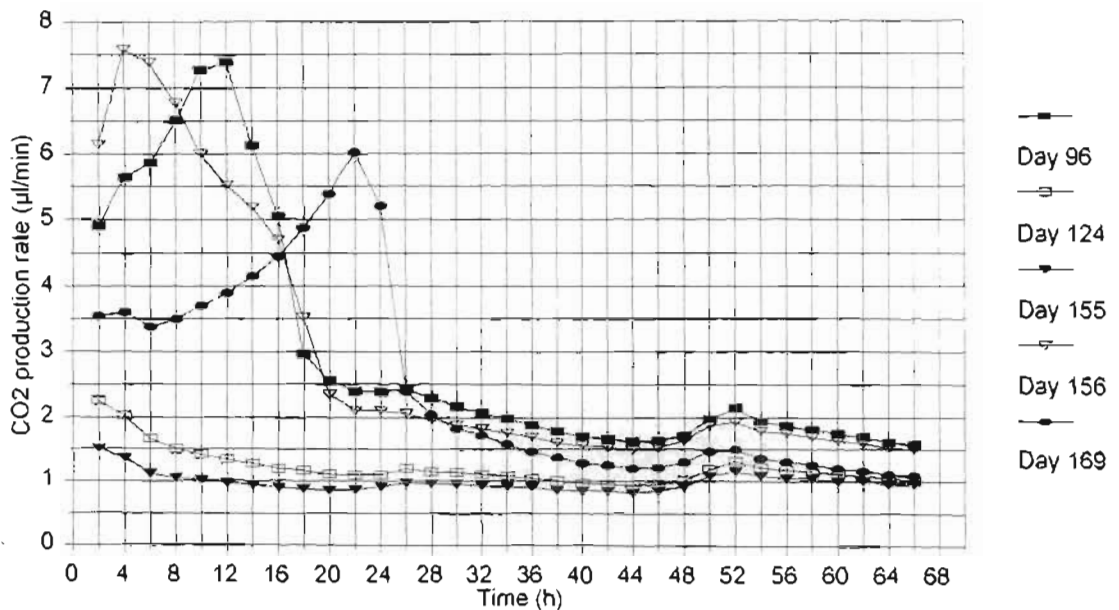


FIG. 7.7 Carbon dioxide production rates (μlmin^{-1}) of soil samples collected from the full-scale remediation site on days 96, 124, 155, 156 and 169.

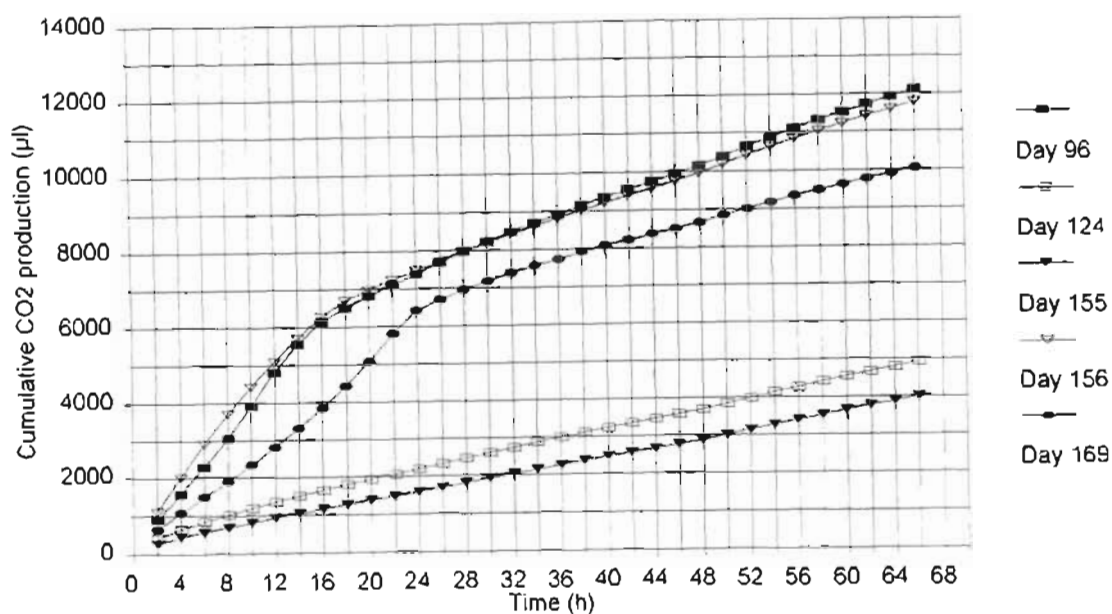


FIG. 7.8 Cumulative carbon dioxide production (μl) of soil samples collected from the full-scale remediation site on day 96, 124, 155, 156 and 169.

7.4 DISCUSSION

The residual TPHC concentrations and population sizes of the hydrocarbon degrading microorganisms during the course of the remediation are detailed in Table 4.2. For the purpose of the discussion, the major trends will be repeated here. During the first 56 days (results from samples taken on days 0, 26 and 56) the average residual contaminant concentration was 12 200 ppm (m/m) TPHC and no marked decrease was recorded.

However, between days 56 and 78, the TPHC concentration dropped significantly to around 4000 ppm. The TPHC concentration then stayed constant at this level between days 78 and

124. A significant decrease followed again between days 124 and 155 to concentrations around 2 900 ppm(m/m). On day 155, anaerobically digested sludge was added and within a fortnight the TPHC had dropped to 1 400 ppm (m/m). Thus, three distinct decreases in TPHC were observed during the bioremediation process. These changes afforded an ideal opportunity to evaluate respirometry as a tool to investigate the effect of an amendment, in this case anaerobically digested sludge, and to assess the correlation between degradation and respiration data.

Due to the limitation of samples which can be analysed by respirometry at any one time, the samples of days 0 to 78, during which the first drop in THPC concentration was observed, were analysed in a separate experiment to the samples from days 96 to 169 of the treatment, when the second and third decreases in the TPHC concentration were observed. The data are discussed as two sets of results.

Figure 7.1 and 7.2 clearly indicate a major increase in the oxygen consumption rate, and therefore, the respiration rate of the soil collected on the 78th day of treatment in comparison with the samples taken on days 0, 26 and 56. The reproducibility between samples was also good (Appendix 2). This is very important for remedial technologists, since analyses of soil samples are difficult and reproducibility normally inadequate to make valid conclusions.

Figures 7.3 and 7.4 further confirmed the results of Figures 7.1 and 7.2 since the carbon dioxide production on day 78 exceeded the productions on days 0, 26 and 56.

Figures 7.4 to 7.8 represent the treatment time between days 96 and 169. The respiration rates (oxygen consumption, Figure 7.5; carbon dioxide production Figure 7.7) of the samples taken at days 96, 156 and 169 were much higher than those of the samples taken on days 124 and 155. These results can be seen more clearly in the cumulative results (Figures 7.6 and 7.8). It seems that the respiration rates of the bacteria in the soil were maintained from at least day 78 to day 96. Between days 96 and 155 the respiration rates and the efficiency of the soil treatment decreased significantly (Figures 7.6 and 7.8; Table 4.2). On day 155 (after the samples were taken), dried anaerobically digested sludge was added to the site as an amendment. Samples collected on day 156 illustrate the impact of the anaerobically digested sludge on the respiration of the soil microorganisms.

Understandably, as the amendment was progressively utilised, the respiration rates of the soil microorganisms decreased slightly during the time from day 156 to day 169.

Using respirometry, the effects and fates of an amendment (anaerobically digested sludge) and the correlation between degradation and respiration could be assessed. Although the correlation is clear in this study, the effect of autotrophic ammonium oxidation (nitrification) in the soil, due to the ammonium that was added as a nutrient should be considered when interpreting the results. It is the author's opinion, however, that the oxygen consumption due to the degradation far exceeded the oxygen consumption due to nitrification. This technique was, therefore, found to be extremely valuable to monitor the activity of the microorganisms during treatment within a short period of time. When compared to the use of r-RNA targeted, fluorescently-labelled oligonucleotide probes (Chapter 6), respirometry is less labour intensive for the value of information obtained.

7.5 CONCLUSIONS

- A correlation between respiration and degradation was observed;
- The experiments were found to be reproducible even with soil samples from a full-scale site; and
- The effect of an amendment could be measured and its fate monitored.

CHAPTER 8

THERMAL BIOVENTING AS AN *EX SITU* REMEDIAL TECHNOLOGY: A LABORATORY INVESTIGATION

8.1 INTRODUCTION

The ongoing contamination of the environment needs attention as all too often hazardous solid wastes are placed into landfills when other treatment options are available. Consensus has been reached that it is a problem in need of a solution. Landfills cannot be used indiscriminately as they have been in the past. Not only has the technology proved expensive, due to the transportation costs, but the availability of suitable landfill sites in the right locations are fast diminishing. Investigation of alternative treatments of hazardous wastes is thus encouraged. The policy of the Oil Industry Environmental Committee also supports this, stating that "no liquid hydrocarbons or soils polluted with liquid hydrocarbons shall be disposed of to a landfill site, waste dump or class 1 or A sites" and follows that "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).

It is not always possible to bioremediate contaminated soil by landfarming which may be encumbered by problems of land availability and has a serious potential for contaminating water, air and soil (Grabowski & Raymond, 1984). Furthermore, not all contaminants

respond readily to landfarming, especially soils which are contaminated with dangerous volatile chemicals, high contaminant concentrations or weathered/heavy hydrocarbons.

In this laboratory study, the feasibility of a new technology called thermal bioventing was investigated. Thermal bioventing was introduced in Chapter 2. Briefly, thermal bioventing is a solid phase, *ex situ*, aerobic remediation technique where catabolism of the contaminant takes place under elevated temperatures due to increased microbial activity.

Thermal bioventing was tested using three soil types common to the Gauteng Province in South Africa. Previous investigations have proved that soil type has an effect on the catabolism of specific contaminants (Litchfield, Gromicko, Dansey & Minkley, 1993). Estrella, Brusseau, Maier, Pepper, Wierenga & Miller (1993) correlated the effects of biodegradation, sorption and transport in saturated and unsaturated soils and highlighted that biodegradation had a significant impact on the transport of 2,4-dichlorophenoxyacetic acid (2,4-D). Therefore, if the catabolism of a contaminant is optimized, this transport could be minimized and the potential for the contaminant to reach the groundwater decreased.

In some cases, the catabolism of contaminants could be hindered due to the soil type. Elektorowicz (1994) reported the necessity of a pretreatment step in the case of contaminated clayey soils. He suggested that the soil's density and transmissibility could be changed by mixing the contaminated soil with wood chips or sawdust. However, this step is included in thermal bioventing regardless of the soil type since effective aeration necessitates the use of a bulking agent.

The study was made with soil which was deliberately "spiked" with three compounds representing a wide range of petroleum hydrocarbons.

8.2 OBJECTIVES

The objectives of this laboratory-scale study on thermal bioventing were to:

- Identify model petroleum hydrocarbons contaminants with which to "spike" the soil;
- Identify three soil forms varying in texture but common to Gauteng Province;
- Establish the feasibility of thermal bioventing in the three soil forms at laboratory-scale; and
- Compare different soil supplements and treatment options.

8.3 MATERIALS AND METHODS

8.3.1 Selection of soil types

The major soil types of the Gauteng region were identified using 1:250 000 scale land type series maps. From these, three soil types, with the highest variance in texture, were chosen. Soil samples were collected and stored at 4°C. The soils were identified and described by the Institute for Soil, Climate and Water (Pedology), Pretoria. Analyses made on the soil samples included mineralogy, Walley-Black organic carbon, Bray 1 phosphorus and total nitrogen, using a digestion and colorimetric method (Standard Soil Testing, 1990).

8.3.2 Selection of contaminants

The contaminants, 3-methyl pentane, toluene and naphthalene, were selected using the following criteria:

- The contaminants should be present in most petrochemical products including diesel, petrol, jet fuel, base oils and lubricating oils;
- The contaminants should be representative of the chemical make-up of petroleum products i.e. there should be both aliphatic and aromatic constituents;
- Both volatile and stable molecules should be present; and
- At least one of the BTEX group (benzene, toluene, ethyl benzene and xylene) should be included.

8.3.3 "Spiking" of the soils

Each soil was dried at room temperature for 48 h and sieved through a 5 mm mesh stainless steel sieve. Naphthalene (320 g) was dissolved in 600 g toluene while the temperature was constantly adjusted to 30°C. This solution and 3-methyl pentane (200 g) were dispersed into 10 kg of soil. A hole was made in the middle of the soil into which a small volume of the contaminant was poured and mixed well with a hand spade. This procedure was repeated until all of the contaminant was dispersed. All three soil types were similarly "spiked". The mixtures were then sealed and left at room temperature to equilibrate for 1 mo.

8.3.4 Reactor design

Figure 8.1 illustrates the reactors which were designed to enable sampling from the side and the top. Air was fed upwards through the soil column after it had been humidified by bubbling through water. The reactors were maintained at a constant temperature of 40°C by means of a water mantle.

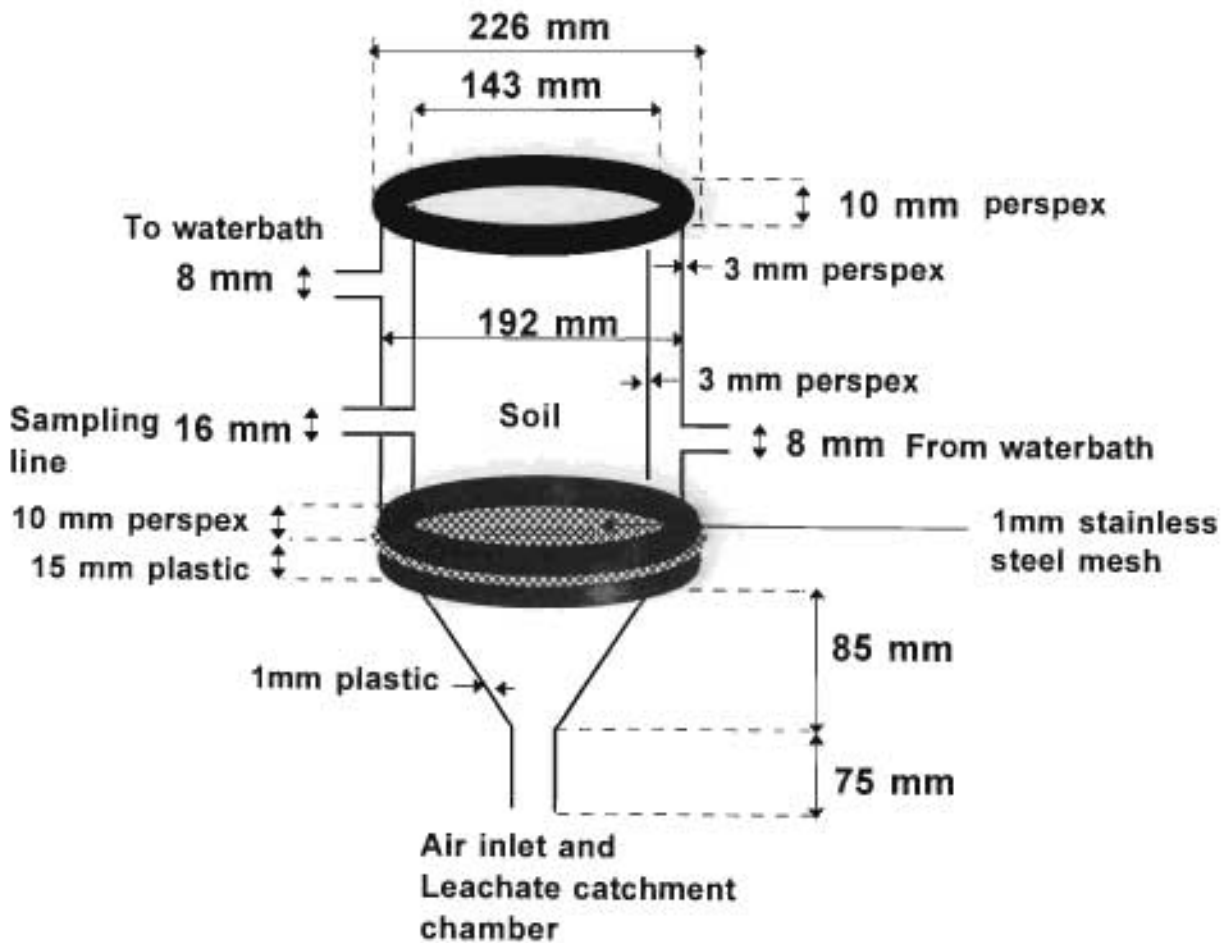


FIG. 8.1 Design of thermal bioventing laboratory-scale reactors. (The vertical length of the reactors were 250 mm).

8.3.5 Experimental design

The experimental design is summarised Table 8.1. The soil mass of each reactor was 1 500 g.

TABLE 7.1. Experimental design showing the different soil types and the different treatments examined

Reactor ^a	Soil type	Treatment	Amendments
L	Hutton - Red sand loam	None	
C	Bonnheim - Clay	None	
S	Longlands - Loam sand	None	
S(A+W)	Longlands - Loam sand	Air, Water	
L(A+W)	Hutton - Red sand loam	Air, Water	
C(A+W)	Bonnheim - Clay	Air, Water	
S(A+W+DS)1	Longlands - Loam sand	Air, Water	Dry sludge
S(A+W+DS)2	Longlands - Loam sand	Air, Water	Dry sludge
L(A+W+DS)1	Hutton - Red sand loam	Air, Water	Dry sludge
L(A+W+DS)2	Hutton - Red sand loam	Air, Water	Dry sludge
C(A+W+DS)1	Bonnheim - Clay	Air, Water	Dry sludge
C(A+W+DS)2	Bonnheim - Clay	Air, Water	Dry sludge
S(A+W+CM)1	Longlands - Loam sand	Air, Water	Chicken manure
S(A+W+CM)2	Longlands - Loam sand	Air, Water	Chicken manure
L(A+W+CM)1	Hutton - Red sand loam	Air, Water	Chicken manure
L(A+W+CM)2	Hutton - Red sand loam	Air, Water	Chicken manure
C(A+W+CM)1	Bonnheim - Clay	Air, Water	Chicken manure
C(A+W+CM)2	Bonnheim - Clay	Air, Water	Chicken manure
L(A+W+CB)	Hutton - Red sand loam	Air, Water	Commercial biosupplement and prescribed nutrients

^a Key to reactor names: L, C and S are the three soil forms loam, clay and sand, respectively. This is followed by brackets which indicate the supplements received: A = Air, W = Water, DS = Dry sludge, CM = Chicken manure and CB = Commercial biosupplement. The number after the bracket indicates the replicate.

i. Air

Compressed, oily, air was drawn from a central source and humidified by bubbling it through distilled water before passing through a rotameter. A linear airflow of $35.86 \text{ mlcm}^{-2}\text{day}^{-1}$ was used for each reactor. Glass capillary tubes of 30 cm length were installed at the entry line of the reactor. These tubes minimized backflow of air and forced the air to pass through the soil columns.

ii. Water

With the exception of reactors L, C and S, which served as undisturbed controls, water (250 ml) was added to all reactors at the start of the study. These reactors were adjusted to field capacity¹ (MacVicar, De Villiers, Loxton, Verster, Lambrechts, Merryweather, Le Roux, Van Rooyen & Von Harmse, 1977) twice weekly, either with the reactor's leachate or distilled water. The applications never exceeded 250 ml. Cooling was minimized through the water mantle surrounding the soil (8.3.4).

iii. Nutrients

Chicken manure (obtained from a private farmer) and dried anaerobically digested sludge (Daspoort sewage works, Pretoria), hereafter referred to as dried sludge, which were dried on sand beds were individually used in a ratio of 5:1 (contaminated soil:supplement). The

¹ Field capacity is the water content of a freely draining soil which has been saturated with water in the field and allowed to drain for 2-3 days.

unsupplemented reactors (Controls) were diluted with uncontaminated soil of the same type as the corresponding experimental reactors. The supplements were mixed thoroughly with the soil. Financial constraints permitted chemical analyses on days 0, 14 and 28, but not for the rest of the experiment.

iv. *Commercial biosupplement control*

Reactor L(A+W+CB) represented a commercial biosupplement and its efficacy was measured against the other treatments. Permission was not granted to disclose the source of the biosupplement. Another biosupplement, which could be disclosed, was, therefore, screened for the pilot-scale study (9.4.1). The treatment was applied according to the manufacturer's specifications. The dry microorganisms (10^{12} cfu m^{-1}) were added at 5% of the weight of the contaminant and the supplied fertilizer was added at 10% of the weight of the dry microorganisms.

v. *Analyses*

Samples were taken weekly and consisted of composites of one sample taken vertically from the top and a second sample taken horizontally from the bottom of each reactor.

Moisture. The moisture content was determined weekly by drying at 105°C overnight and determining the reduction in weight.

Nutrients. Extractable phosphorus, using the Bray 1 method, organic carbon, using the Walkley-Black method and total nitrogen, using a digestion and colorimetric method, were determined every fortnight (Standard Soil Testing, 1990).

Microorganisms. Plate counts were made weekly. A 1 g sample of wet soil was used to prepare a dilution series in physiological saline solution prior to inoculation onto nutrient agar. The plates were incubated inverted for 48 h at 40°C. These results were converted to dry mass plate counts, using the moisture data for that specific week.

Contaminant reduction. A gas chromatograph (Hewlett Packard, HP 5890 Series II) equipped with a flame ionization detector (GC/FID) and HP 5181 122 0 integrator was used for the detection/quantification of the contaminants. A HP 1 (Hewlett Packard) methyl silicone cross linked column (25 m, 0.2 mm, 0.3 µm film thickness) was installed. Samples (10 g wet weight) were extracted with 100 ml of dichloromethane (DCM) (analytical grade) for 5 min in 250 ml extraction vessels. Aliquots (approx 5 ml) of these extracts were passed through a C18 Sep-Pak® cartridge (Waters) for solid phase extraction. A 1 µl sample of each eluate was injected into the GC/FID. Duplicate samples of the same extraction were injected. The details of the GC/FID programme are shown in Table 8.2. Results obtained were corrected for moisture and thus expressed as a dry weight (8.4.5). The weekly results were compared with the GC chromatographs of the corresponding reactors at the beginning of the experiment, with special attention to the appearance and disappearance of peaks.

TABLE 8.2. GC/FID programme for the detection of hydrocarbons in soil samples

Parameter	Setting
Initial oven temperature	50°C
Initial time	3 min
Temperature programme:	
Ramp Rate	10°Cmin ⁻¹
Final temperature	200°C
Final time	4 min
Injector temperature	200°C
FID temperature	300°C
Purge valve	On
Split ratio	70:1
Attenuation	2

8.4 RESULTS

8.4.1 Selection of the soil types

The characteristics of the three soil types are described in Table 8.3. The sampling location was chosen close to urban areas and, where possible, close to major roads with the rationale that hydrocarbon spills are most likely to take place at such locations. Only the horizons² (MacVicar *et al.*, 1977) that were used for the pilot-scale are depicted in this table.

² Processes which form soil have a net tendency to differentiate the material on which they act into horizons.

TABLE 8.3. Descriptions of the three soil types used

Soil form and family	Description
Bonheim Onrus	<p>Melanic A horizon: Dry; very dark grey, moist; black; sandy clay; few fine faint black and brown illuvial iron & humus mottles; moderate medium subangular blocky; hard; slightly firm; common fine pores; common coarse pore; fine cracks, slight effervescence; few clay cutans; surface crust water absorption 4 seconds; common roots; gradual, wavy transition.</p> <p>Pedocutanic B horizon: Moist; moist very dark grey; clay; few coarse prominent grey lime mottles; strong coarse angular blocky; very firm; common fine pores; common coarse pores; medium cracks. moderate effervescence, few slickensides, many clay cutans; water absorption 10 seconds; common roots; gradual, wavy transition.</p>
Longlands Ermelo	<p>Orthic A horizon: Dry; dry brown to dark brown, moist very dark greyish brown; undisturbed; apedal; loose; few fine pores; water absorption 1 second; common roots; diffuse; smooth transition.</p> <p>E horizon: Moist; moist light yellowish brown; undisturbed; apedal; loose, few fine pores; very few sesquioxide concretions; water absorption 1 second; few roots; clear, smooth transition.</p>
Hutton Suurbekom	<p>Orthic A horizon: Dry; dry dark reddish brown; disturbed; apedal; soft; common fine pores; few coarse pores; water absorption 1 second; few roots; gradual, smooth transition.</p> <p>Red apedal B horizon: Dry; dry dark red ; disturbed; fine sandy loam; apedal; soft; common fine pores; few coarse pores; water absorption 2 seconds; few roots.</p>

Soil horizons below 300 mm were not included in this study, since contamination of soils generally do not penetrate deeper than this depth, especially in a fresh spill.

Empirical and theoretical estimates of the ratio of nitrogen and phosphorus necessary to facilitate biodegradation of a known mass of hydrocarbon vary from 600:10:0.75 to 100:10:1 (carbon:nitrogen:phosphorus) (Dibble & Bartha, 1979). More recently a ratio of 100:10:1 has been used with success in the bioremediation of diesel fuel contaminated soil at a former fuelling yard (Troy, McGinn, Greenwald, Jerger & Allen, 1992). The nutrients in the soils used in this study (Table 8.4) were not in this range and thus had to be added to establish the correct ratio. The contaminant carbon was not added at this time. Nutrients were added to establish the correct ratio taking into account the increased carbon concentration due to "spiking".

8.4.2 Selection of the contaminants

Naphthalene, toluene and 3-methylpentane were chosen as representative petroleum hydrocarbons. These molecules represent two-ring, single-ring and branched alkane structures, respectively, with boiling points above 60°C.

TABLE 8.4. Results of chemical and physical-chemical analyses

Soil form and family	Mineralogy (% m/m)	Organic carbon (% m/m)	Phosphorus (mgkg ⁻¹)	Total nitrogen (% m/m)
Clay (Bonheim Onrus)	Smectite 71 Mica 24 Kaolinite 5	2.02	2.0	0.083
Loam-sand (Longlands Ermelo)	Kaolinite 52 Smectite 30 Mica 18	0.86	3.4	0.029
Red sand loam (Hutton Suurbekom)	Kaolinite 84 Mica 10 Smectite 6	1.09	1.8	0.046

8.4.3 Selection of amendments

The amendments considered for evaluation were mature mushroom compost, chicken manure, cow manure and dried (on sand beds) anaerobically digested sludge. These were analysed and the results are shown in Table 8.5.

Chicken manure and dried anaerobically digested sludge were chosen as amendments for this study as they had the highest nitrogen concentrations, which is considered an important nutrient. Furthermore, both are seen as waste products that can be used in a beneficial manner. Chicken manure and dried sludge have the advantage of being freely available in Gauteng and can be obtained at no cost other than transport costs.

TABLE 8.5. Results of analyses of different amendments

Amendment	Mg (% m/m)	P (% m/m)	K (% m/m)	N (% m/m)	C (% m/m)
Cow manure	0.230	0.232	0.140	1.724	41.37
Dry sludge	0.136	0.324	0.140	3.381	29.11
Chicken manure	0.264	1.612	0.587	3.617	35.79
Mushroom compost	0.168	1.113	0.622	1.737	24.48

8.4.4 "Spiking" of the soils

A white layer of residue appeared on the soil surface during the month's equilibration. Plate counts were made on the "spiked" soils 4 and 15 days after "spiking". It was found that the plate counts decreased 10 fold when compared to the initial "unspiked" soil.

8.4.5 Monitoring

The 19 reactors were divided in three groups according to the different soil types and the results are presented accordingly.

i. Contaminant reduction

The contaminant concentrations were normalized in each series by depicting the change in contaminant concentration as (c_1/c_0) where c_1 represents the contaminant concentration at time t_1 and c_0 represents the initial contaminant concentration at time t_0 (Figures 8.2-8.7).

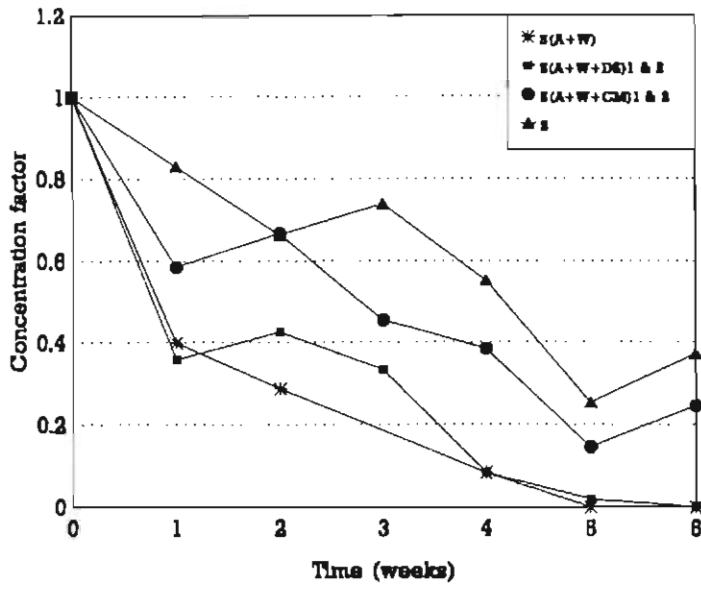


FIG. 8.2 Decrease in naphthalene concentration factor in loam-sand (Longlands Ermelo) subjected to various treatments as detailed in Table 8.1.

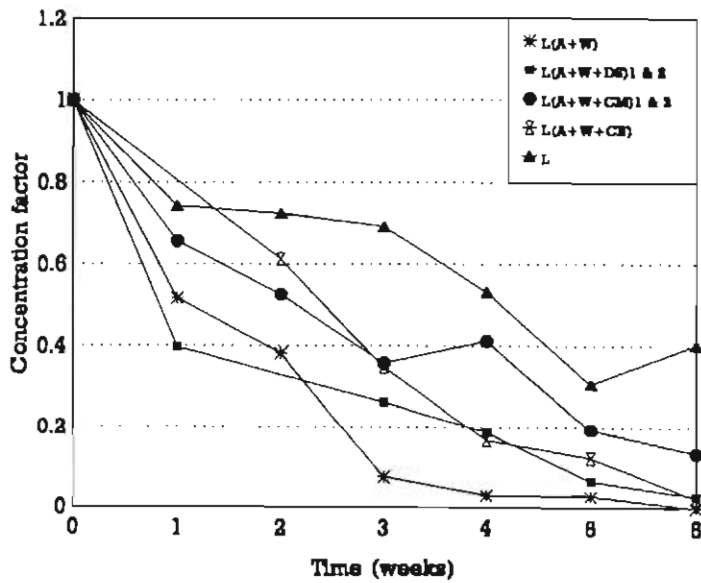


FIG. 8.3 Decrease in naphthalene concentration factor in red sand loam (Hutton Suurbekom) subjected to various treatments as detailed in Table 8.1.

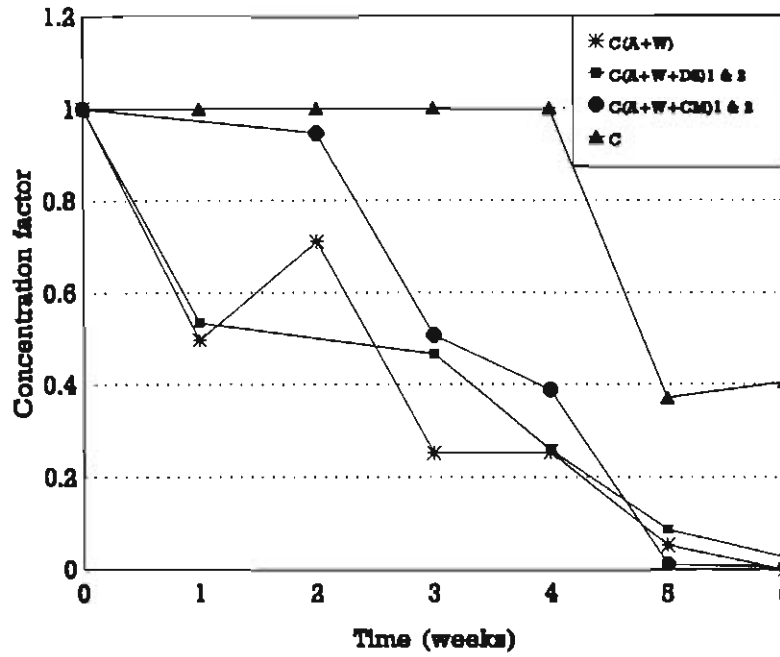


FIG. 8.4 Decrease in naphthalene concentration factor in clay (Bonheim Onrus) subjected to various treatments as detailed in Table 8.1.

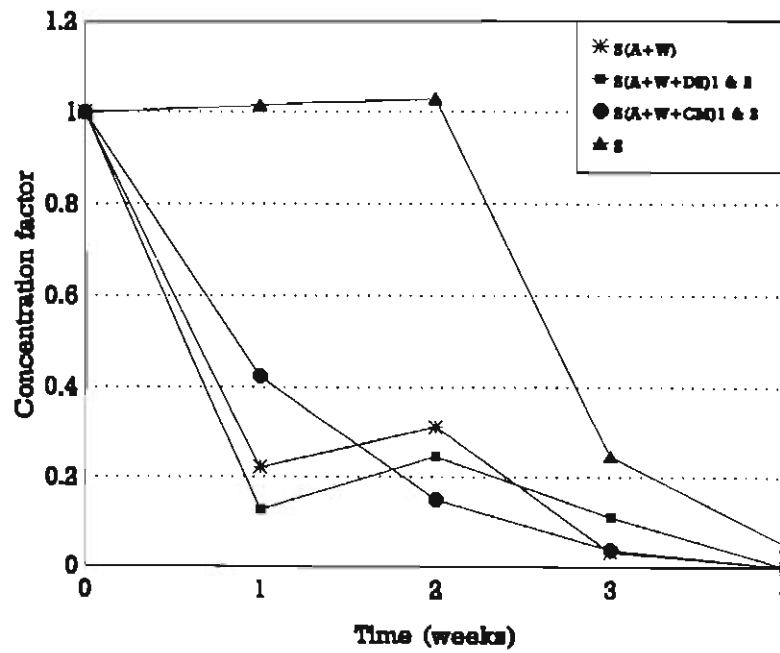


FIG. 8.5 Decrease in toluene concentration factor in loam-sand (Longlands Ermelo) subjected to various treatments as detailed in Table 8.1.

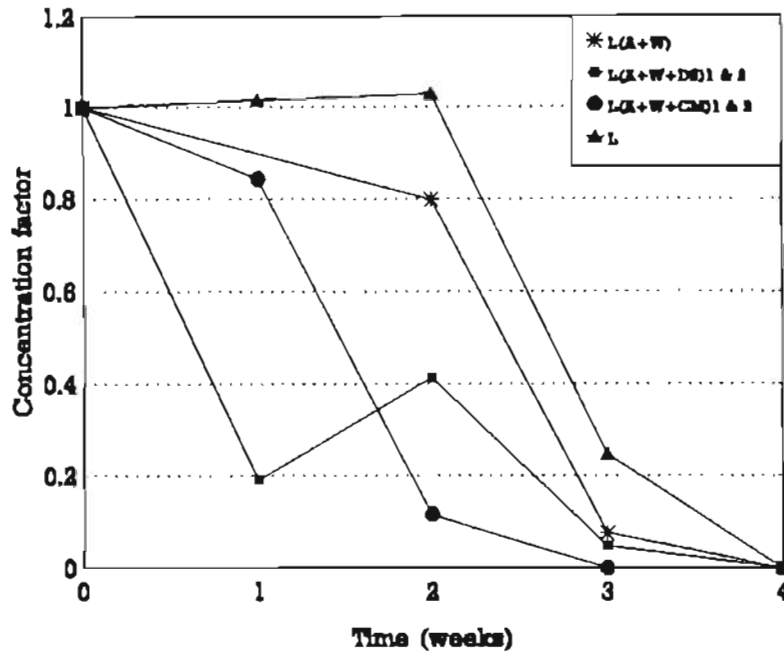


FIG. 8.6 Decrease in toluene concentration factor in red sand loam (Hutton Suurbekom) subjected to various treatments as detailed in Table 8.1.

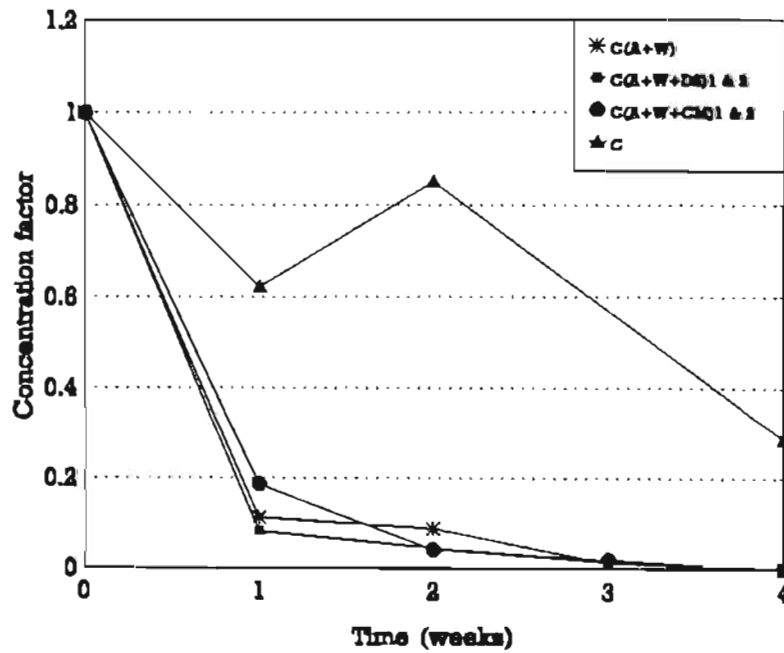


FIG. 8.7 Decrease in toluene concentration factor in clay (Bonheim Onrus) subjected to various treatments as detailed in Table 8.1.

Although all three soil types were "spiked" with 3-methyl pentane, this contaminant was not detected after the equilibration period. Although it is generally accepted that volatile compounds become less volatile once they are soilbound, especially in soils with small particle sizes, this was not substantiated in the experiment.

ii. *Microbiological plate counts*

The results of the microbial plate counts are shown in Figures 8.8-8.10.

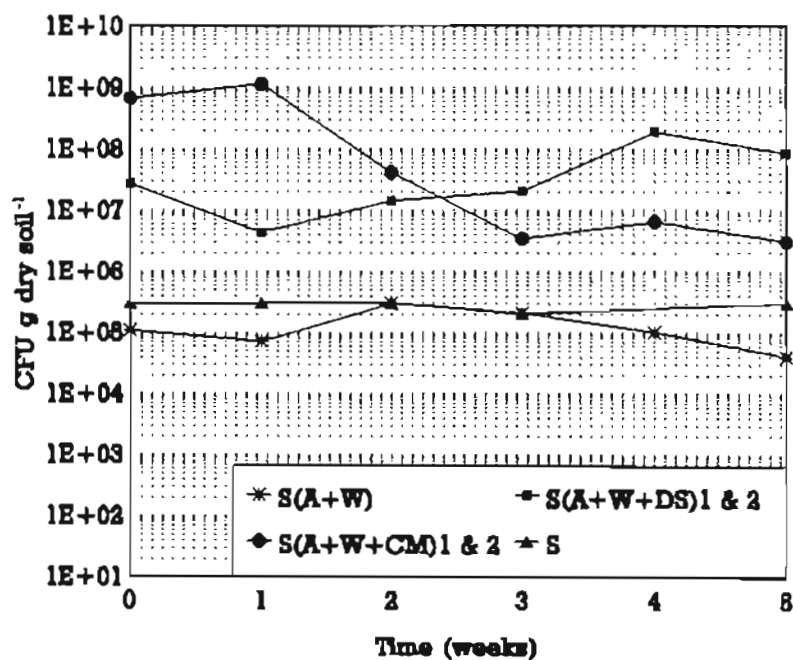


FIG. 8.8 Changes in total colony forming units (CFU) present in loam-sand (Longlands Ermelo) subjected to various treatments as detailed in Table 8.1.

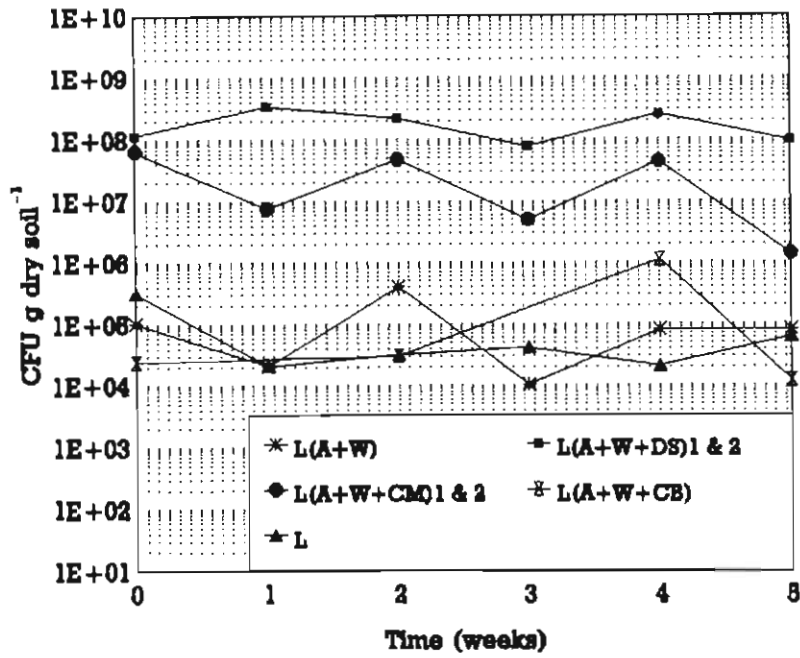


FIG. 8.9 Changes in total colony forming units (CFU) present in red sand loam (Hutton Suurbekom) subjected to various treatments as detailed in Table 8.1.

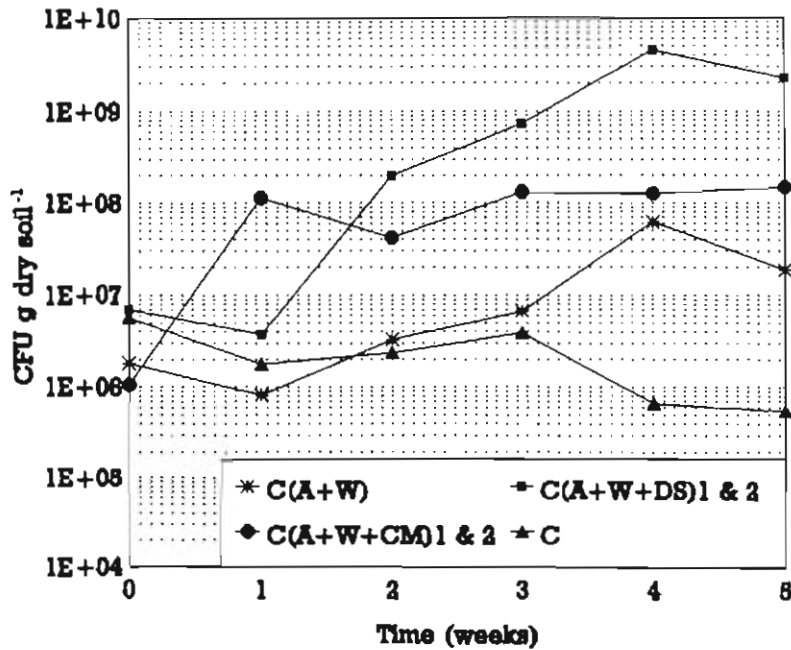


FIG. 8.10 Changes in total colony forming units (CFU) present in clay (Bonheim Onrus) subjected to various treatments as detailed in Table 8.1.

iii. *Nutrients*

The nutrient concentrations are shown in Tables 8.6-8.8. In all cases the carbon concentration was normalised to a value of 100.

TABLE 8.6. Changes in C:N:P ratios of "spiked" loam-sand (Longlands Ermelo) subjected to various treatments as detailed in Table 8.1

Reactor	C:N:P ratio at time 0	C:N:P after 14 d	C:N:P after 28 d
S(A+W)	100:3:0.02	100:7:0.8	100:9:1.0
S(A+W+DS)1	NA	100:57:4.5	100:40:5.0
S(A+W+DS)2	100:49:1.1	100:26:5.0	100:53:4.8
S(A+W+CM)1	100:75:1.0	100:9:0.2	100:20:3.6
S(A+W+CM)2	100:67:1.4	100:34:3.8	100:38:3.8
S	100:1:0.02	100:1:0.03	100:1:0.02

TABLE 8.7. Changes in C:N:P ratios of "spiked" red sand loam (Hutton Suurbekom) subjected to various treatments as detailed in Table 8.1

Reactor	C:N:P ratio at time 0	C:N:P after 14 d	C:N:P after 28 d
L(A+W)	100:4:0.04	100:5:0.01	100:7:0.6
L(A+W+DS)1	100:30:1.0	100:19:3	100:30:2.6
L(A+W+DS)2	100:15:1.0	100:16:3.4	100:32:3.0
L(A+W+CM)1	100:41:1.0	100:31:1.5	100:14:2.5
L(A+W+CM)2	100:32:0.8	100:27:1.7	100:13:2.0
L(A+W+CB)	100:5:0.9	100:5:0.3	100:7:0.3
L	100:4:0.2	100:3:0.03	100:4:0.01

TABLE 8.8. Changes in C:N:P ratios of "spiked" clay (Bonheim Onrus) subjected to various treatments as detailed in Table 8.1

Reactor	C:N:P ratio at time 0	C:N:P after 14 d	C:N:P after 28 d
C(A+W)	100:3:0.01	100:3:0.01	100:44:0.05
C(A+W+DS)1	100:14:0.7	100:11:0.2	100:17:0.6
C(A+W+DS)2	100:21:0.4	100:9:0.2	100:15:0.6
C(A+W+CM)1	100:22:0.7	100:18:0.6	100:30:0.6
C(A+W+CM)2	100:27:0.3	100:23:0.3	100:24:0.5
C	100:3:0.01	100:2:0.01	100:2:0.004

iv. *Moisture content*

TABLE 8.9. The average moisture content of each reactor subjected to various treatments as detailed in Table 8.1 during the 6 wk experimental period

Reactor	Moisture (% m/m)
S(A+W)	4.58
L(A+W)	9.43
C(A+W)	18.58
S(A+W+DS)1	12.32
S(A+W+DS)2	15.15
L(A+W+DS)1	19.79
L(A+W+DS)2	20.72
C(A+W+DS)1	29.05
C(A+W+DS)2	27.73
S(A+W+CM)1	24.97
S(A+W+CM)2	24.92
L(A+W+CM)1	23.87
L(A+W+CM)2	23.25
C(A+W+CM)1	39.26
C(A+W+CM)2	29.98
L(A+W+CB)	11.67
L	4.23
C	9.95
S	2.41

8.5 DISCUSSION

8.5.1 Biodegradation in loam-sand (Longlands Ermelo)

Figure 8.2 shows that the naphthalene concentrations in reactors S(A+W), S(A+W+DS)1 and 2 decreased to target concentrations of $<0.01 \text{ mgg}^{-1}$. All treatments performed better than the control (S). The reactors which received dried sludge (Reactors S(A+W+DS)1 & 2) performed significantly better than the reactors which received chicken manure (Reactors S(A+W+CM)1 & 2). This could be due to toxicity caused by high concentration of ammonia often associated with this product, which could be inhibitory to microbial action. Reactors S(A+W) and S(A+W+DS)1 & 2 reached target concentrations of $< 0.01 \text{ mgg}^{-1}$ within 5 wk from an initial concentration of $35.989 \pm 7.985 \text{ mgg}^{-1}$. The toluene concentration was reduced from an average of $0.357 \pm 0.188 \text{ mgg}^{-1}$ to below the target concentration ($< 0.01 \text{ mgg}^{-1}$) in 4 wk. Figure 8.5 indicates the same trend as Figure 8.2 since dried sludge facilitated better catabolism than chicken manure. However, the difference between reactors receiving treatment in Figure 8.5 seem less significant in comparison to Figure 8.2 which shows greater differentiation between the reactors. Figure 8.5 shows the effect of volatilisation since the concentration decrease in the first week was $> 55\%$ in all reactors receiving forced aeration. To a lesser extent, this effect is also indicated in Figure 8.2.

An activated carbon column fitted to the outlet of Reactor S(A+W) emphasised the role played by volatilisation in the reduction of contaminants. For example, in 21 days, 8.275 g

of contaminant were retrieved from the activated carbon column. When this amount is compared to the decrease in contaminant concentration calculated from the GC chromatograms (17.09 g), it can be seen that volatilisation accounted for 48% of the reduction. This led to the conclusion that the linear air flow rate used for the experiment ($35.86 \text{ cm}^3 \text{ d}^{-1}$) may have been too high. An airflow of $54.75 \text{ cm}^3 \text{ d}^{-1}$ was suggested by Dupont & Lakshmi Prasad (1993) who established a relationship between airflow rates, biodegradation and volatilisation rates for maximising biodegradation and minimising volatilisation. However, in the present study these suggested airflows were accepted without considering the recommended average retention time of 0.5 pore volumes per day. Although an accurate pore volume exchange is not available in this study, a rough estimate of six pore volumes per day was calculated from the available data. Since this could account for the high volatilisation achieved in the study it should be closely monitored in the subsequent pilot-scale evaluation.

The conditions for bioremediation in the reactors subjected to supplements, specifically reactors S(A+W+DS)1 & 2, which received dried activated sludge, and S(A+W+CM)1 & 2, which received chicken manure, were significantly better than the control reactors.

Figure 8.8 illustrates that the treated reactor plate counts were orders of magnitude higher than the plate counts of the control reactors. Table 8.9 also shows that the moisture holding capacity of the sandy soil was significantly increased by the use of both amendments since increases of 9.15% and 20% were recorded in the dried sludge and chicken manure amended reactors, respectively.

Table 8.6 shows the C:N:P ratios with the carbon value fixed at 100 parts. Reactor S(A+W) showed the most favourable ratio at the end of study. However, it must be noted that the carbon source had, effectively, been depleted at this stage and, judging by the low plate counts (as well as evaluating the absolute values in the unprocessed data), it appeared that little of the nutrients had been utilised. The general increase in the phosphorus concentration in reactors receiving amendments suggested that the pool of unavailable phosphorus may have been made more available through some biological system.

8.5.2 Biodegradation in sand loam (Hutton Suurbekom)

Figure 8.3 shows that the reactors supplemented with dried sludge performed better than the reactors containing chicken manure. The contaminant decreased to target concentrations of $< 0.01 \text{ mgg}^{-1}$ within 6 wk in reactors L(A+W), L(A+W+DS)1 & 2 and L(A+W+CB).

For this soil type a further control, reactor L(A+W+CB), was included, which received a commercial biosupplement, for comparison with the other treatments. The plate counts of soil from this reactor were comparable to the reactors which received no amendments (Figure 8.9) and, therefore, it was assumed that the count represented only the indigenous microorganisms and that the biosupplement failed to survive these specific conditions.

Reactor L also showed a substantial decrease in contaminant concentration (Figures 8.3 and 8.6), indicating that the adsorption capacity for the specific contaminants was minimal compared to the clay soil (8.5.3).

Table 8.7 shows the nutrient ratios of the red sand loam reactors at three different times. It must be noted that in reactor L(A+W+DS)1 the C:N:P ratio was correct at time zero, but that the nitrogen and phosphorus concentrations seemed to increase with time. This was possibly because initially unavailable nutrients were made available through biological action.

Table 8.9 shows that the amendments increased the water holding capacity of the soil. The inherent moisture content of the loam-sand was lowest (2.41%) followed by the red sand loam (4.23%) with clay the highest (9.95%), as expected.

8.5.3 Biodegradation in clay soil (Bonheim Onrus)

Figure 8.4 shows the decrease in the naphthalene concentrations. The naphthalene was successfully removed from the clay soil within 5 to 6 wk. Once again, although not as significant, the dried sludge amended reactors were more effective than the chicken manure amended reactors. The results of Reactor C show that the contaminant was stable in this soil for a longer period of time (4 wk) than in the other two soil types (Figures 8.2 and 8.3).

The sharp and similar decreases in toluene concentrations in all the reactors, except Reactor C(A+W) (Figure 8.7), give an indication of the role of volatilisation.

The results of the weekly plate counts for the clay soil (Figure 8.10) show an interesting variation when compared to the plate count results for the other soil types. The plate counts

for the amended reactors were low in the first week, when compared to the control reactors. However, in the supplemented reactors (Reactors C(A+W+DS)1 & 2 and C(A+W+CM)1 & 2), the plate counts increased substantially to values above those of the control reactors. The reactors amended with dried sludge gave higher plate counts in the latter part of the experiment and these could explain the increased rates of contaminant reduction.

8.5.4 General discussion

The results of Reactors S(A+W), L(A+W) and C(A+W) could be spurious since the waterbath serving them increased in the temperature by 5 Celsius degrees. Thus, increased volatilisation could have occurred invalidating direct comparisons. Specific trends were, however, apparent and these are considered in the discussion.

The carbon of the activated carbon column fitted to Reactor S(A+W) was extracted with DCM without much success. Carbon disulphide (CS₂) could be used in further studies, since this is normally a more effective solvent. If the activated carbon is extracted with a high efficiency, a mass balance could be calculated and thus answer some questions.

As discussed, dried sludge performed better than chicken manure as a supplement in all three soil types. However, it could be that the dried sludge increased the water holding capacity to a greater extent than the chicken manure, thus increasing the microbial population and, hence, the rate of the degradation.

Although it was established that volatilisation accounted for at least 40% of the carbon removal, bioremediation must have accounted for the remaining 60%. No catabolic intermediates were detected in any of the gas chromatograms although a comprehensive qualitative analysis was not undertaken. Apart from naphthalene, the contaminants chosen as model contaminants were found to be too volatile for accurate laboratory measurements. It is suggested that heavier hydrocarbons be used when a mass balance is needed to establish the feasibility of a technology at laboratory-scale.

Thermal bioventing at laboratory-scale proved to be a viable and feasible bioremediation technique. However, there are several uncertainties which should still be addressed at pilot-scale. In particular, a mass balance study would provide important information and should be incorporated. Other aspects that need investigation, or at least close monitoring, are the contaminant choice and airflow.

8.6 CONCLUSIONS

- Naphthalene, toluene and 3-methyl pentane were identified as model contaminants. Toluene and 3-methyl pentane was found to be volatile and not ideal for laboratory studies;
- Three soil forms were identified, Hutton (red sand loam), Bonnheim (clay) and Longlands (loam sand). Thermal bioventing proved to be effective in all three soil forms;
- Thermal bioventing proved to be a viable technique for the bioremediation of petroleum hydrocarbons at laboratory-scale and could effect the concentrations from 112 gkg^{-1} to below target concentration of 0.01 mgg^{-1} in 35 to 42 days;
- Volatilisation contributed at least 40% of the reduction. The high exchange in pore volume could have contributed to this high volatilisation. A pore volume exchange of 0.5 to 1 is recommended for use in the pilot-scale evaluation; and
- Of the two supplements evaluated, the dried anaerobically digested sludge promoted higher degradation than the reactors supplemented with chicken manure.

CHAPTER 9

THERMAL BIOVENTING AS AN *EX SITU* REMEDIAL TECHNOLOGY: A PILOT-SCALE INVESTIGATION

9.1 INTRODUCTION

In Chapter 9 the feasibility of thermal bioventing as an aerobic solid-phase bioremediation technique was investigated. This study proved that the technique is a viable option for treating heavily contaminated soils. Application of this technique is envisaged for the treatment of small volumes of soil, contaminated with high concentrations of chemicals which are difficult to degrade in a controlled manner, to minimize the risks of dangerous volatiles. The technique was tested with three soils common to the Gauteng region viz. loam-sand (Longlands Ermelo), red sand loam (Hutton Suurbekom) and clay (Bonheim Onrus) which had been individually "spiked" with 3-methyl pentane, toluene and naphthalene. The initial TPHC value of 112 gkg^{-1} was reduced to below the target concentration of 0.01 mgg^{-1} in 35 to 42 days, in all the reactors which received supplements. However, volatilisation was a major contributor to the reduction of the contaminant concentration in the soil.

This chapter reports on the extension of the feasibility study of thermal bioventing to pilot-scale. An objective of the pilot-scale study was to minimize the volatilisation of the contaminant. During the laboratory study, soil was deliberately contaminated. It was found

that even after an equilibration period, the contaminants could be easily removed by even low aeration flows, thereby volatilising the contaminant. This is not always the case in actual spills where long periods may lapse before the spill is reported and the contaminants weather during this time. A more representative contaminated soil was, therefore, used in the pilot-scale study.

9.2 OBJECTIVES

The objectives of this pilot-scale study on thermal bioventing were to:

- Demonstrate bioremediation of recalcitrant hydrocarbons;
- Establish the feasibility of thermal bioventing at pilot-scale as an in vessel bioremediation treatment; and
- Compare different soil supplements and treatment options.

9.3 MATERIALS AND METHODS

9.3.1 Experimental design

The pilot-scale reactors consisted of three 25 l covered plastic containers [CONT, CONT(B), CONT (W:B)] and four 220 l sealed plastic drums [CONT(W:A:B), EXP(W:A:B:CB), EXP(W:A:B:CM), EXP(W:A:B:AS)]. The latter were each equipped with a leachate drain, sprinkler system, air inlet, air outlet, grid and three sample ports

(Figure 9.1). The purpose of the grid was to prevent the soil from blocking the air inlet and to distribute the air across the base of the reactor. The grid was covered with a geotechnic material (Biddum) to both prevent soil from falling through and enhance airflow through the reactor. The seven reactors were operated in parallel.

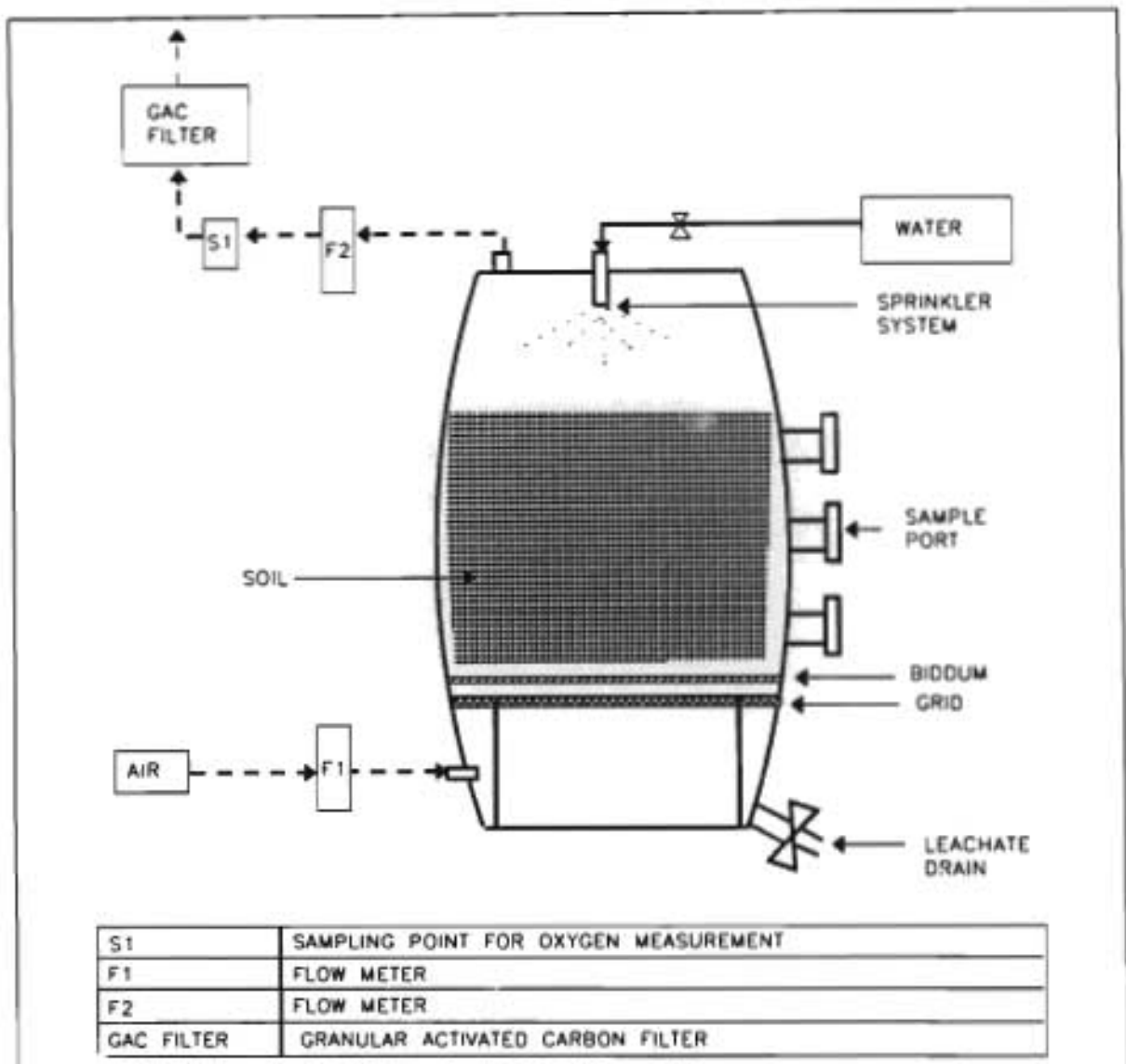


FIG. 9.1 Design of thermal bioventing pilot-scale reactor.

For the pilot study, approximately 320 t of petroleum-contaminated, coarse, loamy/sandy

soil were taken from an area around a waste oil refining company. Corn cobs (milled to 1 cm³ pieces) were used as a bulking agent to prevent compaction of the soil and to provide void space, which is necessary for gaseous exchange throughout the reactor.

Dried anaerobically digested sludge (Daspoort sewage works, Pretoria, which treats domestic effluent) hereafter referred to as dried sludge, chicken manure and a commercial biosupplement were investigated as soil amendments and sources of nutrients within the three experimental reactors. The reactors were aerated and moistened. Inert vermiculite was used to replace the commercial biosupplement, chicken manure and dried sludge within the CONT(W:A:B) reactor and thus maintain a standard volume.

Table 9.1 details the experimental protocol.

TABLE 9.1. Experimental protocol of the contents and operating conditions of each reactor

Reactor	Bulking agent	Air	Water	Amendments			
				Vermiculite	Commercial biosupplement	Chicken manure	Dried sludge
CONT	No	No	No	No	No	No	No
CONT(B)	Yes	No	No	Yes	No	No	No
CONT(W:B)	Yes	No	Yes	Yes	No	No	No
CONT(W:A:B)	Yes	Yes	Yes	Yes	No	No	No
EXP(W:A:B:CB)	Yes	Yes	Yes	No	Yes	No	No
EXP(W:A:B:CM)	Yes	Yes	Yes	No	No	Yes	No
EXP(W:A:B:AS)	Yes	Yes	Yes	No	No	No	Yes

B	=	Bulking agent (Corn cobs)	W	=	Water
A	=	Air	CB	=	Commercial biosupplement
CM	=	Chicken manure	AS	=	Dried anaerobically digested sludge
CONT	=	Control	EXP	=	Experiment

Table 9.2 shows a summary of the ratios of amendment:soil and (amendment + soil):bulking agent used for each reactor. The same sources of soil and bulking agent (corn cobs) were used for all reactors.

TABLE 9.2. Amendment:soil and (amendment+soil):bulking agent ratios for each reactor

Reactor	Amendment : soil	(Amendment+soil) : Bulking agent	Type of amendment added
CONT	No amendment added	No bulking agent added	None
CONT (B)	1 : 4	1 : 0.27	Vermiculite
CONT (W:B)	1 : 4	1 : 0.27	Vermiculite, Water
CONT (W:A:B)	1 : 4	1 : 0.27	Vermiculite, Air, Water
EXP (W:A:B:CB)	1 : 4	1 : 0.27	Commercial biosupplement = 16.9 g dry biosupplement + 3.12 kg Sybron nutrients + Vermiculite, Air, Water.
EXP (W:A:B:CM)	1 : 4	1 : 0.27	Chicken manure, Air, Water
EXP (W:A B:AS)	1 : 4	1 : 0.27	Dried sludge, Air, Water

B	=	Bulking agent (Corn cobs)	W	=	Water
A	=	Air	CB	=	Commercial biosupplement
CM	=	Chicken manure	AS	=	Dried anaerobically digested sludge
CONT	=	Control	EXP	=	Experiment

9.3.2 Operational procedures

i. Moisture

The experiment was initiated by the application of municipal tap water to the reactors through the fitted sprinkler system (Figure 9.1). The water system was calibrated to provide 2.38 l h⁻¹. The sprinkler system was activated one day per week to deliver 4.75 l over a period of 2 h. Proportionally less water was added to the 25 l control reactors, calculated on a volume_{reactor}:volume_{water} ratio based on the 4.75 l of water added to the 220 l reactors.

ii. Air

Compressed air (not humidified) was led through a flow meter into the reactor during the first 19 days (Table 9.3). The flow rates through the reactors were adjusted several times. For days 19 to 24 a 50:50 mixture of compressed air and instrument grade oxygen was led through the reactors. From day 25 to the end of the experiment, pure instrument grade oxygen was led through the reactors. The aim was to determine the optimum gas flow rate and percentage oxygen required to provide sufficient oxygen for microbial degradation of contaminants. The gas flow rate was increased until an oxygen breakthrough was detected by a MSA Oxygen Indicator (Model 246RA). Table 9.3 shows the air/oxygen flow rates through the reactors during the experimental period. The air/oxygen flow rates were the same for all the reactors.

TABLE 9.3. Gas flow regulation during the experimental period

Day	Gas flow rate	Pore volumes displaced day ⁻¹
0	2.0 l h ⁻¹ (Air)	1
4	4.0 l h ⁻¹ (Air)	2
7	8.0 l h ⁻¹ (Air)	4
11	12.0 l h ⁻¹ (Air)	6
20	6.4 l h ⁻¹ (mixture of 50% O ₂ and 50% air)	3
25	4.0 l h ⁻¹ (100% oxygen)	2

The effluent gas was directed first through a flow meter, then through a T-piece which served as the detection point for the oxygen sensor connection, and finally through a

granular activated carbon filter to capture the volatile organic compounds.

9.3.3 Analytical procedure

The soil temperature, pH and moisture content were determined weekly. Microbial plate counts were made weekly on a dilution series prepared from a 1 g soil sample in physiological saline. The cultures were incubated inverted at 30°C for 48 h before counting. Nutrients such as phosphorus and nitrogen were determined by the Institute of Soil, Climate and Water (ARC) (Standard Soil Testing, 1990).

The contaminant concentration was determined gravimetrically and by GC/FID. A 10 g soil sample was extracted by shaking for 5 min with dichloromethane/acetone (50:50) in an extraction vessel. The extract was filtered through anhydrous sodium sulphate (Na_2SO_4). The filtered product was then decanted into a pre-weighed round-bottom flask, concentrated with a rotary evaporator model and air dried. The residue was weighed. This value represented the gravimetric oil concentration.

The residue was dissolved in 10 ml dichloromethane. A small amount (approx of 5 ml) was passed through C18 Sep-Pak® cartridges (Waters) for solid phase extraction and a 1 µl sample injected into a gas chromatograph (Hewlett Packard, HP 5890 Series II) equipped with a flame ionization detector (GC/FID) and HP 5181 122 0 integrator. A HP 1 (Hewlett Packard) methyl silicone cross linked column (25 m, 0.2 mm, 0.3 µm film thickness) was installed. A summary of the GC/FID program is shown in Table 9.4.

TABLE 9.4. GC/FID program for the detection of hydrocarbons in soil samples

Parameter	Setting
Initial oven temperature	50°C
Initial time	2 min
Temperature programme:	
Ramp rate	20°Cmin ⁻¹
Final temperature	320°C
Final time	10 min
Injector temperature	320°C
FID temperature	300°C
Purge valve	On
Split ratio	70:1
Attenuation	2

9.4 RESULTS AND DISCUSSION

9.4.1 Amendments and nutrients

In the laboratory study (Chapter 8), the ratios were determined from the mass ratios. In this study, due to equipment constraints, the ratios were determined volumetrically. These factors were compensated for in the processing of the results for direct comparison with the laboratory study.

The amendment:soil:bulking agent ratios used were chosen to provide sufficient nutrients for bacterial activity and to avoid the possibility of the volumes becoming unpractically high.

Chicken manure and dried sludge were investigated as possible soil amendments to serve both as nutrient and microorganism sources. A commercial biosupplement (ABR Hydrocarbon), formulated specifically for the treatment of hydrocarbon oils, was obtained from Sybron Chemicals (SA) (Pty)Ltd.

Table 9.5 shows the results of the analyses of the chicken manure and dried sludge. Data were not available for the nutrients (Accelerator II, Sybron Chemicals SA) recommended for use with the commercial biosupplement.

TABLE 9.5. Analyses of the different selected amendments

Amendment	N (mgkg ⁻¹)	P (mgkg ⁻¹)	C (mgkg ⁻¹)
Dried sludge	33 810	3 240	291 100
Chicken manure	36 170	16 120	357 900

Chicken manure and dried sludge were also compared in the preceding laboratory-scale experiment (Chapter 8). The results of the laboratory-scale study showed that dried sludge enhanced bioremediation to a greater extent than chicken manure. Chicken manure was used because of a logical extrapolation of its success in accelerating the rate of composting garden refuse. However, this was not found in the case of bioremediation. This again emphasised that generalisations cannot be made across different technologies, even if they are underpinned by the same principles. Table 9.5 shows the nutritional complements of the two amendments, with chicken manure characterised by higher concentrations of nitrogen and phosphorus. Unfortunately, the results of this study did not confirm whether the lower nitrogen and phosphorus concentrations contributed to the improved performances in the

reactors amended with dried sludge. It could be that the dried sludge contained bacterial populations acclimatised to hydrocarbons and, therefore, enhanced/supported the natural hydrocarbon degrading population in the soil.

TABLE 9.6. The C:N:P ratios of the reactors at the beginning and end of the experiment

Reactor	C:N:P ratio (m/m)	
	Day 0	Day 98
CONT	100 : 0.95 : 0.0027	
CONT(B)	100 : 1.46 : 0.0042	
CONT(W:B)	100 : 1.60 : 0.0127	
CONT(W:A:B)	100 : 5.15 : 0.0042	100 : 2.46 : 0.0033
EXP(W:A:B:CB)	100 : 10.67 : 0.0800	100 : 4.99 : 0.0683
EXP(W:A:B:CM)	100 : 4.85 : 0.1328	100 : 4.10 : 0.0705
EXP(W:A:B:AS)	100 : 2.25 : 0.0840	100 : 3.44 : 0.0569

B	=	Bulking agent	W	=	Water
A	=	Air	CB	=	Commercial biosupplement
CM	=	Chicken manure	AS	=	Dried anaerobically digested sludge
CONT	=	Control	EXP	=	Experiment

Table 9.6 lists the C:N:P ratio of each reactor at the start and termination of the study. The commercial biosupplement amended reactor was closest to the "ideal" ratio of 100:10:1. In contrast, the other reactors appeared to have phosphorus deficiencies. However, Table 9.6 shows that even in the most active soil reactors, very little of the nutrients were used after 98 days. It may be speculated, therefore, that the importance of nutrients is over emphasised. Lt Col Ross N. Miller (Air Force Centre for Environmental Excellence, Texas, USA, personal communication) has also expressed his opinion in this regard and stated that: "In over a hundred sites treated by *In situ* bioventing, I have rarely seen a need for the addition of nutrients". However, in Chapter 3 of this thesis, the positive enhancement of

microbial catabolism of the contaminant was clearly demonstrated. The efficacy of the addition of nutrients is, therefore, still not resolved and should be investigated further. It could be that the nutrients are only needed in specific processes or sites.

9.4.2 Operational parameters

i. pH

The pH values of all the reactor soils were stable within 1 pH unit (Table 9.7). No major pH shifts were recorded during the experiment.

TABLE 9.7. Average pH, temperature and moisture content values of the reactors during the experiment

Reactor	pH	Temp (°C)	Moisture (% mass)
CONT	6.5 ± 0.4	22.7 ± 1.6	4.36 ± 0.65
CONT (B)	6.9 ± 0.3	22.8 ± 1.9	3.84 ± 0.50
CONT (W:B)	7.1 ± 0.4	22.9 ± 1.8	16.37 ± 4.92
CONT (W:A:B)	6.7 ± 0.5	24.7 ± 2.6	13.70 ± 4.89
EXP (W:A:B:CB)	7.1 ± 0.4	23.9 ± 1.9	15.38 ± 3.29
EXP (W:A:B:CM)	7.0 ± 0.5	24.6 ± 2.6	16.34 ± 4.64
EXP (W:A:B:AS)	6.2 ± 0.4	25.0 ± 2.7	16.54 ± 5.61

B	=	Bulking agent	W	=	Water
A	=	Air	CB	=	Commercial biosupplement
CM	=	Chicken manure	AS	=	Dried anaerobically digested sludge
CONT	=	Control	EXP	=	Experiment

ii. Temperature

Table 9.7 shows that the reactors which received forced aeration and water, and which were bulked with the corn cobs, showed an increase in temperature. It is suspected, however, that the cold ($\pm 20^{\circ}\text{C}$) air and oxygen used to aerate the reactors had a cooling effect. A larger increase in temperature could, therefore, be expected had the soil been aerated by other means such as tilling. Table 9.8 also shows that the reactors with the highest oxygen consumptions were characterised by the highest temperature increases and the highest microbial plate counts. The best examples of these were recorded with the reactor which received dried sludge.

iii. Moisture

Table 9.7 shows the average moisture contents of all the reactors over the 98-day experimental period. Statistical analysis shows that the standard deviations in moisture content of the reactors which received water were elevated when compared to the other reactors. During the first 3 wk of the experiment, the reactors were subject to moisture content equilibration to prevent contaminant washout when the field capacity was reached. Although Reactor CONT(W:B) recorded the highest moisture content, it should be noted that this reactor was not equipped with a leachate system. If the data of this reactor are discarded, it would be possible to make some deductions for the other reactors. All the reactors which received amendments were found to retain water more efficiently than the control reactor (Cont (W:A:B)) with the dried sludge and chicken manure amended reactors

retaining excess moisture compared with the commercial biosupplement-amended reactor.

iv. *Oxygen*

The objective was to enhance bioremediation conditions but minimize volatilization (Dupont & LakshmiPrasad, 1993). The experiment was initiated with a one pore volume of air per day exchange rate. The oxygen concentration was measured before and after passing through the soil column. Table 9.8 shows the influent and effluent oxygen concentrations for the different flow rates used.

TABLE 9.8. Oxygen concentrations in all reactors subjected to forced aeration

[Influent Oxygen] (%)	Day	PVday ⁻¹ *	[Effluent Oxygen] (%)			
			CONT (WAB)	EXP (WAB CB)	EXP (WAB CM)	EXP (WAB AS)
21	0	1	21	21	21	21
21	4	2	0.2	11.1	0.2	1.2
21	7	4	0.2	9.2	0.1	2.9
21	11	6	0.5	12.5	0.1	0.1
60	20	3	12.6	5.9	10.8	0.2
100	25	2	23	42.3	5.6	0.9

* PVday⁻¹ = pore volume exchange per day

Table 9.8 shows the results of the different aerations tested. The reactors all showed a drop in effluent oxygen concentration 24 h after starting the experiment. This suggested an increase in metabolic activity which was supported by an increase in the microbial plate counts (9.4.4). Table 9.8 also shows that, even with the use of 100% oxygen at a pore volume exchange of two, the oxygen demand of the reactor supplemented with dried sludge

was not satisfied, since it is generally accepted (Crawford *et al.*, 1993) that the oxygen concentration in a thermal bioventing system should be > 5% (1.3.2 *iv*). This could mean that its potential remedial capacity was oxygen limited. Another concern was that 100% oxygen had to be used to satisfy the oxygen demand. Oxygen is costly and in most cases is unpractical for use in full scale projects. The possibility of increasing the bulking ratio together with the use of a higher airflow should, therefore, be investigated. Respirometric tests could also be made to establish the optimal oxygen concentration in relation to the catabolic rate.

9.4.3 Contaminant reduction

The contaminant reduction was measured gravimetrically. The results are shown in Figure 9.2. A sample of the resulting residue was injected into a GC/FID. As a control, some samples were submitted to an independent laboratory for EPA 418/1 analyses. These are tabled in Appendix 3. Comparison of the results of the two analytical methods showed that the catabolism of the contaminant seemed to be more evident when the EPA 418/1 method was used. A possible explanation for this can be found in the different methods of sample preparation. For the EPA method, the primary oily extract is passed through a silica column, which removes all the fatty acids which are normally the first breakdown products in aliphatic compound catabolism. In contrast, the gravimetric method includes the fatty acids and, thus, the gravimetric results should be regarded as very conservative estimates of the degradation.

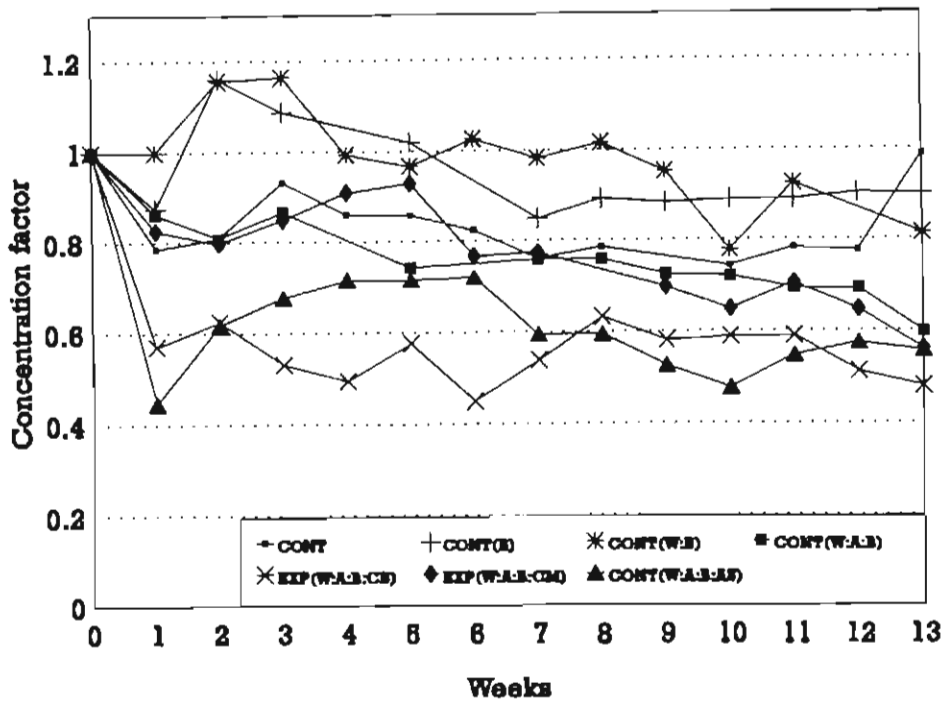


FIG 9.2. Changes in contaminant concentration factors (determined gravimetrically) during pilot-scale thermal bioventing. The reactors were subjected to various treatments as detailed in Tables 9.1 and 9.2.

Before discussing the contaminant reduction, it is important to consider the nature of the contaminant itself. The contaminant was a weathered heavy petroleum oil which consisted of detectable amounts of the C8 to C40 alkanes and zero to four ring polyaromatic hydrocarbons (determined by GC/MS by Arthur D. Little, Inc, Cambridge, U.S.A). The pristane to phytane ratio was found to be 0.49. The total petroleum hydrocarbon concentration, as determined by the EPA 418/1 method, was 17.2% (m/m). With this information, it was envisaged that remedial progress would be slow and it was decided to run the experiment until 50% of the contaminant was degraded. Figure 9.2 shows that a contaminant reduction of at least 50% was achieved at pilot-scale in 13 wks by thermal

bioventing. Two reactors (viz. EXP(W:A:B:AS) and EXP(W:A:B:CB)) showed increased rates of bioremediation. Figure 9.2 also shows that the reactor which received chicken manure (EXP W:A:B:CM) compared well with the control reactor (CONT W:A:B). Although the results pertaining to the chicken manure amended reactor did not compare favourably with those from either the commercial biosupplement or the dried sludge amended reactor during the first 5 wk of treatment, the degradation rate increased substantially after the fifth week to give an average degradation rate of $0.478 \text{ mgkg}^{-1}\text{day}^{-1}$ compared to the earlier $0.200 \text{ mgkg}^{-1}\text{day}^{-1}$. However, if the results of the laboratory-scale study are also considered, chicken manure cannot be recommended as a nutrient supplement in the treatment of oil contaminated soils.

A sample of the residual extract was injected weekly into the GC/FID. From the results it was evident that the contaminant consisted of heavy hydrocarbon fractions with few aliphatic constituents (Appendix 4). The results of the gravimetric analyses generally corresponded with the chromatographs. After 5 wk a general decrease in the hydrocarbon "hump" was evident in Reactor (EXP W:A:B:AS). This effect could also be seen in (EXP W:A:B:CB) but to a lesser extent. Reactor (EXP W:A:B:CM) showed no concentration shift when compared to the control reactors. Although a general hydrocarbon "hump" shift was not expected in the first five weeks of treatment, some peaks disappeared as time progressed. For example, group of peaks with a retention time of between 13.714 and 14.523 min was reduced substantially in the reactors which showed the largest TPHC decreases. After 10 wks the general chromatogram pattern changed substantially in the treated reactors. For example, in the reactor which received a dried sludge amendment, two

peaks, probably the phytane and pristane peaks, became prominent compared with the [CONT] (Appendix 5). Pristane and phytane are both branched alkanes and difficult to degrade. They therefore serve as indicator molecules to determine the progress of bioremediation. These peaks will therefore still be visible when other peaks have disappeared as in the chromatograms in Appendix 5.

9.4.4 Microbial population

Estimates of the total colony forming units were made as the experiment progressed. The microbial population increased from 10^5 cfug⁻¹ to 10^9 cfug⁻¹ soil within the first 13 days in all the amended reactors. This indicated that the conditions were conducive for microbial proliferation and therefore bioremediation. From day 13 the microbial populations increased steadily to 10^{12} cfug⁻¹ soil in these reactors. Possible reasons for these atypically high plate counts in the soil samples are discussed in Chapter 10.

9.5 CONCLUSIONS

- The study proved that a contaminant reduction of at least 50% can be achieved at pilot-scale in 13 wks using thermal bioventing;
- Of the amended reactors, the presence of dried sludge and commercial biosupplement effected the largest contaminant decreases;
- As a possible amendment to increase the rate of bioremediation, dried anaerobically digested sludge performed better than chicken manure. The laboratory-scale experiment gave the same results;
- Gravimetric analyses were found to be a conservative indication of the remediation process; and
- The plate count of all the amended reactors exceeded the plate counts of the control reactors, proving that the amendments were conducive to establishing a favourable environment for bioremediation.

CHAPTER 10

THE USE OF SCANNING ELECTRON MICROSCOPY TO INVESTIGATE THE MICROORGANISMS IN A PILOT-SCALE THERMAL BIOVENTING REACTOR

10.1 INTRODUCTION

Bioremediation takes place in a microbiological world which has extremely complex interrelationships not only between different microorganisms but also between microorganisms and soil and microorganisms and the contaminant. Therefore, it is necessary to investigate microbial behaviour in the soil. One such method is by direct scanning electron microscopy.

In the pilot-scale investigation of thermal bioventing, atypical, high plate counts were observed (9.4.4) compared to normal microbial numbers in soil undergoing bioremediation. The thermal bioventing pilot-scale reactor could be compared to an in-vessel composter. Even these compost based biofilters normally have microbial numbers which seldom exceed 10^8 cfug⁻¹ compost (Lei, Lord, Arneberg, Rho, Greer & Cyr, 1995). The high microbial numbers found in this study were investigated since it was possible that the microorganisms preferentially immobilized in the reactor. Therefore, if a sample contained a biofilm of these microorganisms immobilized to the bulking agent, for example, the plate count would give results which are not representative of the whole reactor.

10.2 OBJECTIVE

To investigate the:

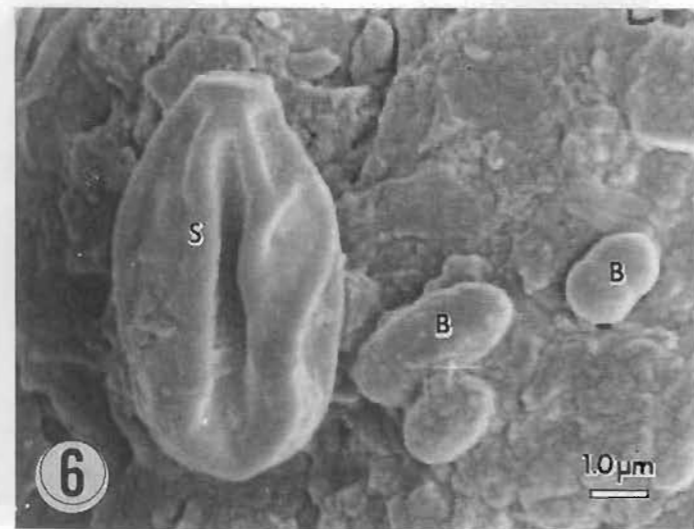
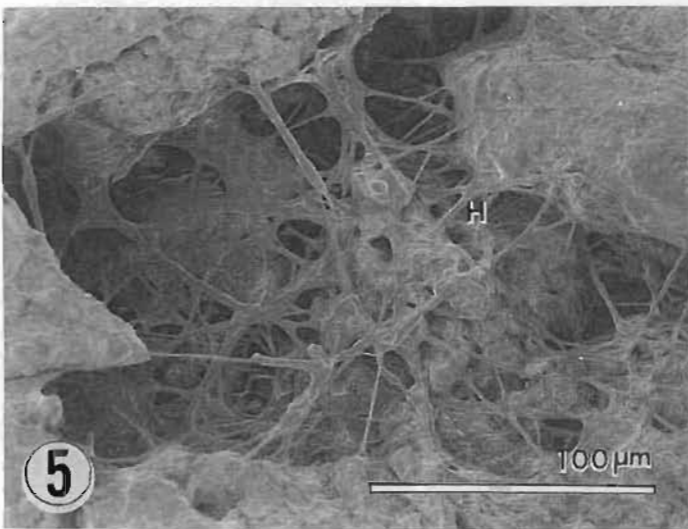
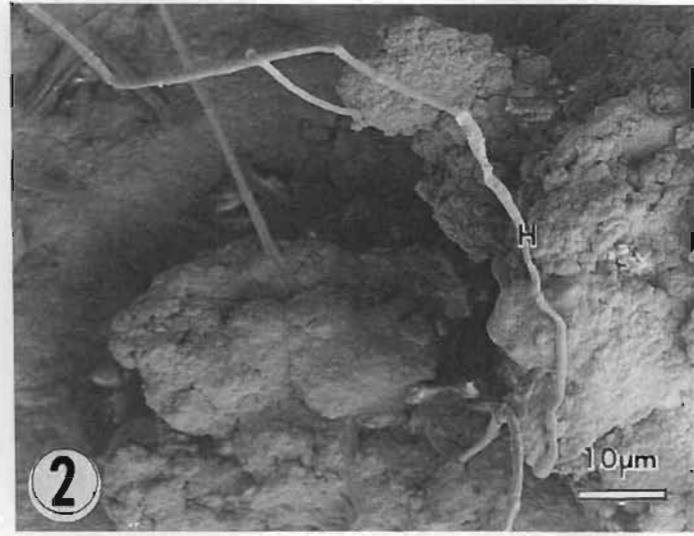
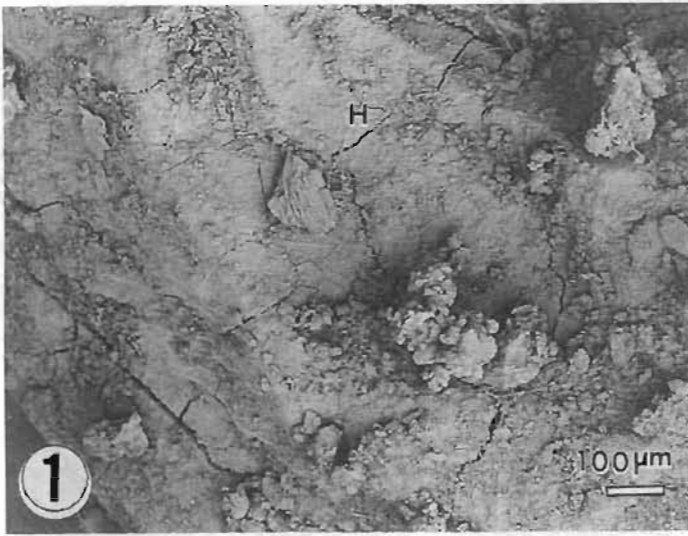
- Potential of scanning electron microscopy (SEM) to examine the microorganisms in soil undergoing bioremediation; and
- Possibility that microorganisms have surface preference when immobilizing themselves in soil undergoing thermal bioventing.

10.3 MATERIALS AND METHODS

Composite soil samples were taken from the pilot-scale thermal bioventing reactors which were subjected to bioremediation treatment (Chapter 9). One gram samples from each reactor were vapour-fixed with 1% (m/v) OsO₄ for 72 h in a closed container. All the specimens were mounted with colloidal silver paint on a large SEM stub which was modified by boring eight circular holes 1 mm deep and 5 mm in diameter. The samples were coated with gold in a Polaron Sputter Coater and examined with a Jeol JSM 840 scanning electron microscope operated at 5 kV.

10.4 RESULTS AND DISCUSSION

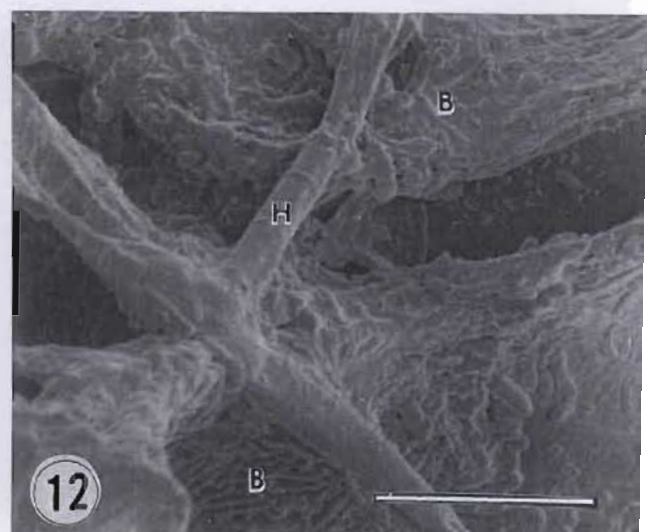
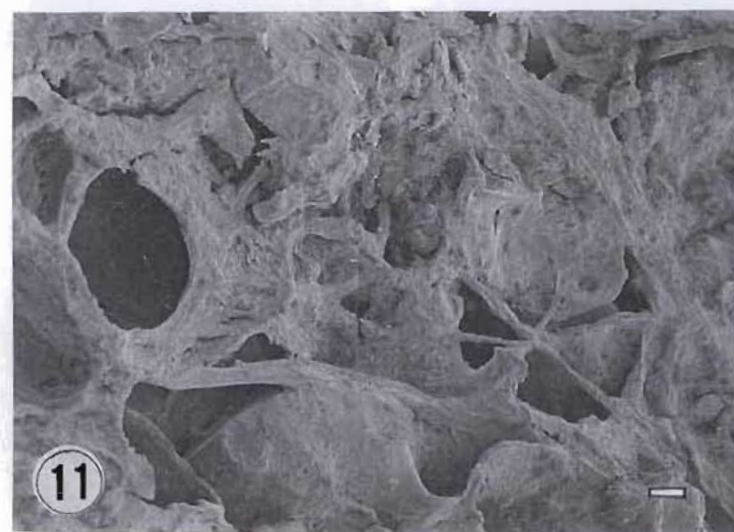
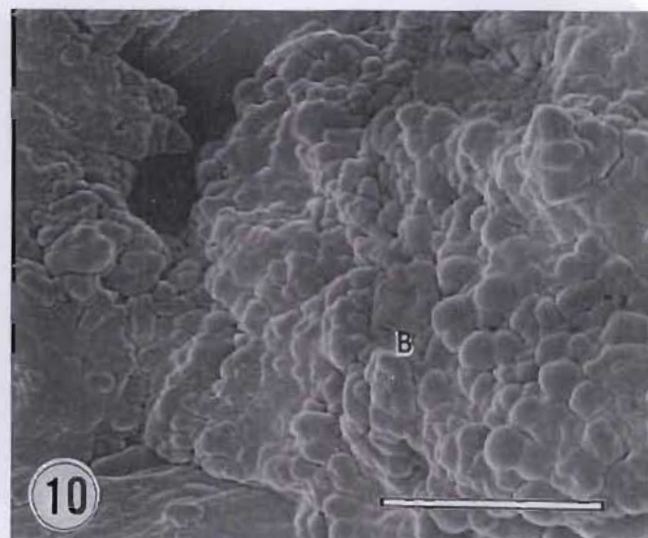
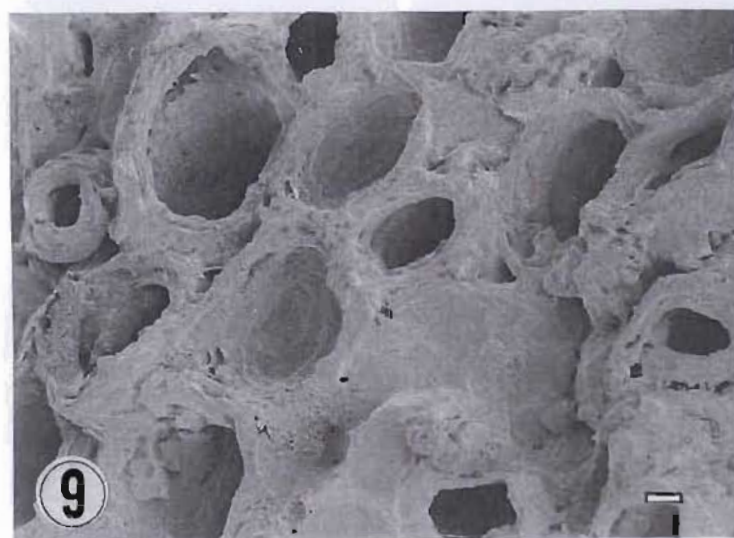
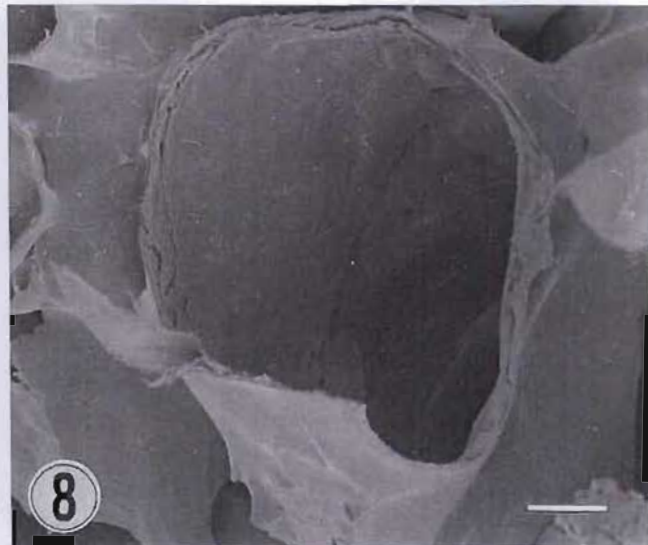
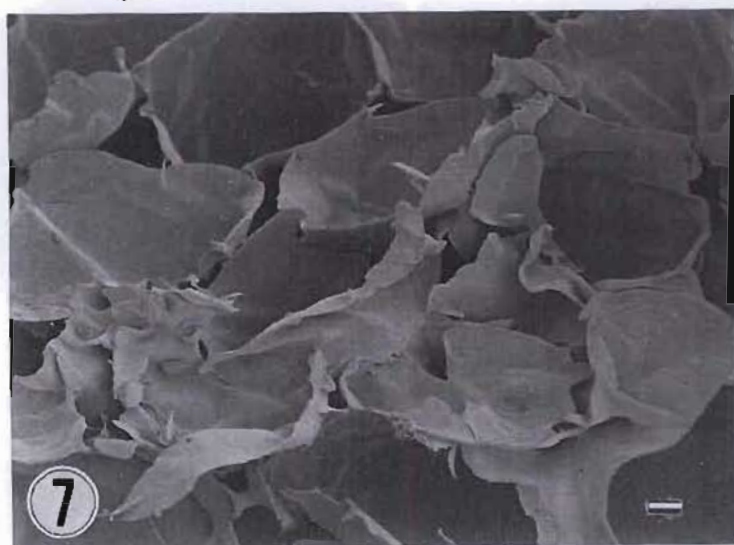
Figures 10.1 to 10.6 illustrate that amendment with the commercial biosupplement and



Figures 10.1-10.6 Scanning electron micrographs of soil samples taken from pilot scale reactors undergoing bioremediation. Figures 10.1 and 10.2 Soil from a control reactor showing hyphae (H) associated with the soil structure. Figures 10.3 and 10.4 Soil from a reactor treated with a commercial biosupplement showing increased amounts of hyphae (H) when compared to the control reactors. Figures 10.5 and 10.6 Soil from a reactor treated with dried anaerobically digested sludge as amendment. Figure 10.5 shows a hyphal mass and, possibly, a bacterial slime layer. Figure 10.6 confirms the presence of bacteria (B). A fungal spore (S) can also be seen in this micrograph.

dried sludge the microbial numbers. The dried sludge-amended reactors showed the presence of both bacteria and fungi (Figures 10.5 and 10.6) while the commercial biosupplement-amended reactors show a fungal dominance. During the routine monitoring of the microbial population, nutrient agar plate counts were normally used which excluded the fungal population. Using both plate counts and SEM, the fungal:bacterial population ratios could be determined and the treatment adjusted accordingly. For example, if a contaminated site had initially a high fungal presence, the microbial population should not be forced to a bacterial dominated one, especially when the fungal population was effectively degrading the pollutant.

Figures 10.7 to 10.12 illustrate that the corn cobs in the reactors amended with chicken manure (Figures 10.9 and 10.10) and the reactors amended with dried sludge (Figures 10.11 and 10.12) were more susceptible to microbial attack than in the control reactors (Figures 10.7 and 10.8). However, the corn cobs did not appear to collapse structurally after 5 weeks of use as a bulking agent. This could indicate that the microorganisms preferentially attached to on the corn cobs which could be to the advantage of the bioremediation process. Due to a limitation of bioremediation vessels, it is often necessary to bioremediate contaminated soil in batches. The bulking agent is re-used in each batch. A small amount of the treated soil is also mixed with the new contaminated batch to establish the same microbial ecology as the previous batch. If it could be established whether the microbial community, immobilised on the corn cobs, was representative of the remediating microbial community, it would not be necessary to mix treated soil with the new untreated batch. This would have significant cost saving implications.



Figures 10.7-10.12 Scanning electron micrographs of corn cob samples used as a bulking agent in soil reactors undergoing bioremediation treatment (bar = 10 μm). Figures 10.7 and 10.8: Corn cobs taken from the control reactors. Figures 10.9 and 10.10: Corn cobs from reactors amended with chicken manure. Figure 10.10 confirming that the layers covering the corn cobs in Figure 10.9 are bacterial (B). Figures 10.11 and 10.12: Corn cobs from reactors amended with dried anaerobically digested sludge. Figure 10.12 shows hyphae (H) and bacteria (B) in large numbers.

10.5 CONCLUSIONS

To conclude:

- SEM confirming that treated and untreated soil differed in respect of microbial numbers;
- Microorganisms preferentially immobilized on the bulking agent in the reactors which were amended; and
- The use of electron microscopy not only aided understanding the bioremediation process but also proved to be a valuable decision support tool towards optimizing treatment methods.

CHAPTER 11

ASSESSING THE BIODEGRADABILITY OF SOIL CONTAMINANTS

11.1 INTRODUCTION

Experience has shown that most petroleum contaminated soils can be remediated. In certain cases the biological treatability of the contaminant should be investigated before investing in the full-scale treatment of the soil. For example, this approach is recommended where soils are contaminated with a weathered hydrocarbon or the presence of heavy metals is suspected. In these cases a treatability study should be conducted in which factors such as the feasibility of bioremediation, contaminant biodegradability and toxicity are investigated. These studies could take months to complete (Rogers, Tedaldi & Kavanaugh, 1993). In Chapter 3 the feasibility of bioremediation could only be established after several weeks of treatment and the analytical difficulties were discussed.

Since industries are under time and budget constraints, they often cannot afford to perform the traditional preliminary tests to establish whether bioremediation is feasible at full scale even though they are aware that the full scale bioremediation could prove more cost effective and environmentally friendly than co-disposal (landfilling). Remedial technologists need an inexpensive screening technique to establish the biotreatability of soil contaminants. The test also has to yield results rapidly (preferably within 10 days). The reason for this is that remedial action must start as soon as possible after the incident to

avoid extra costs, and cannot wait for protracted testing. Normally, the stabilisation and the determination of the extent of contamination on the site is completed within 2 weeks after which the remedial action should follow shortly.

Typically, the biodegradabilities of industrial chemicals are determined by using the three-tiered OECD test system (Struijs & Van den Berg, 1995):

- The ready biodegradability test which essentially relies on the principle that biodegradation is monitored as the degree of mineralization, by means of parameters such as oxygen uptake, carbon dioxide production or elimination of dissolved organic carbon (DOC). The test duration is 28 days.
- The inherent biodegradability test which demonstrates the potential degradability of a compound. Conditions for biodegradation are optimised by high population densities. Inherent biodegradability tests make use of practical conditions which more closely resemble environmental conditions and, therefore, have a high potential for degradation.
- The simulation test which is designed to measure the rate of biodegradation in a specified environmental compartment under realistic environmental conditions. Activated sludge is normally used as an inoculum.

These tests are used worldwide to assess the risks of chemicals to the environment and were

used as a guideline in this study. These tests were, however, designed for assessing the biodegradability of water- and not soil contaminants. In this study a method is suggested which is not designed for use in evaluating exposure concentrations to the environment or ecotoxicity, but simply as a tool for remedial technologists to establish whether or not a specific contaminated site can be remediated biologically.

11.2 MATERIALS AND METHODS

11.2.1 Development

The study was performed in three different phases:

- Phase 1: Desktop development of the biodegradability protocol. A method described in HMSO “Assessment of Biodegradability” (Dick, 1982) was adapted for the purpose of determining the biodegradability of soil contaminants in a short period of time;
- Phase 2: Testing of the method in a water matrix, to establish whether the method produces reliable results; and
- Phase 3: Testing of the method in a soil matrix.

11.2.2 Method Testing

i. Biodegradability of soluble oil in a water matrix

A water soluble oil (4420 mgℓ⁻¹ COD contaminants stock solution) was used as the contaminant. Table 11.1 gives an outline of the experimental setup.

TABLE 11.1. Experimental design of a biodegradability test for soluble oil in water

Notation	Nutrients	Inoculum	Contaminant (mgℓ ⁻¹)	Glucose (mgℓ ⁻¹)
Control 1	+		+ (100 COD)	
Control 2	+	+		
Test-100	+	+	+ (100 COD)	
Test-500	+	+	+ (500 COD)	
Test-1000	+	+	+ (1000 COD)	
Toxicity-100	+	+	+ (100 COD)	+ (100 COD)
Standard	+	+		+ (100 COD)
Blank			Distilled water	

Control 1 was used to give an indication of the biological activity of the contaminant oil. In Control 2 the endogenous respiration rate of the sludge was measured. Toxicity-100 and the Standard indicated the toxicity effects which the contaminant had on the degradation of labile components, such as glucose, in the same solution. The blank was included to monitor laboratory and instrument conditions.

A nutrient stock solution was prepared by mixing 4 ml FeCl_3 (0.25 g l^{-1}), 1 ml each of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (22.5 g l^{-1}), CaCl_2 (27.5 g l^{-1}) and $(\text{NH}_4)_2\text{SO}_4$ (40 g l^{-1}) to 2 ml of the following solution: KH_2PO_4 (8.5 g l^{-1}), K_2HPO_4 (21.75 g l^{-1}), $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (33.4 g l^{-1}) and NH_4Cl (1.7 g l^{-1}). Aliquots (1.8 ml) of the nutrient stock solution were added to each test chamber.

Return activated sludge obtained from Daspoort Sewage Works (Pretoria) was used as the inoculum. The sludge was settled, the supernatant replaced with sterile saline solution (autoclaved at 15 lbsi, 121°C , 20 min) and aerated overnight at 25°C before use. Sludge was inoculated at a concentration of 30 mg l^{-1} MLSS per 100 mg l^{-1} COD of contaminant.

The test chambers, which contained a final volume of 200 ml, were connected to a Micro-Oxymax closed circuit respirometer, and were stirred continuously, in order to measure the oxygen consumption and carbon dioxide production every 2 h for at least 4 days. As a control, the COD values of some channels were measured before and after the respirometry test.

ii. Biodegradability of soluble oil in a soil matrix

The conditions used in the water-based test were repeated except that the dilution water was replaced with 200 g of Longlands loam sand (Table 8.3) soil. Table 11.2 gives an summary of the biodegradability test protocol.

TABLE 11.2. Experimental design of a biodegradability test of soluble oil in soil

Name	Soil	Nutrients	Inoculum	Contaminant (mgℓ ⁻¹)	Glucose (mgℓ ⁻¹)
Control 1	+	+		+ (100 COD)	
Control 2	+	+	+		
Test-100	+	+	+	+ (100 COD)	
Test-500	+	+	+	+ (500 COD)	
Test-1000	+	+	+	+ (1000 COD)	
Toxicity-100	+	+	+	+ (100 COD)	+ (100 COD)
Standard	+	+	+		+ (100 COD)
Blank	+			Distilled water	

In Control 1 the effect of the contaminant on the microorganisms in the soil was measured.

In Control 2 the endogenous respiration rate of the sludge and the soil was measured. The

Toxicity-100 and Standard measured the toxic effects of the contaminant on the degradation of labile degradable components, such as glucose, in the same soil. The blank was included to assess the normal microbial activity in the uncontaminated soil.

The soluble oil was added to the soil 4 h before the experiment was monitored to facilitate binding. As different COD values were tested, different volumes of liquid were added to the soil. The liquid contents of all the reactors were adjusted to the same volume with sterile distilled water.

The respiration rates was measured every 2 h for at least 6 days.

11.3 RESULTS AND DISCUSSION

11.3.1 Biodegradability of a soluble oil in a water matrix

Figures 11.1 and 11.2 illustrate the cumulative oxygen consumptions ($\mu\ell$) and carbon dioxide production ($\mu\ell$) over a 6-day period. A biodegradable compound should at least support respiration rates higher than the endogenous respiration rate of the inoculum. If respiration rates in the test compound chamber are observed which are close to those in the Standard (where glucose is the sole carbon source) the compound is considered to be readily degradable.

The results of the biodegradability test indicated that the soluble oil was indeed readily biodegradable. The reaction vessel which contained a $100 \text{ mg}\ell^{-1}$ COD (Test-100) respired at roughly the same rate as the Standard which contained $100 \text{ mg}\ell^{-1}$ COD in the form of glucose. The toxicity test (Toxicity-100) also illustrated that the soluble oil did not inhibit the biodegradation of other labile components of the mixture since the respiration rate of the vessel which contained glucose and soluble oil ($100 \text{ mg}\ell^{-1}$ COD of each) was approximately double that of the Standard. At these concentrations the soluble oil should not be inhibitory to an activated sludge plant.

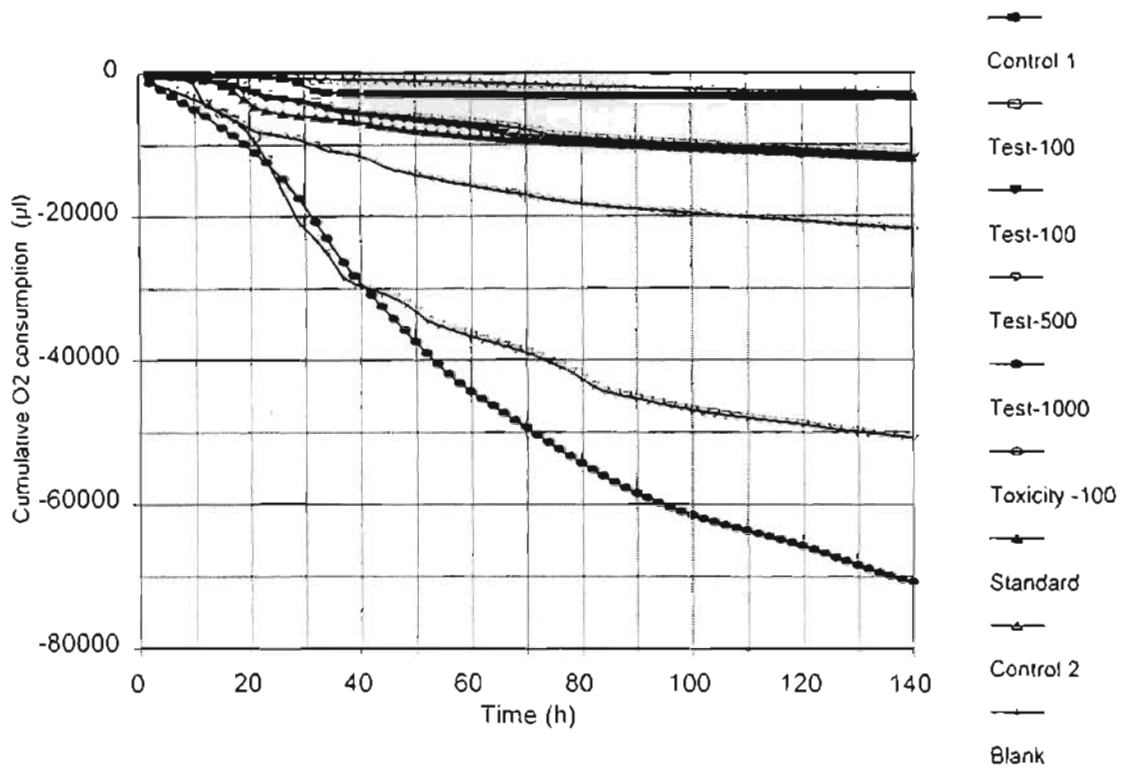


FIG 10.1 Cumulative oxygen consumptions ($\mu\ell$) during biodegradation of soluble oil in water.

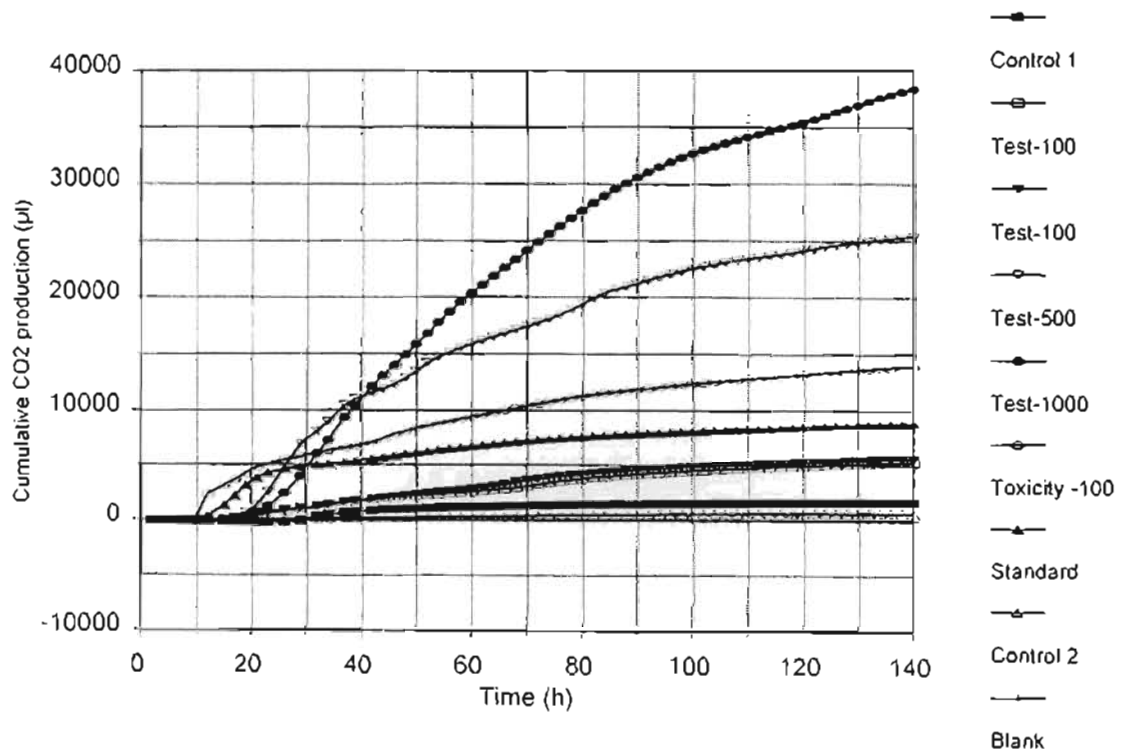


FIG. 10.2 Cumulative carbon dioxide productions ($\mu\ell$) during biodegradation of soluble oil in water.

To confirm the results, the COD decreases of five vessels were monitored over a 6-day period (Table 11.3).

TABLE 11.3. COD ($\text{mg}\ell^{-1}$) of five reaction vessels before and after six days of treatment

Reaction Vessel	Test-100	Test-500	Toxicity-100	Toxicity	Control 2
Time 0	124	667	239	138	29
Time 6 days	52	244	67	47	17

Degradations of between 58 and 63% were achieved, confirming the respiration data.

11.3.2 Biodegradability of a soluble oil in a soil matrix

Figures 11.3 and 11.4 illustrate the results of the biodegradability test of soluble oil in soil. The results obtained in this test were quite different from the results obtained in the biodegradability test of soluble oil in water. The Blank vessel showed higher respiration rates than some of the test vessels. This was expected since the moistened uncontaminated soil would have measurable microbial activity. Uncontaminated soil typically has 10^6 to 10^8 cfug⁻¹ of soil, which should manifest as respiration. Therefore, the respiration of the microorganisms degrading the contaminant has to exceed the respiration of the indigenous microorganisms before degradation rates can be observed. In this specific test, this was only achieved with a $1000 \text{ mg}\ell^{-1}$ COD. However, the clean-up levels for soil are far more lenient

than those of water. The OIEC (South Africa) suggests that soil contamination should be remediated to 1000 ppm above background levels, which means that the suggested biodegradability study would be sensitive enough to detect any contaminant above the clean-up level.

The respiration rates in the vessels inoculated with activated sludge were higher than the Blank and Control 1, which were not inoculated. Activated sludge could, therefore, be a feasible inoculum. However, it could be debated that very few soil contaminants are treated with activated sludge, but rather with indigenous microorganisms or biosupplements. The use of biosupplements poses problems, since these are commercial products which compete against each other, and the use of a single one could raise conflicts between the different parties with accusations of prejudice towards a product.

The reproducibility of results was also affected in the soil matrix and the two Test-100 reactions showed marked differences.

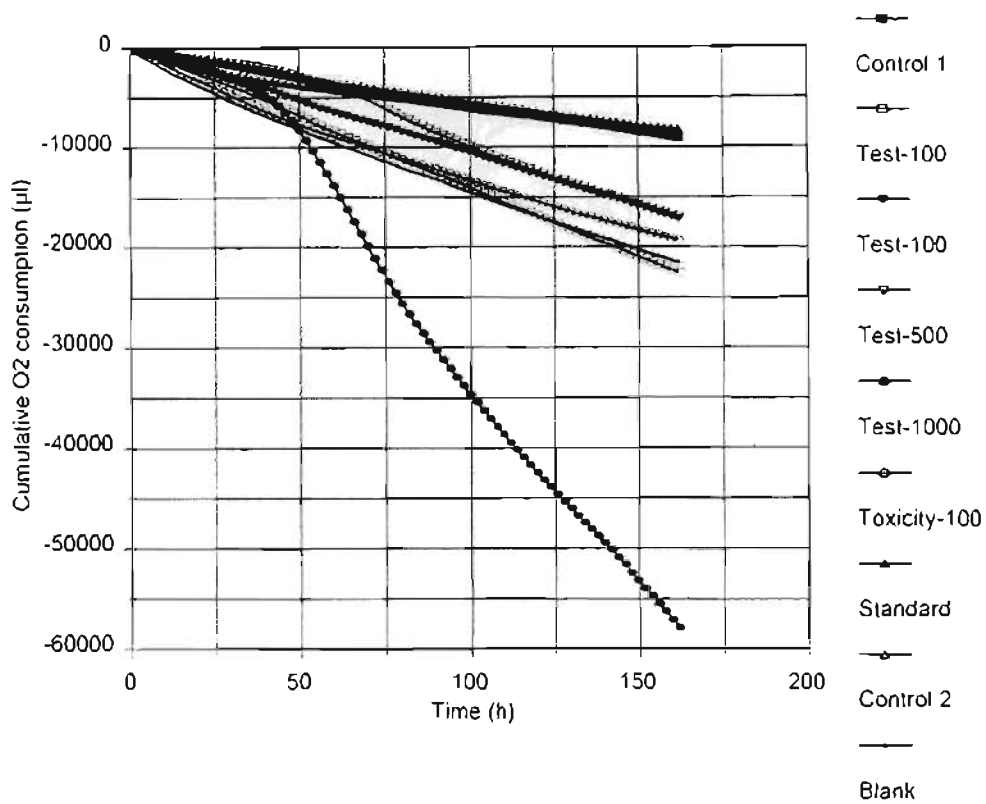


FIG. 11.3 Cumulative oxygen consumptions (μl) during biodegradation of soluble oil in soil.

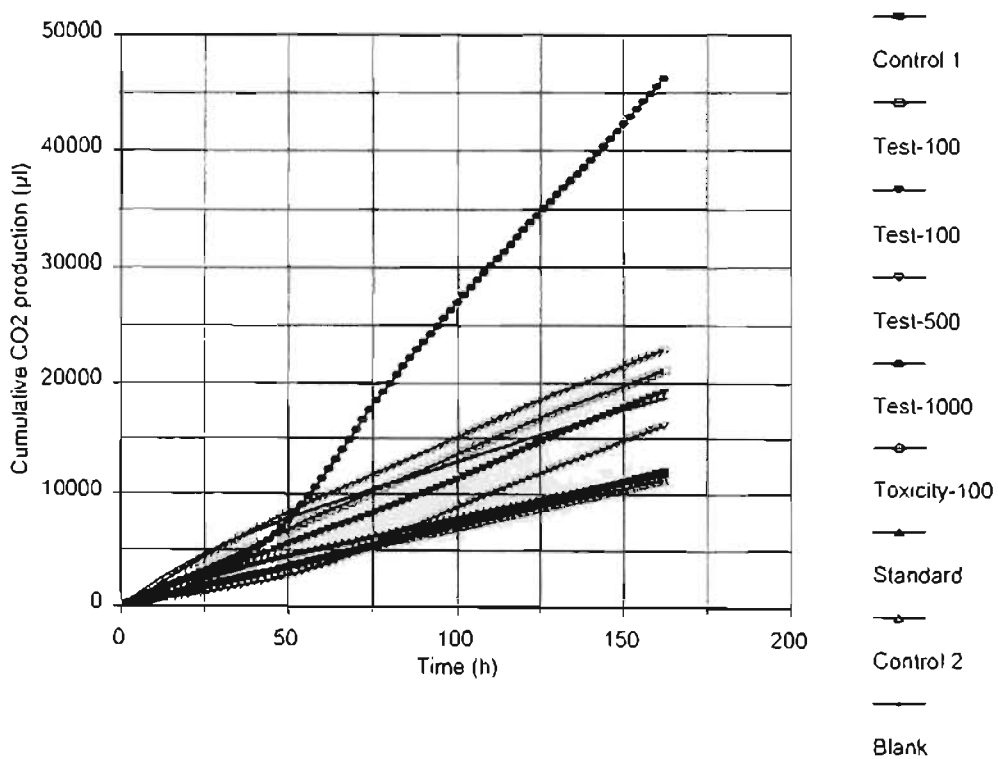


FIG. 11.4 Cumulative carbon dioxide productions (μl) during the biodegradation of soluble oil in soil.

11.3.3 Troubleshooting

Several factors will need investigation before the biodegradability of soil contaminants can be assessed reliably. These include:

- *The time span of the test.* Nutrients are only added at the beginning of the test. Therefore, once the nutrients becomes rate limiting, the test should be terminated.
- *The effect of oxygen transfer.* Maximum dissolved oxygen is facilitated by continuous stirring in the water matrix. This cannot be achieved in soil. The amount of soil used in the test should, therefore, be investigated to limit the effect of anaerobic pockets.
- *Inoculum.* Apart from which inoculum to use, the inoculum size should also be investigated.
- *Water extract.* Most of the problems associated with the test can be eliminated by making a water extract of the soil contaminant. Essentially, the water soluble contaminants are the only compounds which carry the risk of reaching the groundwater. This extract could then be treated in the same way as an effluent.
- *Analysis.* GC/FID were made before and after the biodegradability test. These were found to be unreliable because of extraction difficulties and poor result reproducibility. Once again, the COD decreases in the water extracts before and after the test could overcome this problem.

11.4 CONCLUSIONS

To conclude:

- A method to assess the biodegradability of soil contaminants was suggested and tested;
- The method proved to be feasible at concentration exceeding 1000 mg ℓ^{-1} COD; and
- Several unknowns and shortcomings were identified, which could be resolved in future, leaving remedial technologists with a workable biodegradability/treatability assessment.

CHAPTER 12

GENERAL DISCUSSION

12.1 INTRODUCTION

Bioremediation of oil contaminated soils in South Africa has only recently been considered a viable treatment option by industry. The South African industrial sector is also starting to take responsibility for its actions and to reject the "out of sight, out of mind" solutions such as the co-disposal of the contaminated soils, and the filling up of our limited landfill sites with large volumes of a waste for which alternative treatments are available.

Before critically appraising the results of the project detailed in this thesis, a brief overview of the study, in relation to South African remediation research, will be presented.

Landfarming is one of the key focus areas of most of the environmental divisions of petroleum industries, transport companies and the local telecommunication company. Although these parties invest in the full-scale application of bioremediation, research, focusing on optimisation and development of remedial processes, seems to have a low priority.

This research project was designed to both serve the waste management industry and enhance our knowledge of bioremediation. This project focused on *ex situ* bioremediation

processes while the Natal based research group focuses on *in situ* remediation. The applications of these different approaches were discussed in Chapter 2. The primary objective of these research efforts is to develop or, where necessary, adapt and optimize remediation technologies and processes to best serve industry in treating contaminated sites. In the next section, the two technologies which are central to this thesis will be discussed.

12.2 *EX SITU* REMEDIAL TECHNOLOGIES

12.2.1 Landfarming

Landfarming has been in use in South Africa for a few years, especially for the treatment of oily sludges. Typically, the soil is excavated, treated once with a fertilizer, watered and ploughed irregularly. The author proved that by making a pre-study, treatment optimisations could save some time and money. For example, in the pilot-scale soil treatability study (Chapter 3), it was proved that a relatively simple preliminary experiment, which did not require sophisticated equipment, could save unnecessary capital investment and time. In this specific case, the indigenous microorganisms had the intrinsic ability to catabolize the contaminant. If time is not invested in such pre-study, capital might be spent on a costly biosupplement. Although it did not happen in this case, the indigenous microorganisms may have been unable to degrade the contaminant and a biosupplement would, therefore, be needed. This would also be revealed in a pre-treatment study. The necessity of a pre-treatment study was also stressed by Block *et al.* (1992) who claimed that the only bioremediation projects which failed were those which were not preceded by a

treatability study. However, the time for a 10 to 12 week experiment may simply not be feasible, especially when a company is under pressure by the public, residents, land owners or local law enforcing body to remove the contaminant or treat the contaminated site. Chapter 11 suggested a laboratory test which can be made in one week, which could give an indication of the potential treatability or biodegradability of the contaminant. The effect of a commercial biosupplement can also be assessed by using the same method. The laboratory equipment needed for the test is, however, expensive and small companies could choose to rather use a simulation experiment of the type used in Chapter 3.

Once the treatability study has been completed, the full-scale landfarming operation should be implemented and carefully monitored. In this study, it was proved that, with careful monitoring and regular dosing of water and indigenous bacteria, even a highly weathered contaminant could be degraded to a concentration which met the standards of the DWAF. This study proved that by implementing landfarming intensively, a contaminant could be degraded from a maximum concentration of 23 000 mgkg⁻¹ to a minimum concentration of 820 mgkg⁻¹ within 6 months.

During the full-scale landfarming, three distinct decreases in the TPHC were observed which were accompanied by marked increases in the number of culturable hydrocarbon degrading microorganisms. These changes created an ideal opportunity to study the relationships between microbial numbers, activity, type and hydrocarbon degrading efficiency. Chapters 5 to 7 were dedicated to investigating the factors which underpin the processes which take place in soil. In Chapter 5, the dominant culturable bacteria were

isolated and identified. As expected, the population was dominated by *Bacillus* and *Pseudomonas* species which are known to degrade complex hydrocarbons (Cerniglia, 1984; Antai, 1990). However, the method used to identify the microorganisms is biased towards fast growing culturable microorganisms which represent only a small fraction of the microorganisms present in soil.

As a consequence of this limitation, the method described by Hahn *et al.* (1992) and Amann (1993) was adapted to visualise the microorganisms in soil by *in situ* hybridization with r-RNA-targeted, fluorescently-labelled oligonucleotides (Chapter 6). This method was found to be feasible for the investigation of the total eubacterial population *in situ*.

In Chapter 7, the activity of the soil microorganisms in terms of respiration was investigated at different times in the treatment. A clear correlation between activity and degradation was observed. Furthermore, the effect and fate of an amendment could be assessed and monitored. The results of these experiments led directly to the development of the biodegradability/treatability respirometric test detailed in Chapter 11.

In conclusion, landfarming was identified as a viable technology for the treatment of oil contaminated soils in South Africa provided that a pre-treatment study is made and the full-scale treatment is intensively managed and monitored.

12.2.2 Thermal bioventing

In this project, thermal bioventing was studied at laboratory and pilot-scale. In the laboratory investigation, soil "spiked" with naphthalene, toluene and 3-methyl pentane, was remediated to the extent that the contaminants were not detected in the soil after 35 to 42 days. Bioremediation did not exclusively account for the contaminant concentration reduction since volatilization effected at least 40% of the reduction. Some consultants would not regard this as a problem since air emissions are not, as yet, controlled by law in South Africa. The author is of the opinion that this is a simple displacement of the contamination. Volatilization should be minimized and 100% mineralization should be the ultimate goal of a full-scale bioremediation project.

In the pilot-scale study of thermal bioventing, a highly weathered contaminant was degraded from >10% (m/m) to <5% (m/m) in 13 wks while volatilization was minimized through the reactor design. During the pilot-scale study, the microorganisms were investigated by scanning electron microscopy. Differences between treated and untreated soil samples were clearly visible. Furthermore, the study showed that the microorganisms preferentially immobilized on the bulking agent, which could be financially beneficial. The bulking agent which is normally recycled in a full-scale plant could also serve as the microbial inoculum.

The technology of thermal bioventing is, thus, a powerful tool to degrade petroleum contaminants. Thermal bioventing is, however, expensive and should only be used when a small volume of soil is contaminated with high concentrations of potentially hazardous

petroleum products.

12.3 CONCLUSION

This thesis reports on the processes and microbiology of two *ex situ* bioremediation techniques suitable for South African conditions. It was proven that these technologies are viable and worth pursuing in full-scale soil remediation projects.

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Appendix 1

TABLE A1.1. Culture characteristics of bacteria isolated from oil-contaminated soil

Isolate	Colony shape ^a	Size (mm) ^d	Pigment	Opacity ^b	Elevation	Surface	Edge	Slime	Total count ^c
1	c	1	cream	t	convex	glossy	entire	-	2x10 ⁵
2	c	0.5	pink	t	flat	glossy	entire	-	6x10 ⁵
3	c	<0.5	none	t	raised	glossy	entire	-	54x10 ⁵
4	c	2	peach	t	convex	glossy	entire	-	1x10 ⁶
5	c	1.5	grey	t	convex	glossy	entire	+	8x10 ⁵
6	r	25	white	o	flat	glossy	lobate	+	1x10 ⁵
7	i	7	cream	o	raised	glossy	undulate	+	1x10 ⁵
8	r	5	white	t	flat	dull	undulate	-	1x10 ⁵
9	c	1	orange	t	convex	glossy	entire	-	3x10 ⁵
10	c	0.5	white	t	raised	glossy	entire	-	8x10 ⁵
11	r	12	none	t	flat	rough	rhizoid	-	1x10 ⁵

a = c:circular, i:irregular, r:rhizoid

b = t:translucent, o:opaque

c = total counts on NA as colony forming units per gram dry soil

d = Colony size was measured after 24 h of incubation at 25 °C

TABLE A1.2. Characteristics of bacteria isolated from oil-contaminated soil

Isolate	Gram	Bacilli or cocci	Chain length (cells)	Spores
1	-	Bacilli	4-5	-
2	-	Bacilli	-	-
3	-	Coccobacilli	2	-
4	+	Bacilli	-	-
5	+	Bacilli	-	-
6	-	Bacilli	4-5	-
7.1	+	Bacilli	8	-
7.2	-	Bacilli	-	-
9	+	Coccobacilli	2	-
10	-	Bacilli	-	-
11	-	Bacilli	4	-

Appendix 2

Raw data from two experiments which represent the respiration of the microorganisms between days 0 & 78 and 96 & 169, respectively.

Explanation of abbreviations used in tables:

Time	-	Time (h)
ul/m O ₂	-	Oxygen consumption rate (μlmin^{-1})
ul O ₂	-	Cumulative oxygen consumption (μl)
ul/min CO ₂	-	Carbon dioxide production rate (μlmin^{-1})
ul CO ₂	-	Cumulative carbon dioxide production (μl)
ave	-	The mean value between two replicate samples, for example Channel 1 and 2
STDS	-	Standard deviation

C:\cro\Oxymax\data file v. 5.211
 Mon Nov 27 16 04 02 1995
 Sample interval 03 00 00
 Experiment duration Indefinite
 Number of active channels 9
 Sensor volume 180 ml
 Barometric pressure 650 mmHg
 Normalization units not normalized
 Sensor 1 O2 (1)
 Sensor 2 CO2 (2)
 Sensor 3 CH4 (3)

Experiment comments

Samples (50g) from a full scale landfarming operation

Channel	Head space	Sample
1	338	1 Day 0
2	336	1 Day 0
3	338	1 Day 26
4	338	1 Day 26
5	335	1 Day 56
6	340	1 Day 56
7	341	1 Day 78
8	340	1 Day 78
9	361	1 Blank

Invt	Cnst	Time	ave				STDS				ave				STDS			
			ulim O2	ulCO2	ulim CO2	ulCO2	ulimCO2	ulmaxO2	ulCO2	ulCO2	ulimCO2	ulmaxCO	ulCO2	ulCO2	ulCO2			
1	1	2	-1.281	-230.59	0.177	193.92	-1.2245	0.079903	-220.44	14.35427	1.066	0.015556	191.925	2.821356				
2	1	4	-0.91	-339.74	0.787	288.3	-0.982	0.101823	-338.255	2.100107	0.816	0.041012	289.615	2.142534				
3	1	8	-0.738	-438.27	0.672	368.97	-0.7895	0.072832	-432.96	6.632662	0.702	0.042426	374.07	7.212489				
4	1	8	-0.611	-501.59	0.611	442.31	-0.6565	0.078489	-512.935	16.04425	0.6258	0.020506	449.13	9.644936				
5	1	10	-0.552	-567.83	0.549	508.22	-0.576	0.033941	-582.03	20.08183	0.5535	0.006364	515.545	10.35911				
6	1	12	-0.554	-634.34	0.472	564.85	-0.586	0.045255	-652.38	25.51241	0.47	0.002828	571.97	10.0692				
7	1	14	-0.508	-695.24	0.431	616.58	-0.517	0.012728	-714.395	27.08926	0.429	0.002828	623.445	9.708576				
8	1	16	-0.292	-730.32	0.403	664.92	-0.317	0.035355	-752.465	31.31776	0.4	0.004243	671.43	9.20653				
9	1	18	-0.39	-777.07	0.32	703.37	-0.3915	0.002121	-799.425	31.61474	0.3145	0.007778	709.205	8.251936				
10	1	20	-0.441	-829.95	0.314	741.02	-0.459	0.025456	-854.505	34.72601	0.314	0	748.87	8.273149				
11	1	22	-0.291	-864.88	0.314	778.73	-0.317	0.03677	-892.56	39.14543	0.314	0	784.56	8.244865				
12	1	24	-0.302	-901.16	0.338	819.23	-0.327	0.035355	-931.79	43.31736	0.3405	0.003536	825.41	8.73584				
13	1	26	-0.395	-948.59	0.376	864.39	-0.407	0.016971	-980.66	45.35383	0.382	0.008485	871.245	9.694434				
14	1	28	-0.242	-977.62	0.315	902.2	-0.2715	0.041719	-1013.22	50.33893	0.314	0.001414	908.905	9.482302				
15	1	30	-0.315	-1015.46	0.273	935	-0.3355	0.028991	-1053.52	53.82497	0.2735	0.000707	941.77	9.574226				
16	1	32	-0.189	-1038.78	0.26	966.17	-0.228	0.055154	-1080.89	60.40106	0.2525	0.010607	972.07	8.34386				
17	1	34	-0.138	-1054.66	0.212	991.58	-0.1795	0.05869	-1102.4	67.48627	0.211	0.001414	997.395	8.223952				
18	1	36	-0.28	-1088.25	0.184	1013.65	-0.2985	0.026163	-1138.2	70.6329	0.182	0.002828	1019.215	7.870098				
19	1	38	-0.182	-1110.1	0.163	1033.17	-0.172	0.014142	-1158.85	68.93584	0.169	0.008485	1038.46	8.895403				
1	2	2 2	-1.168	-210.29	1.055	189.93												
2	2	4 2	-1.054	-336.77	0.845	291.33												
3	2	6 200	-0.841	-437.65	0.732	379.17												
4	2	8 200	-0.722	-524.28	0.64	455.95												
5	2	10 200	-0.6	-596.23	0.558	522.87												
6	2	12 200	-0.618	-670.42	0.468	579.09												
7	2	14 200	-0.526	-733.55	0.427	630.31												
8	2	16 200	-0.342	-774.61	0.397	677.94												
9	2	18 200	-0.393	-821.78	0.309	715.04												
10	2	20 200	-0.477	-879.06	0.314	752.72												
11	2	22 200	-0.343	-920.24	0.314	790.39												
12	2	24 200	-0.352	-962.42	0.343	831.59												
13	2	26 200	-0.419	-1012.73	0.388	878.1												
14	2	28 200	-0.301	-1048.81	0.313	915.61												
15	2	30 200	-0.356	-1091.58	0.274	948.54												

3	9 6 900	-0.084	-44.07	0.132	38.78
4	9 8 900	-0.183	-66.05	0.123	53.5
5	9 10 900	-0.156	-84.81	0.137	69.97
6	9 12 900	-0.143	-101.96	0.144	87.26
7	9 14 900	-0.164	-121.6	0.104	99.71
8	9 16 900	-0.082	-131.47	0.099	111.55
9	9 18 900	-0.114	-145.19	0.078	120.87
10	9 20 900	-0.064	-152.85	0.086	131.23
11	9 22 900	-0.056	-159.54	0.076	140.31
12	9 24 900	-0.067	-167.59	0.086	150.6
13	9 26 900	-0.086	-177.9	0.082	160.39
14	9 28 900	-0.087	-188.34	0.067	168.44
15	9 30 900	-0.064	-196.01	0.069	176.71
16	9 32 900	-0.08	-205.6	0.061	184.06
17	9 34 900	-0.041	-210.56	0.065	191.89
18	9 36 900	-0.042	-215.66	0.058	198.89
19	9 38 900	-0.1	-227.67	0.045	204.28

Micio-Oxymax data file (v5 31)
 Fri Dec 01 14:48:24 1995
 Sample interval: 02:00:00
 Experiment duration: Indefinite
 Number of active channels: 9
 Sensor volume: 178 ml
 Barometric pressure: 649 mmHg
 Normalization units: not normalized
 Sensor 1: O2 (1)
 Sensor 2: CO2 (2)
 Sensor 3: CH4 (3)

Experiment comments
 Soil samples (50g) from day 96 to 169 taken from a full scale land farming process

Channel	Head space	No of samples	Sample
1	340	1	Day 96
2	332	1	Day 124
3	334	1	Day 155
4	341	1	Day 155
5	336	1	Day 156
6	337	1	Day 156
7	338	1	Day 169
8	339	1	Day 169
9	360	1	Blank

Intv	Chnl	Time	Temp	RER	ul/m O2	ul O2	ul/m CO2	ul CO2	ave	STDS	ave	STDS	ave	STDS
									O2 rate	O2 rate	CO2 rate	CO2 rate	Cum CO2	Cum O2
1	1	2	25.9	0.68	-7.185	-1293.41	4.916	885						
2	1	4	25.7	0.7	-8.074	-2262.32	5.652	1563.24						
3	1	6	25.4	0.67	-8.716	-3308.29	5.868	2267.41						
4	1	8	25.2	0.7	-9.268	-4420.41	6.52	3049.78						
5	1	10	25	0.7	-10.468	-5676.57	7.276	3922.87						
6	1	12	24.9	0.66	-11.218	-7022.74	7.398	4810.67						
7	1	14	24.5	0.58	-10.63	-8298.4	6.134	5546.79						
8	1	16	24.4	0.6	-8.36	-9301.55	5.055	6153.36						
9	1	18	24.4	0.88	-3.368	-9705.75	2.967	6509.45						
10	1	20	24.4	0.87	-2.958	-10060.7	2.564	6817.17						
11	1	22	24.5	0.85	-2.804	-10397.2	2.394	7104.44						
12	1	24	25.4	0.86	-2.789	-10731.9	2.39	7391.23						
13	1	26	25.4	0.9	-2.708	-11056.8	2.44	7683.98						
14	1	28	25.4	0.85	-2.694	-11380.1	2.302	7960.19						
15	1	30	25.4	0.83	-2.619	-11694.4	2.173	8220.9						
16	1	32	25.4	0.84	-2.476	-11991.5	2.069	8469.13						
17	1	34	25.3	0.86	-2.324	-12270.3	1.99	8707.95						
18	1	36	25.1	0.84	-2.251	-12540.5	1.885	8934.12						
19	1	38	24.9	0.82	-2.171	-12801	1.789	9148.81						
20	1	40	24.8	0.81	-2.095	-13052.5	1.695	9352.2						
21	1	42	24.8	0.84	-1.99	-13291.3	1.664	9551.83						
22	1	44	24.9	0.83	-1.95	-13525.3	1.62	9746.2						
23	1	46	25.1	0.8	-2.029	-13768.7	1.632	9942.05						
24	1	48	26.4	0.79	-2.179	-14030.2	1.717	10148.11						
25	1	50	27.7	0.88	-2.254	-14300.6	1.978	10385.52						
26	1	52	27.3	0.84	-2.566	-14608.5	2.155	10644.14						
27	1	54	27.1	0.74	-2.606	-14921.2	1.94	10876.94						
28	1	56	27	0.77	-2.42	-15211.6	1.871	11101.47						
29	1	58	26.8	0.77	-2.348	-15493.3	1.813	11318.98						
30	1	60	26.6	0.79	-2.226	-15760.5	1.75	11528.96						
31	1	62	26.4	0.79	-2.147	-16018.2	1.687	11731.41						
32	1	64	26.4	0.74	-2.176	-16279.3	1.618	11925.61						
33	1	66	25.6	0.81	-1.952	-16513.6	1.577	12114.86						

28	3 56 300	27	0.85	-1.351	-4144.54	1.144	3623.7	-1.232	0.168291	-3859.08	-403.7085	1.0595	0.119501	3406.1	307.7329
29	3 58 300	26.8	0.83	-1.352	-4308.84	1.121	3758.21	-1.215	0.193747	-4004.88	-427.0359	1.0435	0.109602	3531.31	320.8851
30	3 60 300	26.6	0.8	-1.354	-4469.3	1.087	3888.63	-1.238	0.164049	-4153.41	-446.743	1.0035	0.118087	3651.75	334.9989
31	3 62 300	26.4	0.9	-1.208	-4614.24	1.093	4019.74	-1.0745	0.188798	-4282.35	-469.3704	1.0135	0.11243	3773.33	348.4764
32	3 64 300	26.1	0.76	-1.363	-4777.75	1.034	4143.77	-1.252	0.196978	-4432.57	-486.1582	0.957	0.108894	3888.12	361.5437
33	3 66 300	25.7	0.93	-1.12	-4912.11	1.038	4268.34	-1.034	0.121622	-4556.61	-502.76	0.9585	0.11243	4003.155	375.0282
1	4 2 400	26.4	0.72	-2.108	-379.53	1.51	271.77								
2	4 4 400	25.6	1	-1.337	-540.02	1.331	431.5								
3	4 6 400	25.3	0.88	-1.215	-685.84	1.068	559.7								
4	4 8 400	25.2	0.93	-1.061	-813.11	0.987	678.13								
5	4 10 400	25	0.91	-1.051	-939.24	0.958	793.03								
6	4 12 400	24.9	0.94	-0.978	-1056.63	0.921	903.58								
7	4 14 400	24.5	0.94	-0.932	-1168.44	0.872	1008.18								
8	4 16 400	24.4	0.91	-0.924	-1279.26	0.84	1108.94								
9	4 18 400	24.4	0.96	-0.862	-1382.73	0.83	1208.55								
10	4 20 400	24.4	0.96	-0.844	-1483.97	0.808	1305.52								
11	4 22 400	24.5	0.89	-0.898	-1591.71	0.8	1401.55								
12	4 24 400	25.6	0.89	-0.961	-1707	0.857	1504.39								
13	4 26 400	25.6	1.03	-0.877	-1812.19	0.899	1612.25								
14	4 28 400	25.4	0.9	-0.995	-1931.61	0.891	1719.21								
15	4 30 400	25.4	0.89	-0.986	-2049.94	0.879	1824.71								
16	4 32 400	25.4	0.89	-0.966	-2165.88	0.864	1928.36								
17	4 34 400	25.3	0.92	-0.931	-2277.56	0.852	2030.63								
18	4 36 400	25.1	0.96	-0.872	-2382.16	0.838	2131.21								
19	4 38 400	24.9	0.82	-0.972	-2498.8	0.794	2226.44								
20	4 40 400	24.8	0.91	-0.867	-2602.85	0.786	2320.72								
21	4 42 400	24.8	0.94	-0.837	-2703.25	0.788	2415.28								
22	4 44 400	24.9	0.85	-0.9	-2811.27	0.761	2506.65								
23	4 46 400	25.2	0.87	-0.907	-2920.06	0.792	2601.64								
24	4 48 400	26.5	0.88	-0.989	-3036.35	0.855	2704.22								
25	4 50 400	27.9	0.99	-1.017	-3158.44	1.004	2824.75								
26	4 52 400	27.3	0.95	-1.126	-3293.51	1.084	2952.43								
27	4 54 400	27.1	0.81	-1.221	-3440.03	0.993	3071.54								
28	4 56 400	27	0.88	-1.113	-3573.61	0.975	3188.5								
29	4 58 400	26.8	0.9	-1.078	-3702.92	0.966	3304.41								
30	4 60 400	26.6	0.82	-1.122	-3837.51	0.92	3414.87								
31	4 62 400	26.4	0.99	-0.941	-3950.45	0.934	3526.92								
32	4 64 400	26.3	0.77	-1.141	-4087.39	0.88	3632.47								
33	4 66 400	25.8	0.93	-0.948	-4201.1	0.879	3737.97								
1	5 2 500	26.5	0.77	-8.066	-1452.1	8.251	1125.28	-8.0285	0.053033	-1445.27	9.659079	6.149	0.14425	1108.955	25.91548
2	5 4 500	25.6	0.73	-10.407	-2700.94	7.562	2032.69	-10.518	0.156978	-2707.45	9.199459	7.58	0.025456	2016.545	22.83248
3	5 6 500	25.3	0.69	-10.801	-3997.1	7.411	2922.05	-10.9075	0.150614	-4016.37	27.2519	7.3775	0.047376	2901.87	28.53883
4	5 8 500	25.1	0.68	-9.987	-5195.48	6.793	3737.19	-10.0895	0.144957	-5227.09	44.70329	6.78	0.018385	3715.46	30.73086
5	5 10 500	25	0.86	-9.215	-6301.26	6.056	4463.97	-9.2945	0.11243	-6342.42	58.20903	5.9975	0.082731	4435.205	40.67985
6	5 12 500	24.9	0.64	-8.755	-7351.83	5.606	5136.66	-8.777	0.031113	-7395.64	61.9567	5.5165	0.126572	5097.155	55.86851
7	5 14 500	24.5	0.65	-8.154	-8330.27	5.284	5770.77	-8.182	0.039598	-8377.49	66.77209	5.1925	0.129401	5720.28	71.40364
8	5 16 500	24.4	0.65	-7.23	-9197.84	4.665	6330.57	-7.4635	0.330219	-9273.11	106.4408	4.7035	0.054447	6284.7	64.86999
9	5 18 500	24.4	0.88	-3.199	-9581.71	2.808	6667.56	-4.9155	2.427498	-9862.98	397.7688	3.5295	0.020355	6708.235	57.52314
10	5 20 500	24.4	0.99	-2.272	-9854.41	2.241	6936.5	-2.565	0.414365	-10170.8	447.4147	2.358	0.165463	6991.235	77.40598
11	5 22 500	24.5	0.97	-2.155	-10113.1	2.097	7188.09	-2.1555	0.000707	-10429.4	447.436	2.103	0.008485	7243.55	78.43228
12	5 24 500	25.5	0.96	-2.179	-10374.5	2.098	7439.88	-2.1805	0.002121	-10691.1	447.6905	2.106	0.011314	7496.26	79.73336
13	5 26 500	25.6	0.97	-2.127	-10629.7	2.063	7687.49	-2.161	0.048083	-10950.4	453.5171	2.0635	0.000707	7743.9	79.77579
14	5 28 500	25.4	0.91	-2.142	-10886.7	1.958	7922.44	-2.154	0.016971	-11208.9	455.5889	1.968	0.014142	7980.06	81.48999
15	5 30 500	25.4	0.9	-2.077	-11135.9	1.874	8147.28	-2.122	0.06364	-11463.5	463.261	1.884	0.014142	8206.11	83.19818
16	5 32 500	25.4	0.91	-1.98	-11373.5	1.798	8363.08	-2.025	0.06364	-11706.5	470.919	1.8185	0.028991	8424.345	86.64179
17	5 34 500	25.3	0.91	-1.902	-11601.8	1.733	8571.05	-1.9615	0.084146	-11941.9	480.9811	1.7525	0.027577	8634.645	89.93891
18	5 36 500	25.1	0.93	-1.793	-11817	1.668	8771.23	-1.887	0.132936	-12168.4	496.99	1.69	0.031113	8837.465	93.67044
19	5 38 500	24.9	0.85	-1.842	-12038	1.56	8958.44	-1.889	0.066468	-12395.1	505.0086	1.602	0.059397	9029.735	100.8264
20	5 40 500	24.8	0.91	-1.679	-12239.5	1.527	9141.63	-1.764	0.120208	-12606.8	519.3982	1.561	0.048093	9217.02	108.6176
21	5 42 500	24.8	0.9	-1.673	-12440.3	1.499	9321.56	-1.762	0.125865	-12818.2	534.4808	1.5375	0.054447	9401.525	113.0876

22	5 44 500	24.9	0.87	-1.666	-12640.1	1.451	9495.68	-1.741	0.106066	-13027.1	547.1946	1.4985	0.067175	9581.34	121.1415
23	5 46 500	25.2	0.88	-1.692	-12843.2	1.483	9673.68	-1.7775	0.120915	-13240.4	561.6973	1.53	0.066468	9764.945	129.0682
24	5 48 500	26.6	0.9	-1.757	-13054.1	1.574	9862.61	-1.854	0.137179	-13462.9	578.1305	1.6325	0.082731	9960.855	138.9394
25	5 50 500	28.2	0.94	-1.931	-13285.8	1.814	10080.33	-2.0725	0.200111	-13711.6	602.1085	1.874	0.084853	10185.76	149.1005
26	5 52 500	27.2	0.91	-2.064	-13533.5	1.871	10304.81	-2.218	0.217789	-13977.8	628.2714	1.933	0.087681	10417.68	159.6152
27	5 54 500	27.1	0.83	-2.079	-13783	1.73	10512.36	-2.218	0.196576	-14243.9	651.8252	1.7885	0.082731	10632.27	169.5783
28	5 56 500	27	0.86	-1.953	-14017.4	1.688	10714.95	-2.1025	0.211425	-14496.3	677.175	1.741	0.074953	10841.19	178.5303
29	5 58 500	26.8	0.85	-1.921	-14247.9	1.64	10911.7	-2.0535	0.187383	-14742.6	699.6892	1.69	0.070711	11043.94	187.0156
30	5 60 500	26.6	0.86	-1.839	-14468.6	1.589	11102.42	-1.9815	0.201525	-14980.4	723.8369	1.6415	0.074246	11240.94	195.8898
31	5 62 500	26.4	0.9	-1.719	-14674.9	1.548	11288.19	-1.875	0.220617	-15205.4	750.311	1.591	0.060811	11431.89	203.2154
32	5 64 500	26.3	0.8	-1.844	-14896.1	1.479	11465.71	-1.9575	0.160513	-15440.3	769.5797	1.5305	0.072832	11615.59	211.9623
33	5 66 500	25.9	0.88	-1.649	-15094.1	1.45	11639.71	-1.7895	0.198697	-15655.1	793.4092	1.4975	0.067175	11795.28	220.0021
1	6 2 600	26.6	0.76	-7.991	-1438.44	6.047	1088.63								
2	6 4 600	25.6	0.71	-10.629	-2713.95	7.598	2000.4								
3	6 6 600	25.3	0.67	-11.014	-4035.64	7.344	2881.69								
4	6 8 600	25.1	0.66	-10.192	-5258.7	6.767	3693.73								
5	6 10 600	25	0.63	-9.374	-6383.58	5.939	4405.44								
6	6 12 600	24.9	0.62	-8.799	-7439.45	5.427	5057.65								
7	6 14 600	24.5	0.62	-8.21	-8424.7	5.101	5669.79								
8	6 16 600	24.4	0.62	-7.697	-9348.37	4.742	6238.83								
9	6 18 600	24.4	0.64	-6.632	-10144.2	4.251	6748.91								
10	6 20 600	24.4	0.87	-2.858	-10487.2	2.475	7045.97								
11	6 22 600	24.6	0.98	-2.156	-10745.8	2.109	7299.01								
12	6 24 600	25.5	0.97	-2.182	-11007.6	2.114	7552.64								
13	6 26 600	25.6	0.94	-2.195	-11271.1	2.064	7800.31								
14	6 28 600	25.4	0.91	-2.166	-11531	1.978	8037.68								
15	6 30 600	25.4	0.87	-2.167	-11791.1	1.894	8264.94								
16	6 32 600	25.4	0.89	-2.07	-12039.5	1.839	8485.61								
17	6 34 600	25.3	0.88	-2.021	-12282	1.772	8698.24								
18	6 36 600	25.1	0.86	-1.981	-12519.8	1.712	8903.7								
19	6 38 600	24.9	0.85	-1.936	-12752.2	1.644	9101.03								
20	6 40 600	24.9	0.86	-1.849	-12974	1.595	9292.41								
21	6 42 600	24.8	0.85	-1.851	-13196.1	1.576	9481.49								
22	6 44 600	24.9	0.85	-1.816	-13414	1.546	9667								
23	6 46 600	25.3	0.85	-1.863	-13637.6	1.577	9856.21								
24	6 48 600	26.7	0.87	-1.951	-13871.7	1.691	10059.1								
25	6 50 600	28.3	0.87	-2.214	-14137.4	1.934	10291.19								
26	6 52 600	27.2	0.84	-2.372	-14422	1.995	10530.54								
27	6 54 600	27.1	0.78	-2.357	-14704.8	1.847	10752.18								
28	6 56 600	27	0.8	-2.252	-14975.1	1.794	10967.43								
29	6 58 600	26.8	0.8	-2.186	-15237.4	1.74	11176.18								
30	6 60 600	26.6	0.8	-2.124	-15492.2	1.694	11379.45								
31	6 62 600	26.4	0.8	-2.031	-15736	1.634	11575.58								
32	6 64 600	26.3	0.76	-2.071	-15984.5	1.582	11765.47								
33	6 66 600	25.7	0.8	-1.93	-16216.1	1.545	11950.84								
1	7 2 700	26.8	0.76	-4.77	-858.7	3.627	652.98	-4.761	0.012728	-857.05	2.333452	3.5565	0.099702	640.27	17.97465
2	7 4 700	25.5	0.67	-5.495	-1518.14	3.659	1092.06	-5.4455	0.070004	-1510.54	10.75509	3.6125	0.065761	1073.76	25.88011
3	7 6 700	25.3	0.62	-5.566	-2186.05	3.442	1505.14	-5.5195	0.065761	-2172.88	18.62519	3.385	0.08061	1479.955	35.61697
4	7 8 700	25.1	0.62	-5.774	-2878.9	3.558	1932.1	-5.7285	0.064347	-2860.3	26.31144	3.5065	0.072832	1900.765	44.31438
5	7 10 700	25.1	0.61	-6.121	-3613.46	3.757	2382.88	-6.043	0.110309	-3585.45	39.61919	3.7065	0.071418	2345.53	52.82088
6	7 12 700	24.9	0.6	-6.589	-4404.13	3.949	2856.81	-6.5005	0.125158	-4365.5	54.63814	3.903	0.065054	2813.9	60.6839
7	7 14 700	24.5	0.61	-6.91	-5233.35	4.219	3363.12	-6.8325	0.109602	-5185.38	67.8469	4.1635	0.078489	3313.51	70.15913
8	7 16 700	24.4	0.6	-7.52	-6135.69	4.517	3905.16	-7.418	0.14425	-6075.53	85.07909	4.4595	0.081317	3848.68	79.87478
9	7 18 700	24.4	0.59	-8.313	-7133.31	4.938	4497.7	-8.1945	0.167584	-7058.93	105.1963	4.888	0.070711	4435.25	88.31764
10	7 20 700	24.4	0.6	-9.064	-8221.02	5.456	5152.44	-9.032	0.045255	-8142.75	110.6905	5.39	0.093338	5082.085	99.497
11	7 22 700	24.6	0.6	-10.15	-9439.01	6.047	5878.11	-10.083	0.094752	-9352.7	122.0678	6.0285	0.026163	5805.505	102.679
12	7 24 700	25.5	0.69	-7.225	-10306	4.968	6474.23	-7.8225	0.844993	-10291.4	20.71116	5.2155	0.350018	6431.325	60.67683
13	7 26 700	25.6	0.94	-2.489	-10604.7	2.335	6754.4	-2.6295	0.198697	-10607.4	3.160767	2.4035	0.096874	6719.745	49.00957
14	7 28 700	25.4	0.93	-2.164	-10864.4	2.014	6996.05	-2.2395	0.106773	-10875.7	16.0089	2.051	0.052326	6965.855	42.70218
15	7 30 700	25.5	0.89	-2.042	-11109.5	1.808	7212.96	-2.0905	0.068589	-11126.6	24.22548	1.8265	0.026163	7185.03	39.49898

10	ε 20 900	24.4	1.97	-0.058	-99.22	0.115	98.19
11	ε 22 900	24.7	0.83	-0.144	-116.56	0.12	112.55
12	ε 24 900	25.6	0.56	-0.192	-139.63	0.107	125.37
13	ε 26 900	25.6	1.22	-0.086	-149.94	0.105	137.92
14	ε 28 900	25.5	1.62	-0.057	-156.82	0.093	149.08
15	ε 30 900	25.5	0.37	-0.155	-175.46	0.058	156.04
16	ε 32 900	25.4	84.79	-0.001	-175.56	0.075	165.05
17	ε 34 900	25.3	1.12	-0.039	-180.3	0.056	171.79
18	ε 36 900	25.1	0.53	-0.08	-189.87	0.042	176.83
19	ε 38 900	24.9	1.58	-0.032	-193.7	0.051	182.9
20	ε 40 900	24.9	-13.04	0.003	-193.29	0.045	188.33
21	ε 42 900	24.8	0.57	-0.056	-200.04	0.032	192.16
22	ε 44 900	24.9	1.21	-0.031	-203.78	0.038	196.7
23	ε 46 900	25.5	0.73	-0.046	-209.25	0.033	200.69
24	ε 48 900	26.9	2.62	-0.015	-211.07	0.04	205.45
25	ε 50 900	28.1	1.92	-0.023	-213.77	0.043	210.65
26	ε 52 900	27.2	0.53	-0.065	-221.52	0.034	214.73
27	ε 54 900	27.1	2.58	-0.016	-223.46	0.042	219.73
28	ε 56 900	27	0.47	-0.065	-231.3	0.031	223.39
29	ε 58 900	26.8	0.85	-0.039	-235.94	0.033	227.34
30	ε 60 900	26.6	0.6	-0.048	-241.74	0.029	230.82
31	ε 62 900	26.4	2.92	-0.013	-243.32	0.038	235.43
32	ε 64 900	26.3	0.31	-0.077	-252.57	0.024	238.29
33	ε 66 900	25.9	3.99	-0.009	-253.7	0.037	242.78

Appendix 3

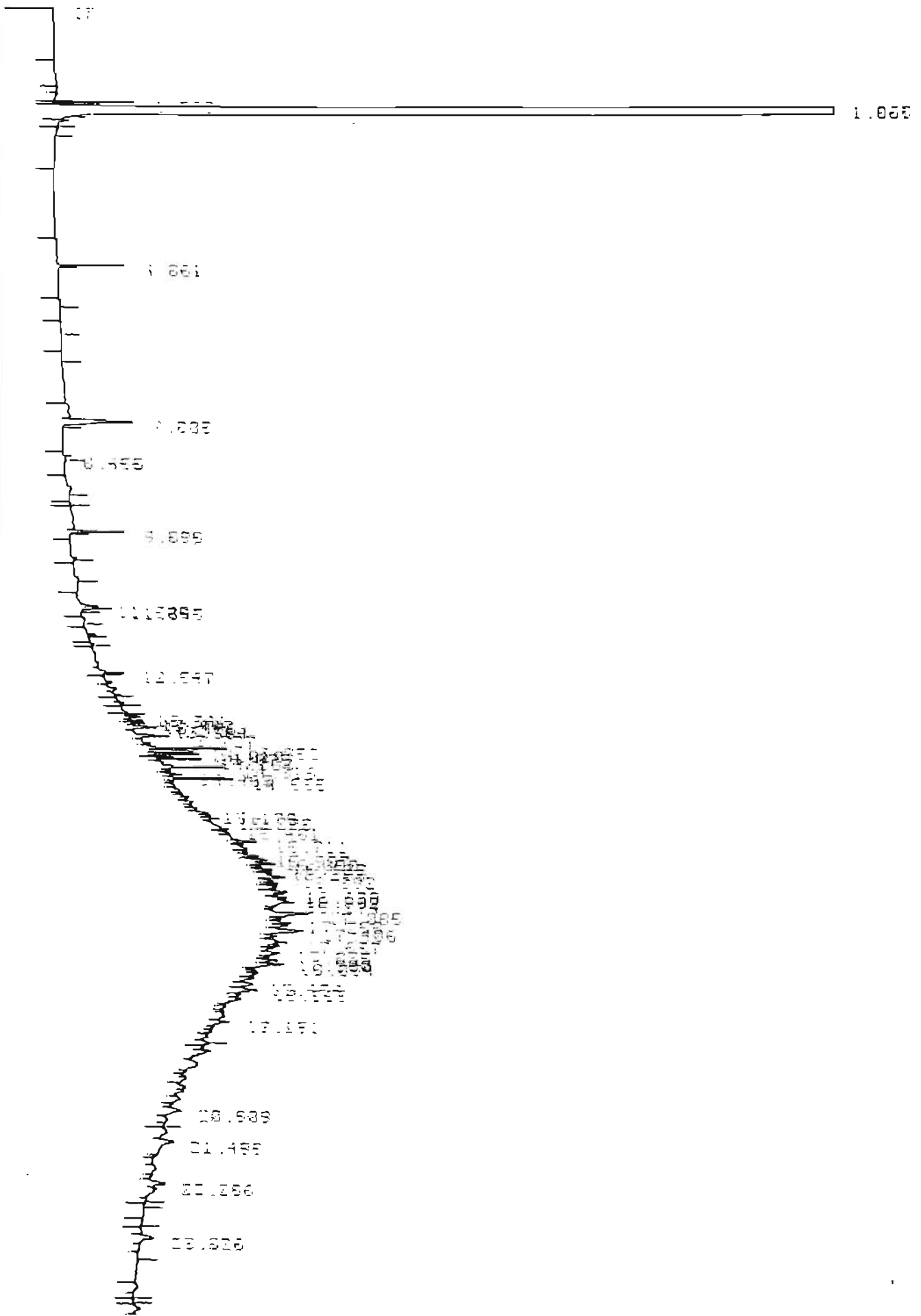
TABLE A3.1. Selected TPHC of samples during the pilot scale thermal bioventing, determined by the EPA 418/1 method

Time (wk)	THPC (% m/m)						
	CONT	CONT (B)	CONT (W:B)	CONT (W:A:B)	EXP (W:A:B:AS)	EXP (W:A:B:CB)	EXP (W:A:B:CM)
0	17.2					17.1	
1						10.1	
2		11.5	9.5	9.7	8.4	10.1	10.5
3						10.1	
4						11.2	
5						11.2	
6	12.3	12.7	8.1	6.2	7.7	9.1	8.7
13		12.5		6.5	4.4	6.1	4.5

B = Bulking agent (Corn cobs)
 A = Air
 CM = Chicken manure
 CONT = Control

W = Water
 CB = Commercial biosupplement
 AS = Dried anaerobically digested sludge
 EXP = Experiment

Appendix 4 A GC/FID chromatogram showing the contaminant with heavy hydrocarbon fractions

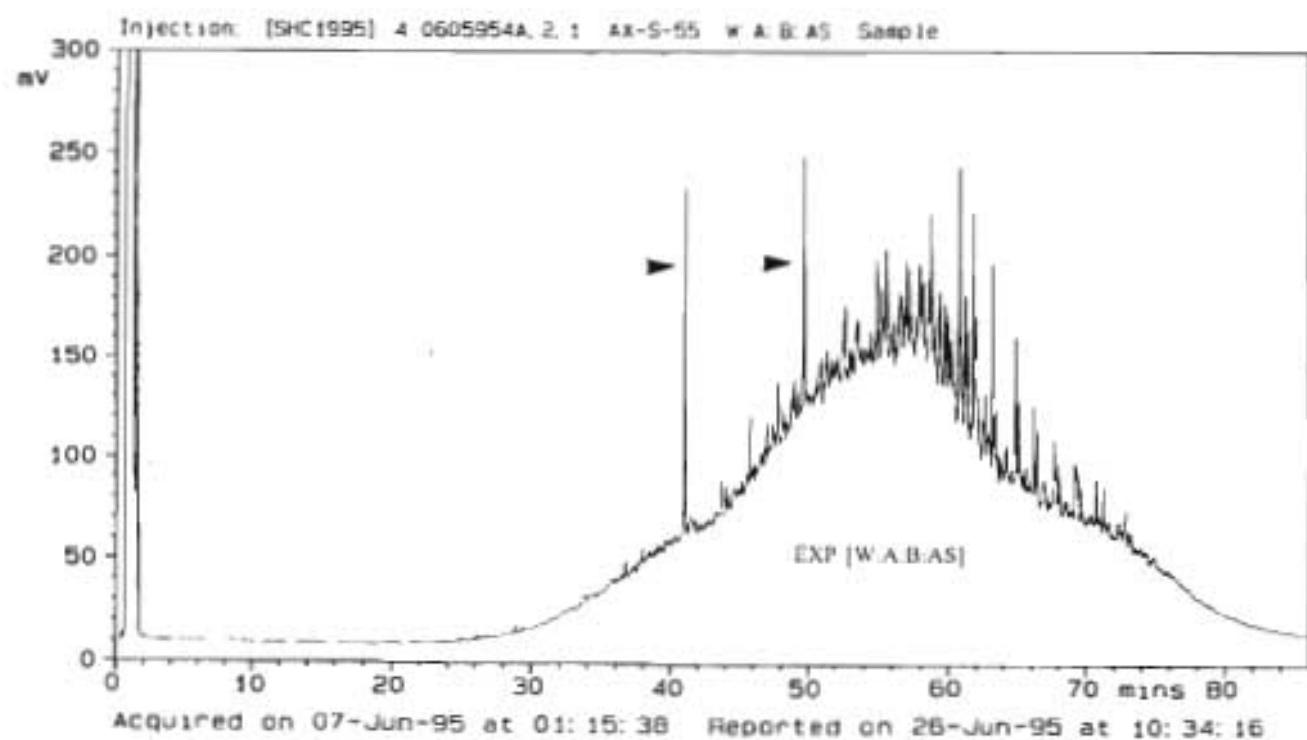
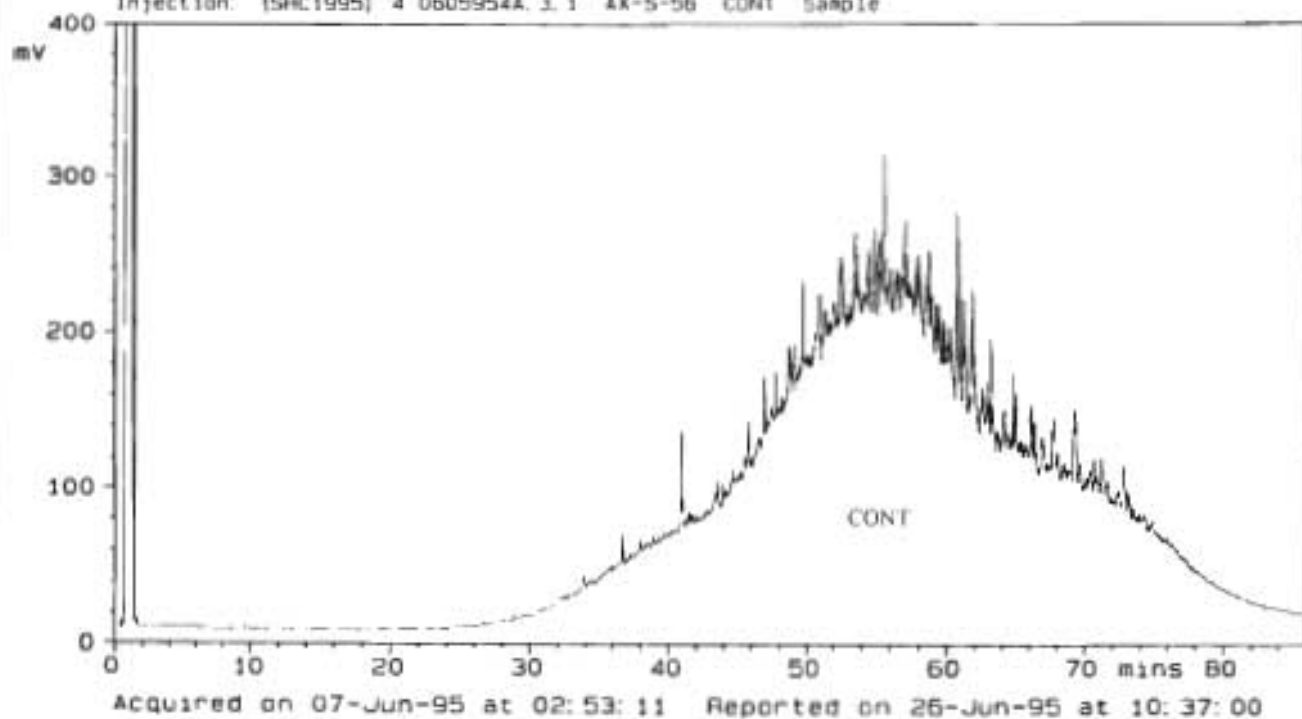


Appendix 5 GC/FID chromatograms of soil sample extracts taken from a reactor which received dried sludge as amendment and a control reactor after 10 wks of treatment

Arthur D Little Inc., Marine Sciences

Method: " RTX-5, 0.32 Co], GC#4, SHC and TPHC Analysis"

Injection: [SHC1995] 4 0605954A, 3.1 AX-S-56 CONT Sample



The arrows show two dominant peaks, which could be the phytane and pristane peaks.