# BIOREMEDIATION OF CREOSOTE-CONTAMINATED SOIL BY MICROBIAL INTERVENTION

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**DECLARATION**

The experimental work described in this thesis was carried out in the Department of Microbiology and Plant Pathology and the Department of Soil Science, University of Natal, Pietermaritzburg, under the supervision of Professor R. J. Haynes and Professor F. M. Wallis. These studies, except where indicated in the text, are the result of my own investigation.

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To determine optimal soil physical and chemical conditions for the bioremediation, 40g contaminated soil (>250 000mg $kg^{-1}$ creosote) in duplicate jars were separately amended with $NH_4NO_3$ and $K_2HPO_4$ to give five C:N ratios ( 25:1, 20:1, 15:1, 10:1 & 5:1) and one C:N:P ratio (10:1:2). The jars were sealed with parafilm to prevent water loss and incubated at 30°C in the dark for six weeks. A C:N ratio of 25:1 was found to be most effective in enhancing biodegradation under the prevailing experimental conditions. The effectiveness of N and P supplementation on microbial growth and degradation of creosote decreased as the ratio of N to C increased.

Pilot-scale landfarming was carried out on 25kg of contaminated soil (>250 000mg $kg^{-1}$ creosote) in each of eighteen reactors. Nine duplicate treatments were set up of which two served as controls. Of the two controls, one was amended with sodium azide to establish a sterile control and the other was set up as a natural control. Both were neither aerated or watered during the experimental period. The remaining seven treatments were watered and aerated regularly. Six of these treatments were amended with mono-ammonium phosphate (MAP) and separately amended with hydrogen peroxide, indigenous soil microbial-biosupplement, cow manure, poultry manure and sewage sludge. The experiment was run for 16 weeks. Although oxygen and moisture were important in the overall microbial degradation of creosote in the soil, addition of MAP, indigenous soil microbial-biosupplement, manures and sewage sludge significantly enhanced the degradation of creosote in the soil. However, there was no significant difference in the degradation of creosote in the treatments amended with indigenous soil microbial biosupplement, cow manure, poultry manure and sewage sludge. $H_2O_2$ did not significantly enhance degradation of creosote. In all treatments the lower molecular mass components of creosote were more readily removed than the higher molecular mass components.

The full-scale landfarming operation undertaken was a modified *in situ* technique in which the contaminated soil was treated in place. The underlying shale was reinforced with clay to increase its impermeability. The excavated topsoil, now with a higher creosote concentration of >310 000mg $kg^{-1}$ because of renewed wood treatment activity at the plant, was spread over the prepared bed and then amended with dolomitic lime, MAP to effect biostimulation, and subsequently with sewage sludge (which was readily available at no cost) to bioaugment the soils microbial population. The treatment was carried out for a period of 10 months. The full-scale landfarming operation reduced the creosote concentration from 310 186.8mg $kg^{-1}$ soil to 1762.5mg $kg^{-1}$ soil over the 10-month period. Although the concentrations of the higher molecular mass components of creosote tested (pyrene, chrysen, fluoranthene and benzo(a)pyrene) were reduced between 75% and 86%, some amount of these compounds persisted until the end of the treatment period. The combination of biostimulation and bioaugmentation significantly enhanced the removal of creosote from the soil. There was a positive correlation between soil microbial respiration rate, microbial population size and rate of creosote degradation. The clean-up target of reducing the initial creosote concentration to below 2 000mg $kg^{-1}$ was achieved by the end of the treatment period.

The persistence of some of the higher molecular mass components of creosote in the soil prompted investigations of other methods to degrade these contaminants. An *ex situ* treatment using indigenous soil fungi isolated from creosote contaminated soil in co-composting the contaminated soil with selected compostable materials was thus investigated. Although the fungi reduced the concentration of the higher molecular mass components in the experimental soil better (between 91% and 96% removal) than did the bacterial treatments (<70%), the fungal population could not be maintained at a large enough size for long enough to effect complete

degradation of the compounds on large-scale application. Also, the profuse production of airborne spores might constitute a health hazard in an open air treatment system.

Co-composting removed the higher molecular mass components effectively ( >99% removal). All the compostable materials investigated supported the growth of creosote degrading microbial populations without additional nutrient supplementation being necessary and there was no significant difference in the level of degradation of creosote by microorganisms present in the different compost materials. However, because of the higher concentration of creosote present in the soil used for this experiment, a longer period of time was required to completely remove the contaminant than was anticipated.

The results obtained from the modified landfarming and co-composting experiments showed that both were viable, cost effective technologies that can be applied to creosote contaminated soils under the KwaZulu-Natal conditions with possible wider application to polluted soils elswhere.

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## GLOSSARY OF TERMS AND ACRONYMS USED IN BIOREMEDIATION

| | |
|---|---|
| Aerobic degradation | The breakdown of organic compounds in the environment by microorganisms in the presence of oxygen. |
| Anaerobic degradation | The breakdown of organic compounds in the environment by microorganisms in the absence of oxygen. |
| Aromatic hydrocarbons | Organic compounds containing one or more benzene rings in their structure. |
| Bioaugmentation | The addition of non-native microorganisms to a site. |
| Biodegradation | Biological conversion of a complex compound to a simpler one. |
| Bioreactor | A reaction vessel that uses biological principles. Usually for treatment of contaminated media. |
| Bioremediation | Processes through which biological principles are used to remedy compromised environment. |
| Biostimulation | The stimulation of biological processes in the environment by the extraneous addition of nutrients and/or amendment of other parameters in the environment. |
| Biotransformation | Microbiologically catalysed transformation of a chemical compound to other products. |
| Bioventing | Circulation of air through the subsurface to remove volatile contaminants and provide oxygen, which stimulates microorganisms to degrade the remaining contaminant. |
| Co-composting | The composting of materials together with other compostable substances. |
| Co-metabolism | The metabolism of a substance by microorganisms growing on another substrate. The co-metabolised substrate may not serve as a growth substrate to the microorganism. |
| Composting | The process by which compost is produced i.e. organic waste materials are biologically converted to humus-like end-product. |

| | |
|---|---|
| Desorption | The dissolution of chemical substances from solid surfaces. |
| DNAPL | Dense non-aqueous phase liquid. |
| Electron donor | The compound or atom that gives up electrons in a chemical reaction. |
| Electron acceptor | Compounds or atoms that accepts electrons during a chemical reaction. |
| EPA | Environmental Protection Agency. |
| *Ex Situ* | The removal of materials from their original location for treatment in another location. |
| Fungal bulking | Large production of fungal mycelia for bioremediation purposes. |
| GC | Gas chromatography. |
| *In Situ* | The treatment of contaminated substances in their original location. |
| Intrinsic bioremediation | Bioremediation that occurs naturally without being artificially engineered. |
| IRS | Infra-red spectrophotometry. |
| Landfarming | An above ground bioremediation process that involves the spreading of the soil and the addition of nutrients to stimulate microbial action. |
| Microorganism | An organism of microscopic size that is capable growth and reproduction through degradation of other substances. |
| Mineralization | The conversion of organic molecules to their inorganic constituents. |
| NAPL | Non-aqueous phase liquid. |
| Oxidation | The transfer of electrons from a compound or atom to another during chemical reaction. Usually involve the addition of atom(s) or removal of hydrogen atom(s). |
| PAH | Polycyclic aromatic hydrocarbons. |
| PCB | Ploychlorinated biphenyls. |
| PCE | Perchloroethylene. |
| PCP | Pentachlorophenol. |
| Sorption | Collection of a substance to a solid surface by physical or chemical |

| | |
|---|---|
| | attraction. |
| Surfactant | Substances that have a hydrophobic and a hydrophilic end, which has the ability to emulsify fat or oil. |
| USEPA | United States of America Environmental Protection Agency. |
| VOC | Volatile organic compounds. |

# GENERAL INTRODUCTION

Creosote, a mid-temperature distillation product of coal tar, is a blend of about 400 individual compounds, including polycyclic aromatic hydrocarbons (PAHs), phenolic compounds and sulphur-containing heterocyclic compounds. Many of these compounds are known carcinogens, mutagens or irritants to the human body (AAFP, 1993; Kastner *et al.*, 1998). Other components of creosote have been associated with different disorders in human beings and other animals, including infertility (Brender *et al.*, 1994).

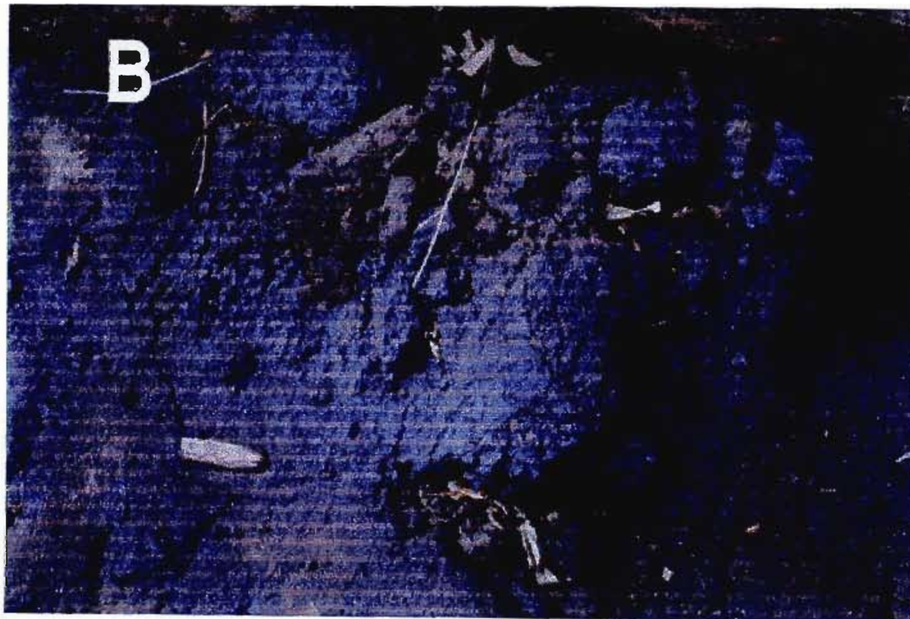
Creosote is commonly used all over the world as a wood preservative. In South Africa, the use of creosote for this purpose is widespread and has survived for decades. In fact the industry is continuing to grow. The proliferation of this industry has not only threatened the future use of creosote-contaminated soils for other purposes but also of nearby surface and groundwater. Contaminated soil have been found with >31% (w/w) creosote around treatment plants. The more water soluble creosote components are readily transported through the environment and may easily pollute surface and groundwater which may compromise the water supply to the local human and farm animal populations. This situation has been observed in a number of places in South Africa (Atagana *et al.*, 2000). In some of the cases, the local authorities cannot afford any sophisticated remediation systems. With this in mind, and the identification of a large number of sites contaminated with different chemical pollutants in the country (Pearce *et al.*, 1995), this study was undertaken to develop a suitable treatment protocol for the restoration of creosote contaminated environments, particularly in KwaZulu-Natal.

Prior to this study, no documented research on the bioremediation of a creosote-contaminated site in South Africa could be found. The data bases of the National Research Foundation (NRF), the Council for Scientific and Industrial Research (CSIR), the Department of Environmental Affairs and Tourism and the KwaZulu-Natal Department of Agriculture and Environmental Affairs provide no information on the biological treatment of creosote contaminated soil or water in South Africa. At the time of this study, there were no set standards for acceptable levels of creosote or its components in soil or water. Target clean-up standards during this study were based on standards set for other organic pollutants in countries such as the USA and the Netherlands.

The aim of this study was to develop a cost-effective remediation technology for the treatment of creosote-contaminated soils in South Africa. It was also aimed at providing information to the authorities, particularly those responsible for regulating environmental issues, and those who deal directly or indirectly with creosote or its components. Results from the present study would constitute a data base for other researchers interested in the remediation of creosote contamination in the environment.

The creosote treatment plant used for this study is located about 20km north of Pietermaritzburg, in the midlands of KwaZulu-Natal Province, South Africa. Apart from a short shutdown period (1994-1998), the factory has been in continuous operation for more than ten years, treating large amounts of wood with creosote. Creosote contamination at the factory site was particularly visible in areas where treated wood was left to dry and surrounding the creosote storage tanks. Another area of visible contamination was in the vicinity of a disused storage tank where a large pool of creosote

Plate 1 (a) Pool of creosote at the base of a disused creosote storage tank at factory site
(b) Congealed creosote in soil used in co-composting experiment at factory site.



(Plate 1a) could be seen and the adjoining land on which large amounts of congealed creosote had been dumped (Plate 1b).

After carefully assessing all the physical, chemical and biological conditions prevailing at the site, it was decided that a bioremediation programme incorporating a specially modified *in situ* landfarming operation would be appropriate and cost-effective. The projected study would include the following:

- an enrichment study to determine the presence of creosote catabolizing microorganisms in the contaminated soil;
- an assessment of the capacity of these organisms to degrade creosote and its components;
- assessing the possibility and cost-effectiveness of using surfactants to enhance the microbiological degradation of creosote;
- determination of the physical and chemical conditions of the soil and their suitability for biological treatment of the contaminated soil;
- implementation of a pilot-scale landfarming experiment to determine what nutrient amendments, if any, might be required for optimum degradation of the contaminating creosote;
- full-scale landfarming operation on the contaminated site using results from the pilot-scale experiment and other preliminary experiments.

# CHAPTER 1

# BIOREMEDIATION : A FUNCTIONAL BIOTREATMENT PRINCIPLE FOR LANDS CONTAMINATED WITH CHEMICAL POLLUTANTS

## 1.1 INTRODUCTION

Bioremediation describes processes whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by law (Mueller *et al.*, 1991; Diehl and Borazjani, 1998). Biological catalysts used to facilitate this process can include indigenous microbes and/or microbial inocula, which may be either enriched from the site or specially selected for their capacity to transform the target molecule (Mueller *et al.*, 1991). Bioremediation is thus a treatment technology involving the application of microbial processes to convert environmental contaminants into harmless substances (Bewley, 1992). It can also be described as the use of microorganisms or plants to detoxify an environment, by transforming or degrading pollutants (Bollag and Bollag, 1995).

Although generally referred to as a new technology, bioremediation has been practised for the past one hundred years or more with the routine use of microorganisms in the treatment and transformation of waste products and in the municipal waste water treatment industry. However, the application of microbiological processes to the remediation of soils, groundwater and sediment can be considered as recent developments (Baker and Herson, 1994; Bollag and Bollag, 1995). Similarly, the role of microorganisms responsible for the decomposition of plant and animal residues in the natural environment has long been recognized as an essential element for

the maintenance of life on earth. Thus the harnessing of these activities for the treatment of wastes that have been released into the environment as a result of man's activities is a natural extension of biotechnological processes which have been successfully practised for many years.

Biodegradation of individual compounds has been the subject of active research and development for more than forty years (Alexander, 1999). The initial interest was in the fate and persistence of pesticides in soil; however, the subject has expanded enormously in recent years to encompass a wide variety of chemicals and a broad array of issues (Alexander, 1999; Iranzo *et al.*,2001). As a rule, contaminant organic compounds are not found in the environment individually, but rather in simple or complex mixtures which may be associated with the release, storage, or transport of many chemicals in surface or groundwaters, waste-treatment systems, soils, or sediments (Alexander, 1999). Today, a large number of chemicals and a large number of different types of mixtures have been identified. Moreover, the concentrations of individual compounds vary appreciably. They may be higher than 1.0 g per litre of water, or per kilogramme of soil, at sites subject to spills from tank cars or trucks, to discharge of industrial wastes, or to leakages from storage or disposal facilities for industrial chemicals. In contrast, the concentration may be lower than 1.0 μg per litre of water, or per kilogram of soil, at some distance from the point of release, spill, or storage. Even at these low concentrations, some chemicals are toxic, and risk analyses suggest that exposure of large human populations to the low levels of these substances will result in deleterious effects to some individuals (Leland *et al.*, 2001;Weber *et al.*, 2001;Cook *et al.*,2002). In addition, some chemicals when present at low concentrations are subject to biomagnification and may reach levels that have harmful effects on human beings, animals, or plants. Technology has been developed that markedly enhances biodegradation or that results in microbial destruction of organic pollutants that otherwise would persist at the sites of

contamination. These bioremediation technologies have led to the clean-up of many polluted groundwater and soils and they have fostered the development of a new bioremediation industry (Alexander, 1999).

Biodegradation is carried out by microorganisms in many different types of environments. Sewage treatment systems, soils, underground sites for the disposal of chemical wastes, groundwater, surface water, oceans, sediments and estuaries are of particular relevance for pollutants and potential pollutants ( Alexander, 1999). Microbial processes in the various kinds of aerobic and anaerobic systems used for treating industrial, agricultural and municipal wastes are extremely important because these treatment systems represent the first point of the discharge for many chemicals into environments of importance to human beings or other living organisms . (Alexander, 1999).

Natural microbial communities or individual species in different habitats have the capability to metabolize, and often mineralize, a large number of organic molecules, including virtually all natural products, regardless of their complexity. This accounts for the lack of accumulation in excessively large amounts of natural organic compounds in the environment over the millions of years of existence of life on earth. While one species may metabolize only a small number of substances, other species in the same habitat are able to make up for its deficiencies. Thus, no one organism is sufficiently omnivorous to destroy a large percentage of natural chemicals that are formed by plants, animals and other microorganisms. For biodegradation to take place in an environment, several conditions must be met (Alexander, 1999; Yateem *et al.*, 2002). These include: (1) an organism must exist that has the necessary enzymes to bring about the biodegradation; (2) that organism must be present in the environment containing the chemical;

(3) the chemical must be accessible to the organism having the requisite enzyme; (4) if the initial enzyme bringing about the degradation is extracellular, the bonds acted upon by the enzyme must be exposed for the catalyst to function; (5) should the enzymes catalyzing the initial degradation be intracellular, the molecule must penetrate the surface of the cell to the internal sites where the enzyme acts; (6) because the population or biomass of bacteria or fungi acting on many synthetic compounds is initially small, conditions in the environment must be conducive to allow for proliferation of the potentially active microorganisms.

Bioremediation is often an appropriate choice of treatment process for many industrial and toxic or hazardous wastes, both before and after they are discharged into the environment. Bioremediation processes are natural and final, but the natural biodegradation rate must be accelerated and optimized at contaminated sites in order to reduce the pollutant concentration to an acceptable level within a reasonable, finite, time period. Anaerobic biodegradation processes are orders of magnitude slower than aerobic processes and are much more difficult to accelerate, optimize and control (Anweiler *et al.*, 2001). In addition, anaerobic processes often terminate at some intermediate metabolic step and are in danger, therefore, of producing terminal intermediate metabolites equally as toxic as the parent pollutant (Carberry, 1994 ).

In order to optimize a biodegradation process, three important properties must be determined: the kind of pollutant(s) that are to be treated, the matrix that contains the pollutant(s) and the type of microbe that will be used to degrade the target chemical(s). A number of factors have been identified to slow the biodegradation of substances in the environment. These include low temperatures, low nutrient concentrations, especially phosphorus and nitrogen, low oxygen concentration, low concentrations of hydrocarbon-utilizing microorganisms and high

concentrations of hydrocarbon contaminants (Baker and Herson, 1994; Alexander, 1999). Water content is important in the biodegradation processes in the soil. Atlas and Bartha (1972) showed that the optimum hydration level for microbial degradation of pollutant compounds was between 50 and 70 % of the water holding capacity of the soil. They showed that higher saturation levels interfere with aerobic degradation processes and that lower saturation levels inhibit water transport.

The process of bioremediation comprises three phases: (1) site characterization and the assessment of feasibility; (2) the management of the remediation process; and (3) the definition of the active remediation end-point. Collectively, this procedure is directed towards reducing the risks associated with the site to an acceptable level and restoring the quality of the site to a level determined by its proposed subsequent use (Smith and Mason, 1999).

## 1.2 MICROBIOLOGY AND BIOREMEDIATION

Microbial degradation accounts for much of the loss of organic chemicals from soil. Whether or not a chemical is adsorbed, absorbed, activated, inactivated, persistent, short-lived, mobile or stationary, or will eventually constitute a residue problem, may depend upon its transformation by soil microorganisms (Kaufman,1983; Buzea and Destefanis, 1999). While many microorganisms are capable of degrading a large number of xenobiotics, many of these chemicals have failed to support microbial growth and are hence resistant to biodegradation. This resistance has led to the introduction of the concepts of molecular recalcitrance, microbial fallibility, and cometabolism (Baker and Herson, 1994; Alexander, 1999; Iranzo *et al.*, 2001).

Biodegradation of organics in the natural environment is carried out mainly by bacteria and fungi. Bacteria represent a widely diverse group of prokaryotes with ubiquitous distribution throughout the biosphere. Bacteria possess several characteristics which allow them to be successful. These characteristics include rapid growth and metabolic rates, genetic plasticity and the ability to adjust rapidly to a variety of environments (Talaro and Talaro, 1993; Baker and Herson, 1994; Prescot *et al.*, 1999). These factors have contributed to the success of microorganisms in bioremediation.

The growth of microorganisms in the natural environment can be limited by numerous factors. Biotic interactions, such as competition between different bacterial strains for the same substrate (Tate, 1995; Alcamo, 1997; Prescot *et al.*, 1999) or predation ( Tate, 1995; Alexander, 1999), can function to keep population numbers low. It has been reported by Goldstein *et al.* (1985) that protozoan predation on a strain of *Pseudomonas* capable of degrading 2,4-dichlorophenol was responsible for the failure of the introduced organisms to persist in non-sterile sewage.

In addition microbial population size may be limited by the abiotic environment . The most important abiotic factors limiting bacterial populations from the perspective of bioremediation, are water content, temperature, pH, the presence of toxic materials such as metals, type and amount of organic material present, electron acceptors and inorganic nutrients such as nitrogen and phosphorus (Baker and Herson, 1994; Bollag and Bollag, 1995; Kuyukina *et al.*, 2001). The growth and reproduction of bacteria depend on an adequate supply of water. The absolute amount of water present in the soil is not the amount of water available for use by soil microorganisms, because part of it may be adsorbed to solid surfaces or may be absorbed by high solute concentrations. Thus, a measure of the physiologically-available water, i.e. water activity

( $a_w$) or water potential, will be more adequate in considering the amount of water available to the microorganisms in soil and the environmental effect of water on microorganisms. Microorganisms require water activity of between 0.900 and 1.000 to survive (Brock and Madigan, 1991), although there are exceptions such as the halophile, *Halobacter*, which is capable of growing in the Dead Sea ( $a_w$ = 0.700) (Oren,1988). Water activity below 0.900 typically limits bioremediation (Baker and Herson,1994; Iranzo *et al.*, 2001).

An increase in temperature typically increases biological activities up to a maximum temperature, above which enzymes are denatured. This leads to inhibition of cell function and death. The temperature response of microorganisms shows a decidedly skewed pattern, with maximum activity occurring at a temperature only slightly below the lethal temperature. Bacteria are known to tolerate a wide temperature range. Microorganisms have been reported to grow at temperatures below $0^{\circ}$C to above $100^{\circ}$C in the presence of an adequate supply of water (Atlas and Barther, 1987). Based on temperature tolerance, microorganisms are classified into three broad groups namely psychrophiles, mesophiles and thermophiles. Psychrophiles are those whose optimum temperatures of activity vary between $5^{\circ}$C and $15^{\circ}$C. Mesophiles have their optimum temperature for activity between $25^{\circ}$C and $40^{\circ}$C and thermophiles have their optimum temperature between $40^{\circ}$C and $60^{\circ}$C. Within the tolerable temperature ranges, microbial activity usually increases by a factor of 2 to 3 for each $10^{\circ}$ C increase in temperature, up to the optimum temperature of the microorganism (Atlas and Bartha,1987). Most bioremediation research has been conducted in mesophilic conditions (Baker and Herson,1994). However, evidence of biodegradation of contaminants in psychrophilic and thermophilic conditions has been documented (Zobell and Agosti,1972; Merkel *et al.*, 1978; Parr *et al.*, 1983; Woodward, 1990). For example, a strain of *Corynebacterium* isolated from petroleum-contaminated soil in the

Antarctica was shown by Kerry (1990) to be able to degrade hydrocarbons at $1^{0}$ C . Williams *et al.* (1988) have also shown that thermophilic microorganisms can degrade explosives in soil-compost systems operating at $55^{0}$ C.

Generally, most microorganisms thrive within the pH range of 6.0 to 8.0, with fungi being slightly more acid tolerant than most common soil and aquifer bacteria (Alexander 1999; Cerniglia *et al.*, 1979). The degradation of petroleum hydrocarbon has been reported to be optimum at a soil pH of 7.8 (Cerniglia *et al.*, 1979; Cerniglia, 1997). Under acidic conditions, soil fungi have an advantage over bacteria in metabolizing contaminants. However, the metabolic pathways for degradation are different in fungi and bacteria (Cerniglia *et al.*, 1979). Fungal decomposition of polycyclic aromatic hydrocarbons (PAH) may produce mutagenic intermediates, as was reported by Cerniglia *et al.* (1979). Liming may thus be required to increase the soil pH so that bacterial growth is favoured and such intermediates are not produced. An increase in soil pH has been found to reduce the availability of calcium, magnesium, sodium, potassium, ammonium, nitrogen and phosphorus, while a decrease in soil pH results in decreasing availability of nitrate and chloride (Sharpley, 1991).

Contaminated sites are frequently impacted by a mixture of waste materials rather than a single compound. In such sites, the presence of high levels of metals, in addition to organic compounds, can be inhibitory or toxic to microorganisms (Beppu and Arima, 1969; Foster, 1983; Baker and Herson, 1994; Schaefer, 2000). Although some metals are necessary in trace amounts to enhance microbial growth, elevated levels of metals can disrupt the cell membrane and denature cellular proteins, leading to cell death (Beppu and Arima, 1969; Foster, 1983). Organic contaminants in the environment are principally degraded by chemoheterotrophic

microorganisms (organisms requiring preferred organic compounds as carbon and energy sources)(Stanier *et al.*, 1986). Within this large group of microorganisms some species are very limited in the number of organic compounds which they can metabolize (e.g. lactic acid bacteria which can metabolize only a few simple organic compounds) while others, such as *Pseudomonas* spp., are capable of metabolizing over 90 different organic compounds as their sole source of carbon and energy (Stanier *et al.*, 1986; Yateem *et al.*, 2002) .

Microorganisms that degrade xenobiotics in natural systems are dependent on fixed forms of nitrogen ( $NH_3$, $NO_3^-$, $NO_2^-$ and organic nitrogen) to meet their nitrogen requirements. These forms of nitrogen are frequently limiting for microbial populations in soil, ground water and surface water (Atlas, 1991). Microbial synthesis of ATP, nucleic acids and cell membranes require phosphorus. However, because of the low solubility of phosphates in water, phosphorus is frequently limiting to bacterial growth in both aquatic and soil environments (Atlas and Bartha, 1972).

Pre-exposure of a microbial population to organic compounds increases the rate of degradation of the compounds by the microbial population. This type of adaptation has been observed in soil and water systems (Spain *et al.*, 1980; Pfaender *et al.*, 1985; Wilson *et al.*, 1985; Aelion *et al.*, 1987; Barkey and Pritchard, 1988; Bourquin *et al.*, 2001). Although the exact mechanism responsible for this adaptation is not known, factors such as enzyme induction, genetic alteration of the population and physiological acclimation to stress conditions have been proposed as important phenomena (Wyndham, 1986; Schmidt *et al.*, 1987; Wiggins *et al.*, 1987). With industrial sites being commonly known to be chronically exposed to low levels of contaminants, it is likely that adaptation of the microorganisms at the site will have already occurred prior to

initiation of a bioremediation project. Therefore the indigenous microorganisms should be capable of a more rapid degradation of contaminants than microorganisms from a pristine site (Baker and Herson, 1994; Baeseman and Novak, 2001).

## 1.3 MICROBIAL NUTRITION AND ENVIRONMENTAL REQUIREMENT

All biochemically synthesized compounds are potentially biodegradable and all sites contaminated with such materials potentially can be treated by indigenous microorganisms (Alexander, 1981; Barry King *et al.*, 1997). The challenge is to harness the metabolic potentials of the microorganisms effectively. For a contaminant to be degraded, environmental parameters and nutritional factors must be considered. The mineralization, or complete degradation, of an organic molecule is mostly a consequence of microbial activity. Only a few abiotic mechanisms in nature totally convert compounds of any kind to inorganic products. Mineralization sequences characterize the microbial metabolism of several classes of synthetic compounds (Alexander,1981; Barry King *et al.*, 1997; Breedveld and Sparrevik, 2000). Much of the organic carbon is usually used up in building cell constituents of the degrading organism, with an increase in biomass and population size. There is a consequent release of energy and $CO_2$. If none of the products of mineralization is toxic or of environmental concern mineralization often leads to detoxification (Alexander,1981; Baker and Herson, 1994; Alexander, 1999; Baeseman and Novak, 2001).

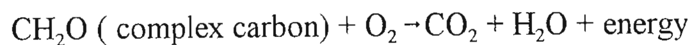
A complex combination of environmental factors in a habitat dictates the survival strategies of its microbial residents. These include nutrient and energy sources, ambient temperature, amount of moisture present, presence or absence of gases, osmotic pressure, pH, presence or absence of

light or irradiation and other organisms (Talaro and Talaro, 1993). For microbial growth to be sustained, a number of elements called essential nutrients must be present in the soil at various concentrations. These nutrients include nitrogen, phosphorus, potassium, sulphur, iron, calcium, manganese, zinc, copper, cobalt and molybdenum. These nutrients are grouped into two categories: the macronutrients, which are required in relatively large amounts by the organisms, and micronutrients, also called trace elements, which are required in trace quantities. Supplementation of soils with trace elements is not often necessary, since they are usually present in sufficient concentrations and are rarely limiting factors. Typically, a bacterial cell will consist of (w/w) 50% carbon, 14% nitrogen, 3% phosphorus, 2% potassium, 1% sulphur, 0.2% iron and 0.5% each of calcium, magnesium and chlorine (Alexander,1977). For nitrogen to be available to soil microorganisms, it must, in most cases, be present in inorganic form (i.e. ammonium or nitrate) (Swindell *et al.*,1988). However, certain species can utilize ammonia or nitrogen gas (Tate, 1995). Phosphorus is available in the form of orthophosphate (Alexander, 1999).

Elemental uptake by microorganisms is by absorption and transportation of solubilised molecules across the cell membrane. Thus the availability of target molecules to the microorganisms is dependent on the amount of water present in the treatment matrix. Optimal water content for aerobic bioremediation of soil is usually between 10 and 20% by mass (Hinchee and Arthur, 1991). Over a wide range of soil water contents (e.g. 30 to 90 % of water holding capacity) biodegradation rates are often little-affected (Hinchee and Athur, 1991). However, water-logging may promote anoxic conditions, thereby reducing aerobic bioremediation efficiency.

## 1.4 PATHWAYS USED BY MICROORGANISMS TO METABOLIZE XENOBIOTICS

Bacteria break down complex organic compounds through a series of coupled chemical reactions called catabolism. Respiratory bacteria use glycolysis, the Krebs cycle and the electron transport system as the central pathways for metabolism (Baker and Herson, 1994). An overall, simplified representation of these reactions can be given as:



$$CH_2O \text{ ( complex carbon)} + O_2 \rightarrow CO_2 + H_2O + \text{energy}$$

This representation shows the complete conversion of a complex carbon compound, called the substrate, to carbon dioxide and water (Baker and Herson,1994).The process is called mineralization. Energy attained in the form of ATP is used for a variety of cellular activities, including synthesis of new cell components, and motility. Incomplete degradation, called transformation, may sometimes occur. This can result in production of substances of more serious environmental concern than the original substrate. An example of this is the production of vinyl chloride, a carcinogen, during the anaerobic transformation of trichloroethylene (Baker and Herson, 1994). Microorganisms may also produce bioemulsifiers or surfactants which can help to increase the mobility and bio-availability of the substrate. These substances play very important roles in the degradation of compounds that are usually strongly sorbed to soil particles (Baker and Herson,1994; Kimball,1994; Sanseverino *et al.*,1994; Dick *et al.*, 2001). The mineralization of a compound is dependent on its convertibility into one of the compounds that is involved in the central metabolic pathways.

Bacteria are very diverse in the mechanisms used to achieve mineralization. In spite of this, the recent understanding of the role of microbial consortia in the degradation of substances in the environment has lead to the development of the theory of 'division of labour' among members

of the consortia, with the metabolic product of one becoming the substrate of another (Baker and Herson, 1994). Co-metabolism, which is the concomitant transformation of a non-growth substrate by a microorganism, also referred to as co-oxidation, has come to be accepted as an important phenomenon in degradation (Baker and Herson, 1994; Alexander, 1999). For example, microorganisms which are not capable of growing on chlorinated aromatic compounds may be able to carry out limited transformations of these compounds in the presence of the non-chlorinated analogues. This phenomenon is most easily explained as a case of fortuitous degradation resulting from a low specificity of degradative enzymes (Baker and Herson, 1994). Thus the structural similarity between the xenobiotic compound which is co-metabolized and the metabolic substrate may be sufficient for enzymes with low specificity to use both as a substrate, resulting in the transformation and partial degradation of the xenobiotic ( Dagley, 1984).

In spite of the fact that many soil microorganisms exist that have the potential to degrade organic contaminants, a large number of such compounds persist in the natural soil environment. The persistence of such compounds in the soil may be attributed either to factors relating to the contaminant itself, such as inherent chemical recalcitrance, concentration and low bio-availability, or to the nature of the recipient environment (physical, chemical or biological) (Bewley, 1992).

Bioremediation technologies can be broadly classified into two groups: *ex situ* and *in situ* (Baker and Herson, 1994). *Ex situ* technologies involve physical removal of contaminated soil to other areas where treatment can be carried out. Landfarming, bioreactors, composting and some forms of solid-phase treatment are examples of *ex situ* techniques. *In situ* techniques involve the treatment of contaminated materials in place, for example bioventing and biostimulation. Some

of the very commonly used bioremediation technologies include:

(i) Bioaugmentation; which is the addition of bacterial cultures to a contaminated medium as is commonly used in bioreactors and *ex situ* systems;

(ii) Biofilters; which involve the use of microbial stripping columns to treat air emissions;

(iii) Biostimulation; which involves the stimulation of the indigenous microbial population in soils or ground water. This can be carried out in both *in situ* and *ex situ* systems;

(iv) Bioreactors; this is biodegradation in a container or reactor which may handle soil slurries or liquid media;

(v) Bioventing; this involves the passage of oxygen through the soil to stimulate microbial growth and activity;

(vi) Composting; this is an aerobic, thermophilic treatment process involving the mixing of contaminated material with a bulking agent in a static pile, aerated pile or continuously fed reactor.

(vii) Landfarming; this is a solid-phase treatment system for contaminated soil which can be done *in situ* or in a constructed soil treatment cell.

The success of all bioremediation techniques depends on having the right microorganisms in the right place with the right environmental conditions. The microorganisms may pre-exist in the environment or may be introduced exogenously. The main elements of bioremediation technology where significant developments have taken place include microorganism application, nutrient addition, addition of other chemical reagents and engineering technology (Baker and Herson, 1994; Bollag and Bollag,1995; Alexander,1999).

## 1.5 TYPES OF BIOREMEDIATION

There are two basic approaches to bioremediation currently in practice: the microbiological approach and the microbial ecology approach. Although the microbiological approach has become more popular, the microbial ecology approach is considered to be more useful for the majority of contaminant situations (Piotrowski, 1991).

### 1.5.1 The microbiological approach

This approach involves the augmentation of contaminated sites with species of contaminant-specific microorganisms, referred to as "superbugs" ( Piotrowski, 1991). This is intended to enhance biodegradation as a result of the increased density of the contaminant-specific degraders. There are two means of achieving this. The first involves the use of prepackaged contaminant-specific degraders that have been specifically selected due to their inherent or induced capability to degrade the contaminants concerned. This type of "pre-packaged superbug" is available from a number of commercial producers. They are usually cultured in large numbers from contaminated sites, stressed to degrade a specific contaminant at elevated concentrations and preserved in stable conditions to allow for easy application to contaminated soil or water. These microorganisms are usually selected through different techniques, including traditional enrichment techniques and mutation by use of mutagenic or ionizing radiation and genetic engineering (Piotrowski, 1991).

The second means of achieving the microbiological approach is the selection, culture and

application of site-specific strains that exhibit desirable biodegradative qualities. In this method, contaminated soil or water is sampled periodically for microbiological analysis to select strains with enhanced degradative potentials on the contaminant involved (Poitrowski, 1991). Studies of nutritional requirements of the selected strains are also carried out. Mass culture of the selected strains is followed by their subsequent dispersion at high density into affected sites, along with the required nutrients. This method has the advantage of using site-specific strains, which are presumed to be capable of persisting at the site better than pre-packaged strains which may have been derived from another location (Piotrowski, 1991).

The microbiological approach has the major advantage of rapidly increasing the density of contaminant-specific microorganisms in the affected medium (Zitrides, 1978; Barles *et al.*, 1979; Kilbane *et al.*, 1983). However, the use of pre-packaged and site-specific "superbugs" have their shortcomings. Prepackaged microorganisms have been found to have difficulty in becoming established in contaminated sites in competition with pre-existing microbial populations (Piotrowski, 1991; Baker and Herson, 1994; Alexander, 1999). Low contaminant concentration in the field relative to the concentration that the added species are adapted to in the laboratory, predation by the indigenous species and the presence of naturally occurring compounds that inhibit the growth of the added microorganisms are some of the factors that have been observed to limit the establishment of the microorganisms (Lewandowski, 1986). The indigenous species are also thought to out-compete the added species for the available organic substrate. These limitations can lead to repeated applications being necessary and this increases the cost of remediation (Lewandowski, 1986). In some cases, "Superbugs" have also been observed not to produce significant increases in biodegradation, compared with indigenous species (Lewandowski, 1986; Hill *et al.*, 1989). It has also been observed that the length of time a

microorganism stays in isolation reduces its fitness and its potential to readapt when it is reintroduced into the natural environment (Piotrowski, 1991).

The limitations of the pre-packaged "superbugs" led to the practice of using site-specific strains. This method, however, has its own disadvantages, some of which are similar to those of the pre-packaged microorganisms (Piotrowski, 1991). Isolation conditions and media produce isolates that become laboratory adapted and may not fit or effectively carry out the function required in the contaminated environment, upon reintroduction. It is also known that plating does not yield an accurate assessment of the entire community as only 10% of the total microbial population in a soil sample is recovered on plating (Alexander, 1977). This situation can give opportunistic species an advantage. Another limitation in the use of "superbugs" is that in the natural environment microorganisms coexist with numerous species in a consortium , with the different organisms acting as competitors and predators, or synergistically with respect to the metabolic activities necessary for degradation of the pollutant organic compounds. Microorganisms are also known to attach to aquifer sediment surfaces, thus making them unavailable (Hill *et al.*, 1989). Most often, a large number are localized at the region of injection because of their attachment to the surface (Lewandowski, 1986; Hill *et al.*, 1989).

Regulatory legislation has also taken its toll on "superbug" application. Both "superbugs" and genetically engineered microorganisms (GEM) are thought to have negative impacts on, and create hazards in the environment. As a result, regulations and permits are required for their release into the environment (Piotrowski, 1991).

### 1.5.2 Microbial ecology approach.

This is basically the identification and adjustment of certain physical and chemical factors that may impede the rate of biodegradation of the contaminant by a naturally occurring microbial community in the environment. Adequate adjustment of these parameters has been shown to result in degradation at satisfactory rates (Piotrowski, 1989). In this approach the emphasis is on limiting factors in the environment rather than on the identification of the microbiological content of the medium (Piotrowski, 1991). Because this approach does not involve the application of microorganisms, it is of little or no legislative significance (Piotrowski, 1991). It also avoids the common drawbacks of the microbiological approach such as competition, fitness, and persistence. Furthermore, it suits *in situ* aquifer remediation since it only involves the addition of chemicals such as oxygen, hydrogen peroxide or inorganic nutrients through the aquifer. It is also cost effective. However, there are several limitations to the approach. It may take some time for the indigenous species to develop a metabolic capability to degrade the contaminating molecules. Contaminant concentration may be too high and become toxic to the microorganisms in new spills (Dibble and Bartha, 1979; Bartha and Bossert, 1984; Sims *et al.*, 1986; Baker and Herson, 1994; Lajoie and Strom, 1994; Alexander, 1999). In other situations, the level of the contaminant in the soil or water may be too low for effective degradation, yet higher than local or regional regulatory bodies permit. With the recommended maximum allowable contaminant concentrations generally becoming lower (with increasingly lower detection limits), the microbial ecology approach may become unacceptable for remediation (Piotrowski, 1991). Because of slower action, this method may also be unable to meet the requirements for urgent clean-up, since the time required by this method is often not predictable and usually longer than the microbiological approach.

Regardless of the exact nature of the treatment technology, all bioremediation techniques depend on having the right microorganisms in the right place, with the right environmental conditions for degradation to occur. The right microorganisms are those bacteria or fungi which have the physiological and metabolic capabilities to degrade the contaminants. In many instances these organisms will already be present at the site as an indigenous community. In other situations there may be the need to introduce the microorganisms exogenously, as is the case with bioreactors handling high concentrations of toxic materials. Once the right microorganisms are present in the right place, the environmental conditions must be controlled or altered to optimize the growth and metabolic activity of the microorganisms. Such environmental factors as temperature, inorganic nutrients ( primarily nitrogen and phosphorus), electron acceptors (oxygen, nitrates and sulphates) and pH can be modified to optimize the environment for bioremediation.

Bioremediation as a treatment option has a number of advantages over other remediation techniques such as land-filling and pump-and-treat methods. One advantage is on-site applicability. This not only reduces costs as a result of eliminating transportation but also minimizes site disruption, thus allowing continued use of the site while treatment is being carried out. The waste is completely decomposed to carbon dioxide and water, thus permanently eliminating the waste from the environment and also the long-term liability associated with non-destructive treatment methods. However, some chemicals are not readily susceptible to biodegradation e.g. highly chlorinated compounds and metals. Sometimes microbial biodegradation may lead to the production of more toxic or mobile substances than the parent compound. For example, under anaerobic conditions, trichloroethylene (TCE) undergoes a series of biologically mediated reactions, resulting in the sequential removal of chlorine atoms from the molecules by reductive dehalogenation to produce vinyl chloride (VC), a carcinogen (Baker

and Herson, 1994). Thus, if bioremediation is applied without an understanding of the microbial processes involved, it could worsen environmental conditions. As a result of this, the initial cost for site assessment, characterization and feasibility assessment may be higher in bioremediation than in other methods.

## 1.6 BIOREMEDIATION TECHNOLOGIES

Different technologies are available for the remediation of contaminated sites. Some of the technologies have more applications than others, while some are specialized or contaminant-specific. Treatment technologies are governed by a number of factors. These include the nature of the contaminant, the extent of contamination, site characteristics, clean-up goals and economics (Piotrowski, 1991). Thus, no one remedial technique can be suitable for all sites, contaminants or situations. Each site needs to be evaluated according to its requirements. The proper design, implementation and operation of a bio-engineered treatment process requires a multi-disciplinary approach to effectively characterize the hydrology, geology, chemical and microbiological characteristics of the locale (Piotrowski,1991). Biological treatment offers the distinct advantage of partial or complete destruction of the contaminants, as opposed to simply transferring the pollutant to another phase of the environment (Troy *et al.*,1994). As previously stated, bioremediation technologies are generally practised under two broad groupings. These are the *in situ* techniques, which involve treating the contaminated soil at the point of contamination, and *ex situ*, which involves moving the soil to another location for treatment.

### 1.6.1 *In situ* bioremediation technologies

*In situ* bioremediation is usually carried out where it is uneconomical or impossible to excavate soil or move the contaminated material to another location for treatment. Typically, the objective of *in situ* bioremediation is to manage the potential of the contaminated site, including the biological entities present in the soil and the nutrients available to them, to achieve bioremediation with minimal disturbance to the site. The success of an *in situ* operation depends on the characteristics of the site such as its hydrology, climatic factors, soil type and properties, microbiological presence and concentration of the pollutant. Hydrological characteristics that are important in *in situ* bioremediation include heterogeneity of the soil, direction and rate of flow of ground water and the depth to the water table (Sims *et al.*, 1990).

#### 1.6.1.1 Preliminary site investigation

A preliminary study of the site is aimed at acquiring information on the history of the pollution. This information includes, among other things, the type of chemical contaminating the soil, the age of the contamination and the type of soil being contaminated. This historical assessment is followed by bioassessment testing, which is a feasibility study for the bioremediation operation. The objective of the present study is to determine a number of factors that will promote or inhibit bioremediation in the soil. These factors will include microbial population, nutrient availability, soil factors such as pH, soil type, moisture content, temperature and availability or use of electron acceptors (Baker and Herson, 1994; Alexander, 1999). The most common form of *in situ* treatment is the bioremediation of contamination within the saturated zone of the soil (Wilson and Jones, 1993). Some of the *in situ* bioremediation methods that have been used include soil washing,

low temperature thermal treatment, soil venting, bioventing, enhanced bioreclamation, bioslurping and passive remediation (Wilson and Jones, 1993).

**(i) Soil Washing:** This entails the injection of a synthetic surfactant or solvent into the contaminated zone to promote release of hydrophobic contaminants to the aqueous phase (Hoeppel *et al.*, 1991). The success of this method depends on the choice of surfactant, as some surfactants will hamper degradation of certain pollutants since they are preferred as growth substrates.

**(ii) Low Temperature Thermal Treatment:** This involves the injection of heated gas or steam into the soil. These methods are costly due to the energy and equipment involved and are therefore not routinely used to treat hydrocarbon spillages ( Hoeppel *et al.*, 1991).

**(iii) Soil Venting:** This also known as soil vapour extraction or *in situ* soil stripping. It involves the controlled flow of air saturated with the volatile and semi-volatile contaminants from the soil under the influence of a vacuum applied to the vadose zone. Soil venting increases the rate of diffusion into the subsurface which, in turn, helps to satisfy the vacuum. Consequently, the air is able to diffuse through the soil, displacing the fractions with higher vapour pressures and stimulating aerobic degradation of poorly volatile compounds (Hinchee *et al.*, 1991). This is particularly significant in soils with low water permeability because of the greater diffusivities of gases compared with liquids. Since air contains > 200 000 mg $L^{-1}$ of oxygen, soil venting can overcome the oxygen deficits that often occur in heavily contaminated soils ( Connor, 1988; Lees, 1996). On escape from the soil, the gas may be treated either to recover or to destroy the contaminants, for example by condensation, adsorption by activated carbon, biofiltration or

thermal treatment (Hoeppel *et al.*, 1991; Goldfarb and Vogel, 1994). Its low cost and potential to remove volatile organic compounds has made it attractive to operators.

**(iv) Bioventing:** Although soil vapour extraction and bioventing are described as the same technology, there are considerable differences in the processes. Bioventing is an improvement on vacuum extraction since the flow of air through the soil is increased to speed the removal of organic compounds. Degradation is, therefore, enhanced by the indigenous microorganisms *in situ* (Hoeppel *et al.*, 1991; Torma, 1994). Unlike soil vacuum extraction technologies, bioventing attempts to stimulate the biodegradative activity while minimizing stripping of volatile organics (Sayles *et al.*, 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-gases. The limitations of bioventing are mainly issues of control factors. Overpressurizing the sparge system constitutes a major concern, as it can displace both vapours and water and cause the dispersion of contaminated vapours to uncontaminated or low pressure areas such as building basements (Brown *et al.*, 1993). Thus the design, implementation and control of an air sparging system must be executed with the utmost care.

**(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 (Hoeppel *et al.*, 1991). Two methods are commonly used: landfarming and bioaugmentation. These techniques can also be employed *ex situ* (Snyman, 1996). Landfarming as an *in situ* technology attempts to enhance the activity of naturally-present microorganisms by the addition of suitable nutrients which are limiting and/or increasing their numbers (Bradford

and Krishnamoorthy,1991; Mason *et al.*,1992). Bioaugmentation involves the introduction of specially adapted or engineered microorganisms, selected for their ability to degrade a particular compound, into the contaminated soil (Mason *et al.*, 1992). Engineered microorganisms are seldomly used in remediaton practices because of legislation against their use by many governments and the fact that indigenous microorganisms are known to have the potential for catabolizing many contaminants. Landfarming is simple and cost effective, but has disadvantages of the ever-increasing problem of availability of land, particularly for off-site treatment, and a serious potential for contaminating water, air and soil (Savage *et al.*,1985).

**(vi) Bioslurping:** This is a new approach to remediation at petroleum-release sites which have free-phase light non-aqueous-phase liquid contamination (Snyman,1996). Bioslurper systems are designed to recover the free product via vacuum-enhanced pumping, while simultaneously initiating the remediation of the vadose zone soil via bioventing ( Kittel *et al.*,1995).

**(vii) Passive Remediation:** This is also called intrinsic bioremediation. It is born out of the recognition that biological, physical and chemical processes are constantly operating in nature. In nature, many contaminants undergo degradation over a period of time and reach innocuous levels without artificial enhancement. Passive remediation should not be confused with "no-action" alternatives, since it entails substantial site reviewing and assessment, analytical investigations and monitoring (Nelson, 1994).

**(viii) Anaerobic *in situ* Bioremediation:** Few attempts have been made at commercial anaerobic bioremediation. This is because aerobic biodegradation is more energy-efficient. However, it is obvious that anaerobic conditions are prevalent in soil and aquifer environments and a wide array of reaction mechanisms, including reduction, hydrolysis, dealkylation and dehalogenation, are

possible in the absence of molecular oxygen (Lees,1996). Research indicates that anaerobes are much more nutritionally diverse than previously imagined and are capable of catalyzing novel biotransformations which are of environmental and commercial interest ( Suflita *et al.*, 1988). Many compounds such as polychlorinated biphenyls (PCBs), chlorinated solvents and pesticides have been shown to be degraded only under anaerobic conditions. Thus, anoxic conditions may be of potential use in bioremediation programmes where the redox potential could be selectively adjusted to favour the degradation of a particular contaminant and/or where pH adjustment may be made to promote sulphate reduction and denitrification (Lee *et al.*,1988; Ramanand *et al.*, 1993).

In typical aerobic systems, oxygen constitutes the major electron acceptor. Thus the growth of heterotrophic microorganisms in such a system, using the contaminating organic molecule as the sole source of carbon, results in the rapid depletion of oxygen. This situation generates a selection pressure which encourages the use of alternative electron acceptors such as nitrates, sulphates and carbonates in the resulting anoxic condition (Hoeppel *et al.*, 1991). As a consequence of self-generating redox gradients, a spatial separation of dominant metabolic processes can be observed, depending on the availability of electron acceptors, the presence of suitable microorganisms and the energetic benefit of each process to the microbial community. Typically, nitrate reduction occurs first, followed by sulphate reduction and then methanogenesis (Hoeppel *et al.*, 1991). Different types of biodegradative activities can be observed within depth-related redox zones. Research has shown that it may prove possible to stimulate desirable metabolic sequences through the intentional introduction of electron donor and/or acceptor combinations (Kaake,1992; Ramanand *et al.*, 1993).

*In situ* anaerobic treatment may be very slow and not very efficient in meeting remediation targets. However, it is believed that anaerobic biodegradation of pollutants can offer a potential advantage over aerobic bioremediation approaches ( Semprini *et al.*, 1991). The cost-effectiveness of aerobic biotreatment methods is accounted for by the cheap provision of air, ozone, hydrogen peroxide, or pure oxygen. Unfortunately these treatments are also known to produce biofouling. Anaerobic processes, by contrast, are low energy producers and therefore generate less biomass, which limits biofouling of the system. In addition, anaerobic biotransformation sometimes results in metabolic products which are less toxic and more amenable to subsequent aerobic metabolism (Lees,1996).

### 1.6.2 *Ex situ* bioremediation 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 contaminated intertidal zones on a coast would be rapidly washed away from the site of the pollution. In some cases it may be necessary to remove the contaminated soil or 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* treatment can be made *ex situ* by excavating the contaminated soil and treating it elsewhere (Snyman,1996). There are cases where vessels or reactors such as slurry reactors or soil columns are used. 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 *et al.*, 1994). Britto *et al.* (1992), for example, developed an effective continuous flow bioreactor treatment for petroleum-contaminated soils.

#### 1.6.2.1 Bioreactors

Bioreactors represent the most highly engineered, and hence most highly controlled approach to bioremediation. Bioreactor technology is broad in application and is competitive and innovations are still being made. The quest of researchers in this field is the development of a universal bioreactor which is capable of coping with all bioremediation situations. The bioreactor is thus a reaction vessel which has a system for delivering oxygen and nutrients and devices for thorough mixing and the adjustment or maintenance of pH. The reactor could be run in a batch or continuous mode (Piotrowski, 1991).

Bioreactors depend on water to achieve an aqueous matrix which facilitates their delivery systems. They can provide primary, secondary or tertiary treatment. They have the added advantage of being mobile and can be used on site. Two of the major disadvantages of bioreactors are that excavation of soil or pumping of ground water is necessary and, during treatment, they all produce a certain amount of sludge or biomass, and/or a volume of off-gas which must be correctly disposed of. These, unfortunately, considerably increase treatment costs (Piotrowski, 1991; Lees,1996).

There are a number of bioreactor types used in commercial soil and groundwater treatments. They include:

**(i) Slurry Reactors:** In this system, the contaminated soil is excavated, mixed with water to form a slurry and treated in a constructed reactor. The reactor contents are agitated to promote breakdown of soil aggregates, enhance desorption of contaminants from soil solids, increase contact between the wastes and microorganisms and enhance oxygenation of the slurry ( Baker and Herson, 1994). Sometimes, soils are pretreated before introduction into the reactor. The soil may be physically sized and graded to a small enough mesh size to allow for maintenance of an acceptable slurry. Silts, clays and sludge are most suitable for this type of treatment, as the soil particles must be smaller than 60 mesh (King *et al.*, 1992), so that an adequate slurry with at least 30% aqueous medium can be maintained against gravity. This reduction in size not only reduces the costs associated with mixing and agitating the final slurry, but may also reduce the total volume which needs to be treated. This is because many organic contaminants such as PAHs have a high affinity for clay minerals and consequently sizing and grading can increase the rate ofbiodegradation of the contaminants ( Portier, 1989). Such homogenization and other treatments have been reported to enhance efficiency in slurry reactors ( Bachmann and Zehnder, 1988; Black *et al.*, 1991).

Since bioreactors operate at near-ideal conditions for biodegradation, contaminant removal proceeds at rapid rate and therefore offers significant advantages compared with conventional techniques such as landfarming (Catallo and Potier, 1992). However, as with other methods, the biokinetics depend upon the type of contaminant, its concentration and the treatment standard which must be attained. The degree of treatment of any one chemical is a function of its solubility in water and its rate of biodegradation. Typical treatment times in a slurry bioreactor vary from less than one month to more than six months (Ross, 1991).

**(ii) Thermophilic Bioreactors:** These operate at temperatures ranging from 50 to 80$^{O}$C, which are not usually considered in the application of aerobic biological treatment, although recent reports have suggested that high temperatures offer potential benefits for the destruction of organic molecules. High temperature conditions increase the aerobic metabolic rate of the system and consequently reduce the size required for an operational reactor. Secondly, thermophiles have less than one tenth the normal generation time of mesophiles, thus reducing significantly the amount of biomass sludge for disposal (Usinowicz and Rozich, 1993). This system can be used as an alternative to aerobic biological treatment for sludges or sediments that are highly contaminated with organic compounds, hot streams containing biodegradable organics and groundwaters heavily contaminated with organics. As bacterial metabolic activities are typically exothermic, research has shown that aerobic reactors operate in a thermophilic range as long as the chemical oxygen demand (COD) is at least 30 000 mg L $^{-1}$ , the reactor is insulated and covered to capture the heat generated and a relatively efficient aeration system is provided (Usinowicz and Rozich, 1993).

**(iii) Vapour Phase Bioreactors (Biofilters) :** Biofiltration has become an accepted technology for treating volatile organic compounds such as gas emissions during *in situ* bioremediation, composting and bioreactor off-gas and odour-containing industrial exhausts ( Duncan *et al.*, 1982; Prokop and Bohn, 1985). The technology has been proved to be economical for high volume emissions with low contaminant concentration and it has low energy consumption and low maintenance requirements. In a typical biofilter, microorganisms are immobilized as biofilms on the packing of solid support materials. The vapour carrying the pollutant is drafted through the colonized support matrix and the microorganisms catabolize the organics in the vapour. The system has the dual advantages of being an effective, destructive process, without the problems

associated with typical landfilling or regenerative methods. There are two types of biofilter, namely soil filter and the treatment bed or disc (Holusha,1991). In soil filters, contaminated air is passed through a nutrient-supplemented compost pile which facilitates catabolism by indigenous mesophilic bacteria. In the treatment bed, the waste air stream and filter are humidified, as gas is passed through one, two or more beds of compost, wood chips, refuse, sand or diatomaceous earth. Spent filters can be used as fertilizer as they present no hazard. Biofilters are in use throughout Europe for receiving and detoxifying emissions containing different organic compounds (Standefer and van Lith, 1993).

**(iv) Submerged Fixed-Film/Plug Flow Reactor:** This type of reactor is effective and adaptable. It can be used to treat low concentrations of organics and concentrations as high as 1000mg $L^{-1}$ (Hamoda and Al-Haddad, 1989). Its success lies in its design flexibility. It can withstand extreme fluctuation in organic loading while maintaining an active biomass for long periods of time (Lewandowski, 1990). The critical factor is the C:N:P ratio, which ideally should be 100:5:1 to effect biodegradation ( Lees, 1996).

## 1.7 RECENT DEVELOPMENTS IN BIOREMEDIATION OF ORGANIC POLLUTANTS

Recent advances in the understanding of how bacteria biodegrade organic pollutants under aerobic conditions has led to the development of remediation systems that can dramatically reduce clean up costs of contaminated sites (Nishino and Spain, 2001). The enhancement or acceleration of bioremediation has been the subject of active research and discussion in the last five years. The following areas of have attracted attention: enhanced aerobic and anaerobic bioremediation and phytoremediation. Of these areas, aerobic bioremediation has been most

studied probably because of the understanding that the initial steps of biodegradation of organic compounds by microorganisms, including bacteria and fungi, involves the oxidation of the substrates by oxygenase and by recognition of oxygen as a limiting factor in many natural environments (Duffy *et al.,* 1999). This notwithstanding, some advances have been made in the area of anaerobic bioremediation of different contaminants in the environment (Koenigsberg and Farone, 1999; Annweiler *et al.*, 2001; Sullivan *et al.*, 2001).

The delivery of molecular oxygen, being the electron acceptor in bioremediation under aerobic conditions, has become the focus of many bioremediation research (Alshawabkeh, 2001; Murray, 2001; Zahiralesamzadeh, 2001). The methods of delivery that have been studied include aeration of well pores, injection of hydrogen peroxide, venting and air sparging (Muniz *et al.,* 2001). Previous studies have shown that down-gradient of the source area, elevated dissolved oxygen levels and intrinsic aerobically-degrading microbes appeared to promote natural attenuation processes of chemical degradation of organic compounds to non-toxic end points such as carbon dioxide (Larson and Voegeli, 2001).

Researches on the delivery of molecular oxygen has led to the development of special formulations designed to slowly but continuously release oxygen for the oxidation of contaminated aquifers and soil. One of these developments is the Oxygen Release Compound (ORC)$^{(R)}$(Buzea and Destefanis, 1999; Muniz *et al.*, 2001). ORC is a formulation of magnesium peroxide that produces a slow and sustained release of molecular oxygen when in contact with soil moisture or groundwater. Thus naturally occurring microorganisms that aerobically degrade pollutants thrive in the oxygen rich environment. The use of ORC to restore contaminated groundwater is more cost-effective than other alternative technologies (Hicks *et al.*, 2001; Larson and Voegeli, 2001; Muniz *et al.*, 2001).

As a result of the relatively few successes associated with anaerobic bioremediation, researches in this area has been slow as compared with aerobic bioremediation. However, some advances have been made in anaerobic bioremediation (Londry *et al.*, 1997; Koenigsberg and Sandefur, 2000; Shrout and Parkin, 2000). A recent development in this area is the development of the Hydrogen Release Compound (Koenigsberg and Farone, 1999). The compound, a polylactate ester formulated to slowly release lactic acid upon hydration, is used to stimulate rapid degradation of chlorinated solvent contaminants often found in ground water and soil (Bourquin *et al.*, 2001; Murray *et al.*, 2001; Zahiraleslamzadeh and Bensch, 2001).

The bioremediation technologies that have received wide attention recently include landfarming (Hansen *et al.*, 2000; Harris, 2000; Kuyukina, *et al.*, 2001) and compost bioremediation (Goldstein, 2001; Leland *et al.*, 2001;Meyer and Steinhart, H, 2001). These two technologies have been employed in the treatment of soils contaminated with large concentrations of pollutants under different environmental conditions.

**(i) Landfarming**

Landfarming has proved to be successful in reducing the concentration of contaminants in the soil to accepted levels on different occasions (USEPA, 1998). Kuyukina *et al.* (2001) employed a combination of landfarming and slurry-phase biological treatment techniques to reduce the concentration of crude oil in contaminated soil from 200 000 mg $kg^{-1}$ to about 1500 mg $kg^{-1}$ in about fifteen weeks. Hansen *et al.* (2000) used landfarming techniques to reduce a complex mixture of PAHs and PCP from about 14 500 mg $kg^{-1}$ to about 8500 mg $kg^{-1}$ in six months. The wide disparity in the results of the two experiments can be largely attributed to the complexity

of the substrate, a wide range of environmental factors and the experimental procedures employed. Harris (2000) reported the achievement of Record of Decision (ROD) level of 4.5 mg $kg^{-1}$ PAHs and PCP from initial concentrations of 200 mg $kg^{-1}$ and 220 mg $kg^{-1}$ in ninety days. Landfarming has been applied to the remediation of sites contaminated with different chemical pollutants, such as explosives and chlorinated hydrocarbons, with varying degrees of success(USEPA, 1997, 1998; Pachon, 2001). However, many higher molecular mass PAHs continued to persist in reasonable quantities at the end of each treatment operation (USEPA, 1998). The total removal of these compounds from contaminated soils and groundwater has continued to be the subject of further research in the past five years. The present research is aimed at attempting to address this problem.

**(ii) Composting**

The ability of the composting process to degrade soil contaminants has been demonstrated in projects and research trials for a number of years (Woodhull *et al.*, 1999; Goldstein, 2001). Most of the reports on compost biorenmediation of contaminated soil has been focussed on contaminations of relatively low concentrations (Potter *et al.*, 1999; Reid *et al.*, 1999). However the potential of the compost system to breakdown complex chemical pollutants has continued to be explored, even at higher concentrations (Barnes *et al.*, 2000; Gray *et al.*, 2000). The inclusion of composting as one of the treatment processes for remediation of superfund sites by USEPA office of Solid Waste and Emergency Response is a clear recognition of the potentials of composting as a bioremediation process. In 1999, the composting of 200 000 tons of soil contaminated with TNT, DNT, Tetryl and RDX was commissioned at the Joliet Army Ammunition Plant in Illinois (Goldstein, 2001). Soil contaminated with 140 000 mg $kg^{-1}$ of

refined petroleum products was treated by the Brown's Compost Company and a remedial level of 4000 mg $kg^{-1}$ was achieved in 20 days (Goldstein, 2001). Other reports on the use of composting in remediating contaminated soil include the composting of soil contaminated with 3155 mg $kg^{-1}$ of the herbicide toxaphene (Gray *et al.*, 2000) and the composting soil contaminated with 10 000 mg $kg^{-1}$ TNT within 18 days to remedial levels of 3.8 mg $kg^{-1}$(Barnes *et al.*, 2000).

The direct application of non-composted materials in a compost system, referred to as co-composting has attracted attention in the bioremediation industry (McGugan, 1997). Current research by researchers in Olds College in Alberta is focussed on co-composting of drilling mud contaminated with about 14% (w/w) oil with fresh manure (Goldstein, 2001). The challenges of co-composting are however, the slow establishment of the microbial population in some cases where slow degrading compost materials are used and the toxicity of the contaminant chemical to the slowly establishing microbial population. However, the presence of readily available, degradable substrate on which the microbial population can grow while adapting to the contaminating compound makes the technology an attractive one for further investigation.

**(iii) Use of surfactants in bioremediation**

Other developments in the bioremediation industry include the use of surfactants or emulsifiers to increase availability of pollutants to the degrading population. Higher molecular mass PAHs present in petroleum and other hydrocarbon complexes such as creosote, have continued to be of concern in the environment as their availability for microbial action is relatively limited due to their inherent low aqueous solubilities and resistance to microbial degradation (Cort and

Bielefeldt, 2000 a & b; Murphy *et al.*, 2000). This property leads to mass transfer limitations concerning desorption of such highly hydrophobic contaminants from media surfaces. The application of surfactants to contaminated soil and groundwater has been found to enhance the desorption and degradation of chemical contaminants (Cort and Bielefeldt, 2000 a & b; Murphy *et al.*, 2000; Shiohara *et al.*, 2001).

Certain surfactants have been reported to inhibit degradation of some organic compounds at certain concentrations. For example between 0-1500 mg $L^{-1}$ TNP10 (a nonionic surfactant) was reported to inhibit the degradation of PCP at 300 mg $L^{-1}$ and $10^{O}C$ (Cort and Bielefeldt, 2000). In this study it was observed that micelle sequestration was the predominant mechanisms for TNP10 inhibition of PCP biodegradation in aqueous systems. This model predicts that similar rates of PCP degradation will be observed for similar free aqueous PCP concentrations. The initial degradation rates for solutions containing 0-1500 mg L-1 TPN10 will begin to overlap if PCP sequestration in the micelle is accounted for. This is consistent with the sequestration model (Cort and Bielefeldt, 2000). Below the treshold of contaminat toxicity, the surfactant can inhibit its biodegradation rates because the compound partitioned into the micelle is unavailable to the degrading microorganisms. The rate then becomes dependent on partitioning rates of the organic substrate between micelle, aqueous and cell phase (McAllister *et al.*, 1996; Cort and Bielefeldt, 2000; Baron *et al.*, 2001). Surfactant-enhanced bioremediation applications hence depend on a faster overall biodegradation rate of the target compound (Diehl and Borazjani, 1998; Shiohara *et al.*, 2001).

**(iv) Fungal bioremediation**

Fungi produce a range of enzymes of low specificity which are used to degrade a wide range of naturally occurring recalcitrant organic compounds including lignin and cellulose (Clemente *et al.*, 1999). Because of the irregular nature and recalcitrance of lignin and the presence of compounds, such as phenol, biphenyls, anisoles, diarylethers in its substructure, bioremediation researchers have postulated that this non-specific lininolytic system could be used by the same fungi to degrade recalcitrant organic compounds such as PAHs, PCP and non-aromatic chlorinated compounds (Kay-Shoemake and Watwood, 1996; Clemente *et al.*, 1999). Fungi have since been applied to the degradation of a wide array of organic pollutants in the environment including PAHs, phenolic compounds, explosives and oils (Bogan and Lamar, 1999; Kotterman *et al.*, 1999; Launen *et al.*, 1999; Novotný *et al.*, 1999). Although non-lininolytic and non-basidiomycetic fungi have been shown to grow on organic compounds of petroleum origin (Launen *et al.*, 1999; Clemente *et al.*, 1999), more attention has been focussed on the white-rot fungi because of the nature of their enzyme system. White-rot fungi such as *Pleurotus, Phanerochaete, Agaricus* and *Trametes* have been recently studied for their capability to grow on and metabolise hydrocarbon substrates (Bazelel *et al.*, 1997; Rodriguez *et al.*, 1999; Harmsen *et al.*, 1999). Various degrees of success have been achieved by different workers including Novotný *et al.* (1999) who reported the reduction in concentration of a number of PAHs by between 41 and 93% in 60 days. Bogan and Lamar (1999) reported reduction in concentrations of PAHs between 43 and 90% in 30 days. Various concentrations of anthracene, phenanthrene and tannic acid were reported to have been degraded by 27, 90 and 100% in ten days by Clemete *et al.* (1999). The activity of various enzymes including those of lignin peroxidase, manganese peroxidase and laccase were detected during the study. Generally the mechanism of enzyme

activity of the white-rot fungi during degradation of organic substrates is similar to those of mammalian enzyme system (Cerniglia, 1997).

**(v) Phytoremediation**

Phytoremediation is the use of green plants and their associated biota and soil amendments to remediate contaminated sites (Al-Zalzala *et al.*, 2001). In phytoremediation trees are the active organisms that facilitate the remediation process. However, other plants are involved in different stages of the process. (Conklin, 2001). Plants and trees interact with soil to produce and area around the root called rhizosphere. This area is characterised by high microbial activity, low pH, increased organic matter, high carbon dioxide and lower water and nutrient content (Conklin, 2001; Campanella *et al.*, 2002).

Phytoremediation focuses on two areas viz the enzymatic action of pollutant degradation and the bacterial action which simultaneously degrade the pollutant and stimulate plant growth. Although the mechanism of the action has not been fully elucidated, its likely that pollutant transformation may be catalysed through reactions mediated by lipid peroxidase (Bogan *et al.*, 2000). The inoculation of bacteria such as *Burkholderia* and *Sphingomonas* into soil seeded with alfalfa has been found to promote plant germination, survival and/or growth. The peroxidase secreted by the alfalfa roots has been reported to oxidise guaiacol and 2,2'-azinobis(3-ethylbenzothiazolin-6-sulfonic acid), and some model PAHs (Bogan *et al.*, 2000). The combined actions of plant mediated degradation of organic pollutants in the environment and bacterial stimulation of plant growth offers a promise area of further research in contaminated site remediation (Gramss *et al.*, 1998, 1999).

The discussion above has shown that bioremediation has advanced reasonably over the past ten years. It also show that many more bioremediation techniques have been found that can partially or wholly reclaim contaminated environment. It has become apparent that many recalcitrant compounds such as higher molecular mass hydrocarbons present in petroleum, coal tar and creosote nd many other compounds of environmental concern can be degraded in the environment by the careful selection of methodologies. It is therefore the aim of this study to examine some of these technologies for application to creosote-contaminated soil with a view to developing a cost effective remediation technology for the South African and other similar conditions.

## 1.8 CONCLUSION

Hydrocarbon-contaminated soils have been identified in many parts of the world and ongoing attempts are being made to remediate such sites in Europe and America, with different degrees of success. In the Third World, where uncontrolled waste disposal continues unabated, virtually no effort has been made to identify polluted sites and little is being done to remedy the situation. There is, therefore, a need to investigate technologies appropriate for Third World communities, commensurate with the prevailing economic conditions in developing countries.

As conventional remediation technologies such as incineration and land-filling become increasingly unpopular, bioremediation is now the most plausible option for remediating contaminated soils. Under normal soil conditions, most contaminant hydrocarbons are biodegraded by microorganisms already present in the environment. Persistence of hydrocarbons in soils is generally due to unavailability to the microbiota and also to their non-degradative

nature. Although a number of generalizations are made, it is apparent that microbial degradation of hydrocarbon pollutants is a complex process and that environmental factors have a great influence on the fates of organic pollutants in soil. However, the ability to adapt these environmental conditions, to the advantage of the bioremediation process, constitutes one of the major achievements of the technology.

Bioremediation can mineralize waste products and hazardous chemicals into water, carbon dioxide, biomass or other innocuous products (Baker and Herson, 1994; Bollag and Bollag, 1995; Alexander, 1999) and thus removes the need to relocate the contaminated medium. Although sometimes time-consuming, its advantage as a natural and final process makes it more attractive than other faster technologies such as incineration. Bioremediation technologies offer cost-effective, ecologically friendly and permanent solutions to the clean-up of hydrocarbon-contaminated soils. With better knowledge of the interaction between the environment, the contaminants and the microorganisms mediating the degradation, improved results should be achieved.

Despite the many reports in the literature describing the success of bioremediation technology for treatment of hydrocarbon-contaminated soils, its full potential is yet to be achieved. Many more applications of this technology are sure to be discovered in the future. Considering the heterogeneous nature of many wastes and the nature of the contaminated media, the success of future bioremediation application will depend on an interdisciplinary approach involving distinct specializations such as microbiology, ecology, geology, soil science, chemistry and engineering. The treatment of recalcitrant and hazardous compounds in the soil environment presents a challenge to future bioremediation technologists. One such substance is creosote, the removal of which from the soil at a contaminated wood treatment plant is the objective of the present investigation.

# CHAPTER 2

# CREOSOTE AND ITS DEGRADATION IN THE ENVIRONMENT

## 2.1 INTRODUCTION

Creosote, a mid-temperature distillation product of coal tar, is a blend of about 400 individual compounds, 85% of which are polycyclic aromatic hydrocarbons (PAH). Phenols constitute 10% and sulphur heterocyclics 5% (Arvin and Flyvbjerg, 1992; Lajoie and Strom, 1994; Hughes *et al.*, 1998). The more significant compounds by mass found in creosote include trimethylbenzenes, naphthalenes, acenaphthalenes, fluorene, phenanthrene, fluoranthene and pyrene (Thompson, 1991). It is difficult to assign specific physical properties to creosote because of the variation in the actual composition of the compound. However, certain ranges are discernible, e.g. the boiling point is accepted to be between 200 and 400$^{0}$C and the density between 0.910 and 1.17 g/cm$^3$ at 25$^{0}$C (Arvin and Flyvberg, 1992). The PAHs that are the main constituents of creosote are characterized by carbon atoms that are jointly shared by ring structures which also share a common aromatic pi-electron cloud (Dyreborg and Arvin, 1995).

### 2.1.1 Uses of creosote

Creosote has been distilled from coal tar for more than one hundred years in Sweden and in the U.S.A. for more than one hundred and fifty years (Carriere and Masania, 1995). It was used principally for wood preservation and this has remained the most important use of creosote. Wood treated with creosote is used for telephone and electricity poles, in fencing and in timber

for building construction. Superficial treatments such as brushing or painting, dipping or spraying are used for wood destined for garden fences and garage buildings. Creosote-treated wood is also used for railway sleepers and lock gates. Less common uses of creosote are treatment of tuberculosis, psoriasis (a dermatological disease) and other related skin diseases. Advantages of the use of creosote-treated wood include creosote's resistance to intense heat and its low electrical conductivity, in addition to its pesticidal effects (von Rumker *et al.*, 1975).

As a result of the wide-spread use of creosote around the world, production rose tremendously, with the U.S.A. alone producing about 4.5 million tons per annum ( von Rumker *et al.*, 1975). Consequently, ground water and soil contamination increased in different parts of the world, with Denmark having as many as 124 contaminated sites, Germany over 1000 and Canada more than 56 sites in the province of Ontario alone (Dyreborg and Arvin, 1995).

### 2.1.2 Toxicity of creosote

Studies of creosote toxicity to human beings are relatively few; however, existing data do show that the substance is toxic. Skin irritation and cancer are the most obvious toxic effects (Van Rooij *et al.*,1993; Hughes *et al.*, 1998; Bouchard *et al.*, 2001). Exposure to creosote and ultra violet (UV) radiation causes eczema (Van Rooij *et al.*,1993) and other skin rashes (Brender *et al.*,1994). This condition also doubles the risk of cancer (Karlehagen *et al.*, 1992). Workers in establishments producing or using creosote are most seriously at risk, with high incidences of skin and respiratory irritation, pitch warts and skin discoloration and tearing (Heikkila *et al.*, 1987; Warren *et al.*, 2001).

Polycyclic aromatics, which constitute the bulk of creosote, are known to be highly carcinogenic and toxic (Thakker *et al.*, 1985; Swartz *et al.*, 1990; Vogelbein *et al.*, 1990; Mueller *et al.*, 1991; AAFP, 1993; Hughes *et al.*, 1998). PAHs, which are known to volatilize when creosote is exposed to high temperature and pressure, have been demonstrated to traverse the uterine placenta and therefore are capable of affecting the foetus (AAFP, 1993). They may reduce the number of oocytes, thereby causing infertility (Brenden *et al.*, 1994). They have also been detected in breast milk (AAFP, 1993). PAHs are normally low in toxicity to human beings but their metabolites and derivatives are strong mutagens and are carcinogenic when they interact with DNA (AAFP,1993; Kästner *et al.*, 1998; Stapleton *et al.*,1998). PAHs increase the chances of carcinomas in the internal organs of human beings. They greatly affect rapidly multiplying cells (meristem)(AAFP,1993). Creosote causes photosensitivity of the eye, coughs, bronchitis, lymphoma, haematuria and kidney cancer (AAFP,1993; Schaefer, 2000). In infants, creosote fumes have been known to cause methamoglobinemia, resulting in hypoxia and cyanosis (Dean *et al.*,1992). Toxicity to fish is very well documented. This can include bioaccumulation, genotoxicity and cytotoxicity (Gagne *et al.*,1995), pancreatic cancers and hepatocellular lesions (AAFP,1993). The 4-ethylguaiacol component in creosote has been found to prevent contraction of the rat intestine (AAFP,1993). In other mammals, PAHs are thought to have effects on haematopoietic and immune systems, producing reproductive and developmental defects (AAFP,1993).

Creosote is toxic to some wood-destroying fungi (Nicholas,1973), its mode of fungicidal action possibly paralleling those which cause teratogenic or carcinogenic effects in animals (AAFP, 1993). The most volatile components are the most strongly fungicidal substances (Nicholas,1973). There are, however, three organisms which are tolerant to creosote: *Lentinus*

*lepidus* (a fungus), *Laminaria tripunctata* (a marine borer) and *Coptotermis* sp. (a termite) (Nicholas,1973). Termites tend to tolerate creosote unless it is combined with 2% pentachlorophenol or another toxicant (Nicholas,1973).

### 2.1.3 Fate of creosote in the environment

Creosote usually contaminates water, sediment and biota. It does not contaminate air readily, since it is not very volatile due to the presence of high molecular mass PAHs (Godsy *et al.*, 1992; Broholm and Arvin, 2001). The movement of creosote through the environment depends on a number of factors which are specific for each of its components. Its solubility in water and sorption coefficient constitute the most prominent factors contributing to its mobility (Fowler *et al.*, 1994; Hughes *et al.*, 1998). The more soluble components are more readily transported through the environment, be it groundwater or soil, and these constitute the most likely threat down-site. The more soluble components include the phenols, the cresols and the N-heterocyclics, which are usually found on the edge of the moving plume of the aquifer. The biodegradation of this initial pulse can deplete the dissolved oxygen in the aquifer, making any further degradative action anaerobic (Fowler *et al.*, 1994). The higher molecular mass PAHs are less mobile because of their relative low solubility in water and they are thus localised around the initial point of contamination (Arvin and Flyvbjerg, 1992). Apart from observing toxicity on living organisms, small concentrations (ppm) of creosote can easily be detected in water because of the odour imparted to the water (Arvin and Flyvbjerg, 1992).

## 2.2 BIODEGRADATION OF CREOSOTE

Biodegradation of creosote involves many different organisms using a wide variety of

degradative pathways. This is due to the large number of chemicals in creosote (about 400) and the large number of conditions under which degradation can take place (Arvin and Flyvbjerg, 1992). A large number of the aromatics are degradable and the order of degradation depends on which one most effectively supports microbial growth (Baker and Herson, 1994). The biodegradability of the PAHs is inversely related to the number of aromatic rings and the number of alkyl groups present. Both factors affect PAH solubility and thus its bioavailability (Baker and Herson, 1994). Naphthalene, anthracene and phenanthrene are readily degradable aerobically, whereas pyrrole and the pentamethyl carbazoles degrade more slowly (Arvin *et al.*,1988; Flyvbjerg *et al.*,1993 ).

The mechanism of aerobic degradation varies among the PAHs, but principally involves the incorporation of oxygen into the ring structure, ring cleavage and the production of intermediates that feed into the citric acid cycle (Gibson and Subramaniam, 1984). Dibenzofuran, pyridine, quinoline and their alkyl derivatives are also degraded relatively rapidly ( Arvin *et al.*, 1988). Insufficient electron acceptors (oxygen) at the site significantly limits the rate of degradation, thus retarding the breakdown of creosote in anaerobic environments (Arvin *et al.*, 1988; Dyreborg and Arvin,1995). In such environments, degradation is slower or non-existent. Some of the reactions that are catalysed by soil and water micro-biota are actually transformation reactions and do not cause complete biodegradation. Sometimes the intermediate products are more toxic than the original substances (Baker and Herson,1994; Alexander,1999; Schaefer, 2000).

The transformation of oxygen-containing aromatics may occur under methanogenic, denitrifying and sulphate reducing conditions (Arvin *et al.*,1988; Dyreborg and Arvin,1995;Anweiler *et al.*, 2001). The non-oxygenated aromatics xylene, toluene and benzene are known to degrade under denitrifying and fermentative conditions (Arvin *et al.*,1988). The first step in anaerobic

degradation of creosote compounds is oxidation by hydroxylation with water (Arvin *et al.*, 1988), before cleavage of the ring structure, which is then followed by pathways similar to those for aerobic biodegradation. Organisms that have been implicated in creosote degradation include *Rhodococcus* spp., *Pseudomonas* spp, *Mycobacterium* spp, and the white-rot fungus *Phanerochaete chrysosporium* (Baker and Herson,1994).

### 2.2.1 Biodegradation pathways of some of the important compounds present in creosote

#### 2.2.1.1 Phenol and phenolic compounds

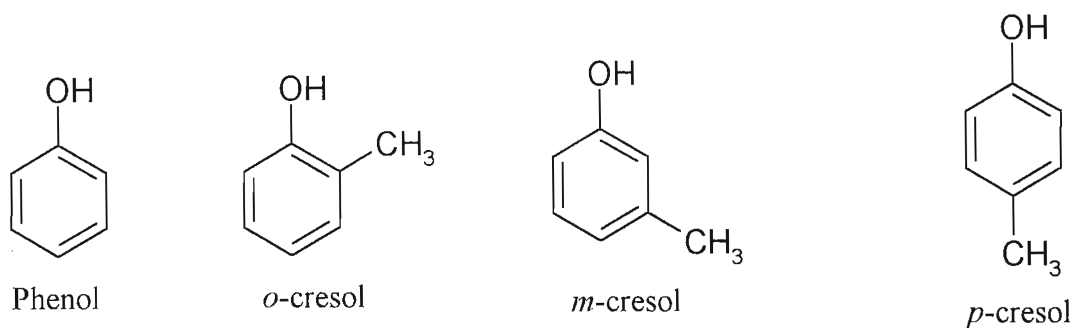



Fig. 2.1. Some phenolic compounds of environmental concern present in creosote.

Some important phenolic compounds present in creosote are shown in Fig. 2.1. Phenol is a toxic organic component often found in wastes from oil refineries, petrochemicals and wood treatment plants (Vipulanandan *et al.*,1994; Subramanian *et al.*,1995). Phenol is listed as a priority pollutant by many international organizations and government agencies, including the U.S. Environmental Protection Agency (Vipulanandan *et al.*,1994). It has a solubility of 6.7g in 100ml of water and its lethal dose for human beings, is reported to be between 5-10 mg $kg^{-1}$ body mass. Being toxic, phenol, depending on the concentration, can either inhibit growth of, or kill, degrading microorganisms (Vipulanandan *et al.*,1994;broholm and Arvin; 2001).

Phenolic compounds are of great interest to microbiologists because of their extensive use and their presence in most hazardous wastes. Phenols have been extensively studied in biodegradation experiments by many researchers for over forty years (Vipulanandan *et al.*, 1994). Interest continues unabated, particularly in the biodegradation of phenol at higher concentrations, to address such problems as shock loading during treatment of highly contaminated wastewater and leachates from hazardous waste sites.

In an early report on the microbiological treatability of phenol, Mohlmann (1929) showed that low levels of phenol can be successfully treated by activated sludge. Since then there have been extensive reports on strategies for biotreatment of phenols at contaminated sites (Vipulanadan *et al.*,1994; McAllister *et al.*, 1996; Cort and Bielefeldt; 2000). The maximum concentration of phenol for microbial activity has been recorded as 1000 ppm, at which level the initial lag phase of the culture was excessively prolonged (Vipulanadan *et al.*,1994). Concentrations above this level were found to be toxic to the microorganisms (Vipulanandan *et al.*,1994; Hansen *et al.*, 2000). Below this level, researchers have recorded some noteworthy successes in biological treatment of phenolics, with removals of up to 90 - 95 % from refinery wastes containing 100mg $L^{-1}$ of phenol recorded (Coe,1952). Biological treatment of phenol has proven to be very successful(Subramanian *et al.*, 1995), in spite of the fluctuation in phenol levels (50 - 500 mg $L^{-1}$) that occur in influent waste streams (Benger,1966; Vipulanadan, 1994).

Some microorganisms have been identified that have the ability in pure and mixed culture to degrade phenol. These include *Comamonas, Achromobacter,* (Jones *et al.*,1972,1973), *Pseudomonas putida* (Yang *et al.*, 1975), *Staphylococcus, Citrobacter, Proteus and Escherichia coli* (Holladay *et al.*,1978). Lewandowski *et al.* (1988) also identified *Klebsiella pneumoniae, Pseudomonas putida* and *Serratia liquefacens* as being able to utilize phenol as sole carbon

source. Gurujeyalakshmi and Oriel (1989) showed that complete degradation of phenol can be achieved at 950ppm using *Bacillus stearothermphilus* and that above this concentration only partial degradation occurred. Although sorption and stripping processes may play a role in the degradation of phenol, biodegradation is generally believed to be responsible for the removal of up to 90 % of phenol ( Kincannon *et al.*, 1983).

### 2.2.1.2 Polycyclic aromatic hydrocarbons (polyaromatic hydrocarbons(PAHs)

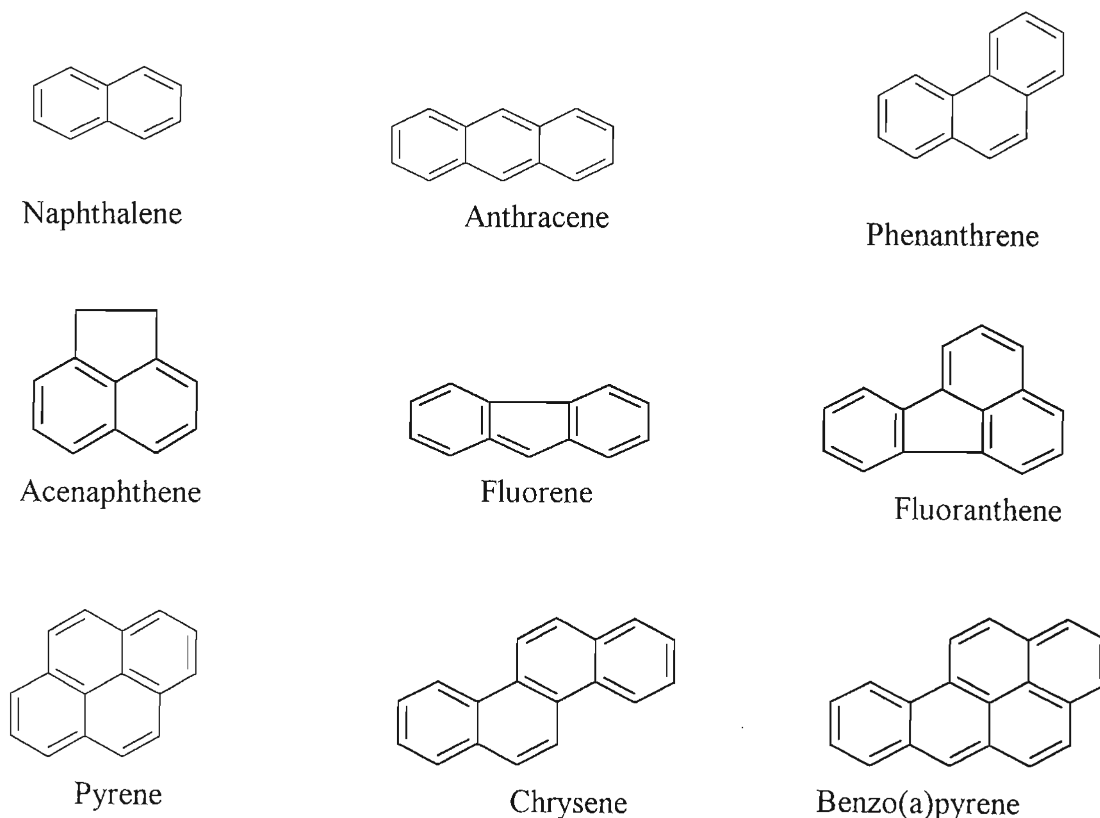



Fig. 2.2. Some polycyclic aromatic hydrocarbons (PAH) of environmental concern present in creosote.

Some PAHs which constitute a large percentage of creosote include naphthalene, anthracene, phenanthrene, pyrene, chrysene, fluorene, fluoranthene, benzo(a)pyrene and pyrrole, which,

despite its five-ring structure, behaves very much like the polyclic aromatic compounds. Polycyclic aromatic compounds or polyaromatic hydrocarbons (PAH) and polynuclear aromatics are synonyms used to describe compounds containing two or more benzene rings. The structures of some of the more important PAHs occurring in creosote are shown in Fig. 2.2. They are also found as major constituents in many hydrocarbon-based complexes such as petroleum.

The use of creosote in wood preservation has led to extensive environmental contamination by these PAH compounds. Rosenfeld and Plumb (1991) reported that acenaphthalene, naphthalene, fluorene, phenanthrene, fluoranthene, pyrene and anthracene have been detected in ground-water in more than twenty percent (20%) of the wood treatment sites tested. Other PAHs were also detected at wood treatment sites, but with less frequency. The concern over PAH contamination in soil and ground-water stems from the fact that many of them are known or suspected carcinogens (Kingsbury *et al.*, 1979; Weissenfels *et al.*, 1990; AAFP, 1993; Boonchan *et al.*, 2000).

Many aerobic bacteria are known to degrade two and three ring aromatic compounds such as naphthalene, anthracene and phenanthrene. The initial reaction in the degradation of PAHs involves the introduction of molecular oxygen into the ring structure, with the ultimate production of intermediates that feed into the Krebs cycle (Gibson and Subramanian, 1984). The number of rings in a PAH is inversely correlated to its susceptibility to microbial degradation (Gibson and Subramanian, 1984; Baker and Herson, 1994). The decrease in the rate of degradation from three to five rings is a function of water solubility, which consequently determines its bio-availability. Thus the greater the number of rings, the less bio-available and the less biodegradable are the PAHs (Baker and Herson,1994).

Despite the constraint, in terms of soluble carbon source encountered in culturing microorganisms, recent studies have shown that a number of aerobic bacteria, both in pure culture and in consortia, have the ability to degrade many of the high molecular mass polyaromatic hydrocarbons (Anweiler *et al.*, 2001; Broholm and Arvin, 2001; De Wildeman *et al.*, 2001). For example, Mueller *et al.* (1989) reported a mixed microbial community with seven morphologically distinct bacterial strains capable of degrading fluorene, anthracene, anthraquinone, fluoranthene and pyrene. It is therefore no surprise to find microbial consortia capable of degrading other PAHs and complex mixtures of PAHs (e.g. creosote) in contaminated sites (Mueller *et al.*, 1991; Dyreborg and Arvin, 1995; Diehl and Boranzjani, 1998; Hansen *et al.*, 2000).

Reports of degradation of PAHs under anaerobic conditions are limited. Mihelcic and Luthy (1988) showed that, under denitrifying conditions, naphthalene could be degraded but under strict anaerobic conditions it could not. However, it was shown that naphthol, which contains a hydroxyl group, could be degraded under both these conditions. Thus the presence of hydroxyl groups apparently makes PAHs susceptible to anaerobic degradation.

Fungi and algae have been known to degrade PAHs under aerobic conditions, but the mechanism of oxidation differs from that used by bacteria (Gibson and Subramanian, 1984). Although the role of fungi in the degradation of PAHs in natural environments is unclear, there is evidence of fungal degradation of PAHs ranging from two to five rings, including benzene and phenol (Gibson and Subramanian, 1984). The white-rot fungi have extensive biodegradative capabilities associated with enzymes involved in lignin biodegradation (Bumpus and Aust, 1987; Aust, 1990; Kennes and Lema, 1994; Boonchan *et al.*, 2000). The production of ligninase or lignin peroxidase by *Phanerochaete chrysosporium* is dependent on the nutritional status of the

organism. Bumpus (1989) has demonstrated that *Phanerochaete chrysosporium* is capable of degrading many PAHs found in anthracene oil, a coal tar derivative. The fungus has been implicated in the degradation of twenty-two different PAHs (Gibson and Subrabramanian,1984; Sutherland *et al.*,1991). Sanglard *et al.*(1986) showed that *Phanerochaete chrysosporium* is capable of mineralizing benzo (a) pyrene, although the intermediates were not identified. Sutherland *et al.*(1991).suggested that the initial oxidation of phenanthrene, and presumably other PAHs, may not be solely dependent on the presence of ligninases. Rather fungal monooxigenases and epoxide hydrolases are thought to catalyse the initial formation of unstable PAH arine oxides, which are subsequently isomerized to phenols or hydrolyzed to form trans-dihydrodiol (Baker and Herson, 1994). A generalized summary of the fungal degradation of PAHs is shown in Figure 2.3.

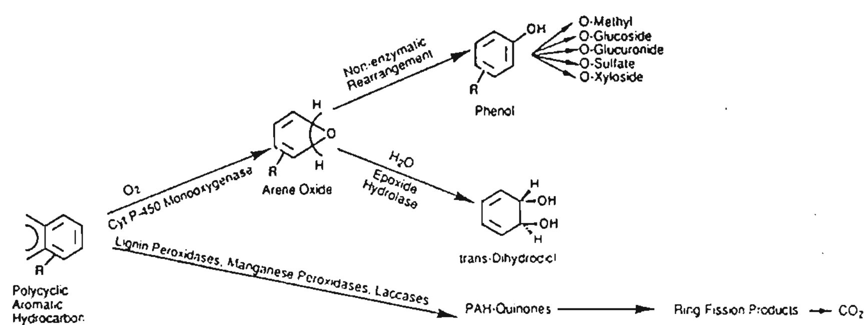



Figure 2.3. Proposed pathways for the fungal metabolism of polycyclic aromatic hydrocarbons (Cerniglia, 1997).

**2.2.1.2(i) Biodegradation of naphthalene**

Microbial utilization of naphthalene has been extensively studied and documented in the literature (Gibson and Subramanian, 1984). The proposed pathway for naphthalene degradation

by bacteria, as summarized by Gibson and Subramanian (1984), is shown in Figure 2.4. The initial reaction in the bacterial oxidation of naphthalene involves the formation of dihydrodiol intermediates. Walker and Wiltshire (cited by Gibson and Subramanian,1984) reported that an unidentified gram-negative bacteria oxidised naphthalene to D-*trans*-1,2-dihydroxy-1,2-dihydronaphthalene (*trans*-naphthalene dihydrodiol) . Subsequent studies by Treccani, Walker and Wiltshire (cited by Gibson and Subramanian,1984) led to the detection of *trans*-naphthalene dihydrodiol in culture filtrates of three *Psuedomonas55* strains and a *Nacardia* strain. A mutant strain of *P. putida* that also oxidized naphthalene to *cis*-naphthalene dihydrodiol was isolated in 1971 by Gibson *et al.*(Gibson and Subramaniam,1984).

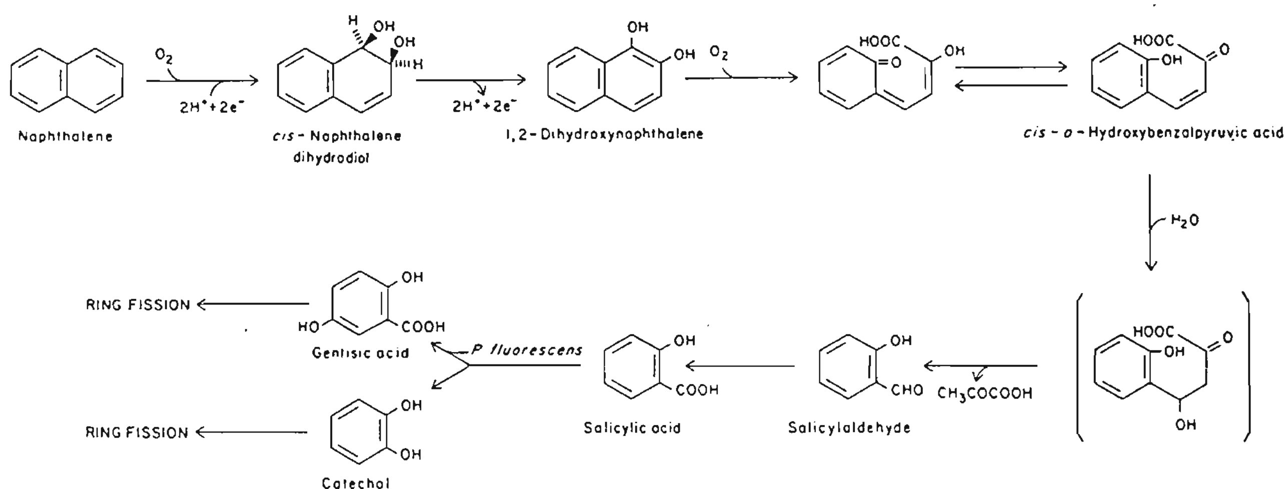



Fig.2.4. Proposed pathway for the degradation of naphthalene by bacteria (Gibson and Subramanian, 1984).

This metabolite has also been reported to be reduced by hydrogen in the presence of palladium to (-)-2(S)-hydroxy-1,2,3,4-tetrahydronaphthalene. This helped to establish the structure of the bacterial dihydrodiol as (+)-*cis*-1(R),2(S)-dihydroxy-1,2-dihydronaphthalene (Gibson and Subramanian, 1984). Both atoms of oxygen in *cis*-naphthalene dihydrodiol have been found to be from a single molecule of oxygen (Gibson and Subramanian,1984). Bacteria have also been

shown to utilize a dioxygenase reaction to initiate the degradation of naphthalene, a reaction which is further catalysed by dehydrogenase to give 1,2-dihydroxynaphthalene (Gibson and Subramanian, 1984; Sutherland *et al.*, 1995). Studies by Davies and Evans and Patel and Barnsley (cited by Gibson and Subramanian, 1984) suggested that ring cleavage occurs between carbon atoms 1 and 9 and that cyclization to 2-dihydoxychromene carboxylic acid occurs before release from the enzyme. Although it is generally accepted that salicylate undergoes oxidative decarboxylation to yield catechol as the substrate for fission of the aromatic nucleus, gentisic acid has also been implicated as an intermediate in naphthalene degradation by *Pseudomonas fluorescens* (Gibson and Subramanian, 1984).

Studies have shown that naphthalene can be degraded by fungi in a manner similar to its degradation by mammals (Cerniglia and Gibson, 1977; Cerniglia *et al.*, 1978; Gibson and Subramanian, 1984; Sutherland *et al.*, 1995). *Cunninghamella bainieri* has been shown to oxidize naphthalene to *trans*-1,2-dihydroxy-1,2-dihydronaphthalene, 1-naphthol, and 2-naphthol (Gibson and Subramanian, 1984). The fungal oxidation of naphthalene is not restricted to only a few species. Indeed, other species, including *C. elegans*, have been shown to oxidize naphthalene to a number of different intermediates. Cerniglia *et al.* (1979) isolated 47 species from 34 genera that had the ability to oxidize naphthalene. Some species of some members of the order *Mucorales*, including *Mucor*, *Cunninghamella* and *Syncephalastrum*, have been shown to oxidize naphthalene appreciably (Gibson and Subramanian, 1984).

**2.2.1.2 (ii) Biodegradation of phenanthrene**

Many species of bacteria found in soil are capable of utilizing phenethrene as a growth substrate and the pathway showing its oxidation is seen in Figure 2.5. The degradation of this compound

by bacteria usually starts with oxidation at the 3,4-position to form dihydrodiol (Figure 2.5). Intermediates of phenanthrene metabolism have been isolated from culture filtrates of

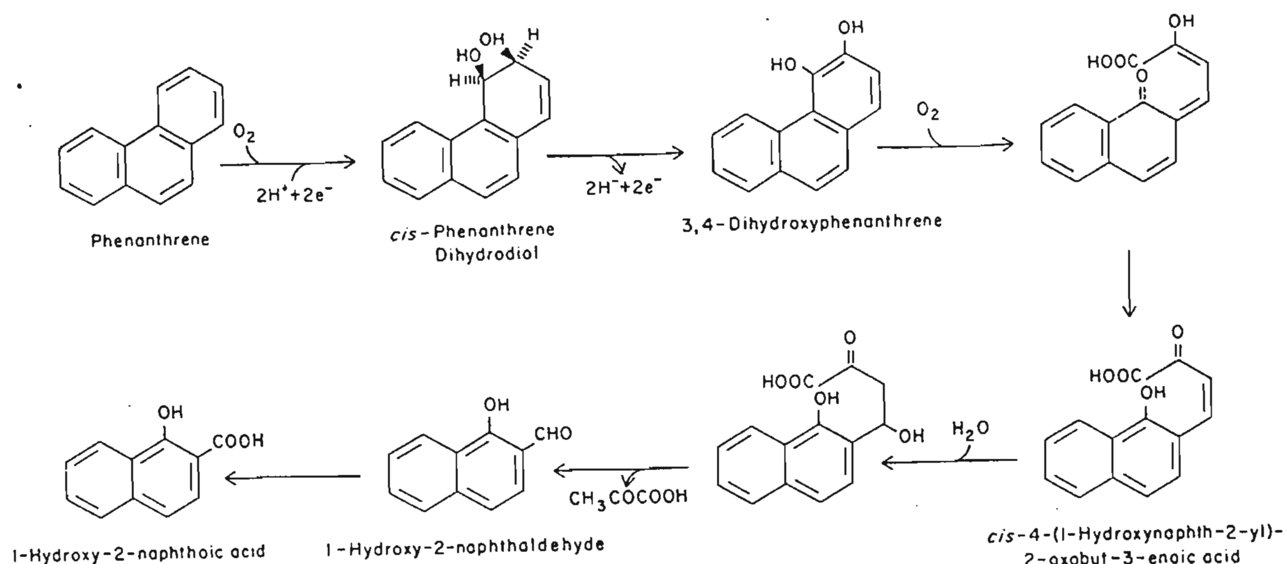



Fig. 2.5 Proposed pathway for the bacterial oxidation of phenanthrene (Gibson and Subramanian, 1984).

*Flavobacterium* grown with phenanthrene as a carbon source. It was suggested by Evans *et al.* (cited by Gibson and Subramanian, 1984) that bacteria oxidize phenanthrene to *trans*-3,4-dihydroxy-3,4-dihydrophenanthrene which, in turn, undergoes enzymatic dehydrogenation to form 3,4-dihydroxyphenanthrene. *Pseudomonas putida and Beijerinckia* species have also been implicated in the conversion of phenanthrene to two dihydrodiol intermediates (Jerina *et al.*, 1976; Gibson and Subramanian, 1984; Sutherland *et al.*, 1995). Although a number of different intermediates have been reported, the true intermediate is thought to be 3,4-dihydrodiol (Gibson and Subramanian, 1984). Other oxidative pathways which involve the compound being oxidized to 1-hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde, o-phthalic acid and protocatechuic acid have also been suggested (Gibson and Subramanian, 1984).

### 2.2.1.2 (iii) Biodegradation of anthracene

The solubility of anthracene in water is 75 μg $L^{-1}$ (Gibson and Subramanian, 1984; Alexander, 1999; Eriksson *et al.*, 2000). This relatively low solubility tends to affect its bioavailability and consequently the number of organisms capable of utilizing it as a growth substrate. Only the initial stages of its bacterial degradation are well-known and these are summarized in Fig. 2.6. The identification of 1,2-dihydroxy-dihydroanthracene led to the suggestion that the initial reactions in the bacterial degradation of anthracene involve the formation of *trans*-1,2-dihydroxyanthracene prior to ring fission (Gibson and Subramanian, 1984).

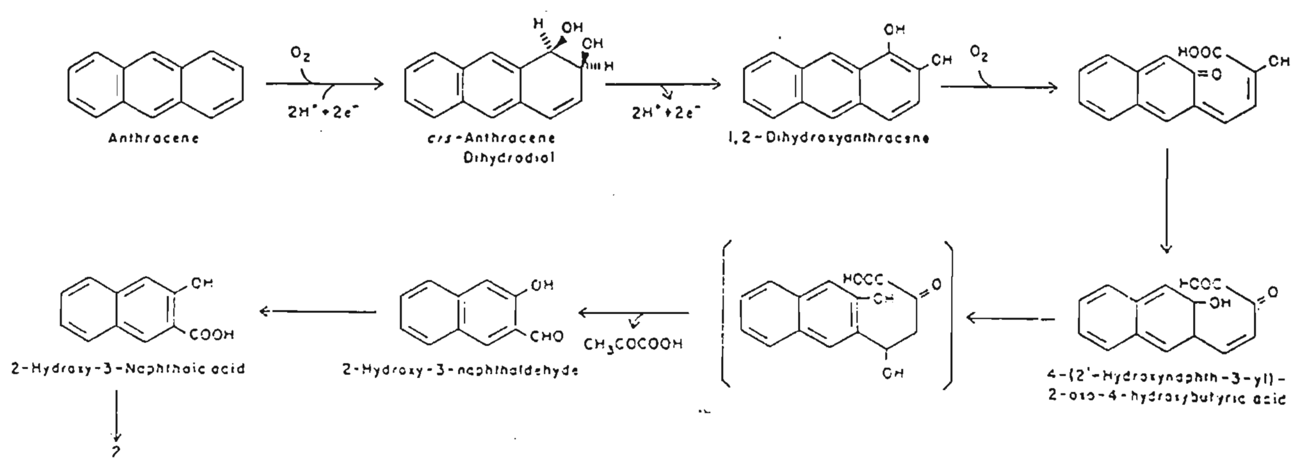



Fig. 2.6. Proposed pathway for bacterial degradation of anthracene (Gibson and Subramanian, 1984)

Further studies revealed that *Pseudomonas putida* strain 119 and *Beijerinckia* sp. strain B-836 oxidized anthracene to (+)-*cis*-1,2-dihydroxy-1,2-dihydroanthracene. Soil pseudomonads have been shown to further metabolize 1,2-dihydroxyanthracene to 2-hydroxy-3-naphthoate, salicylate and catecol (Dagley and Gibson, 1965, Yamamoto *et al.*, 1965). The compounds 1,2-dihydroxyanthracene and 2-hydroxy-3-naphthaldehyde were also found to be rapidly oxidized by cells grown in anthracene (Gibson and Subramanian, 1984). Cells grown in a medium of naphthalene are also known to oxidize anthracene, 1,2-dihydroxyanthracene, and 2-hydroxy-3-

naphthaldehyde (Gibson and Subramanian, 1984; Sutherland *et al.*, 1995). Diluted cell extracts from naphthalene-grown cells were shown to convert 1,2-dihydroxyanthracene to *cis*-4-(2-hydroxynaphth-3-yl) 2-oxo-but-3-inoic acid, whereas the same (undiluted) cell extracts converted 1,2-dihydroxyanthracene to a product whose chromatography, fluorescence and absorption properties were identical to those of 2-hydroxy-3-naphthaldehyde (Gibson and Subramanian, 1984). Although 2-hydroxy-3-naphthoic acid can undergo oxidative decarboxylation to 2,3-dihydroxynaphthalene, further metabolism of this intermediate has not been established (Gibson and Subramanian, 1984; Meyer and Steinhart, 2001; Sullivan *et al.*, 2001).

**2.2.1.2 (iv) Biodegradation of benzo(a)pyrene**

As with naphthalene, the reactions utilized by fungi to oxidize benzo(a)pyrene are very similar to those described for mammals (Gibson and Subramanian, 1984). Cell-free extracts of *Cunninghamella bainieri* and *Saccharomyces cerevisiae* have been shown to have benzo(a)pyrene hydroxylase activity (Gibson and Subramanian, 1984). These results suggest that benzo(a)pyrene is oxidized to *trans*-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene,3-hydroxybenzo(a)pyrene, and 9-hydroxybenzo(a)pyrene (Cerniglia and Gibson, 1979; Cerniglia *et al.*, 1992). Cerniglia and Gibson (1977) further showed that *C. elegans* oxidizes benzo(a)pyrene to almost the same spectrum of metabolites that are formed by mammals, including *trans*-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene, *trans*-9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene, benzo(a)pyrene-1,6-quinone, benzo(a)pyrene-3,6-quinone, 9-hydroxybenzo(a)pyrene and 3-hydroxybenzo(a)pyrene. Although the metabolism of benzo(a)pyrene by the fungus *C. elegans* and certain mammals shows similarities, the fungus does not oxidize the hydrocarbon at the 4,5-position (Gibson and Subramanian, 1984).

#### 2.2.1.2 (v) Biodegradation of pyrene

Many microorganisms have been isolated that have the capability of utilizing four ringed aromatic hydrocarbons such as pyrene (Heitkamp *et al.*, 1988; Meyer and Steinhart, 2001). Although the likelihood of pure bacterial cultures using pyrene as sole carbon source seemed limited, Walter *et al.* (1991) isolated a *Rhodococcus* sp., strain UW1, capable of growing on pyrene as sole carbon source. This organism was found to mineralize up to 72% of pyrene to $CO_2$ within two weeks. Three percent of the labelled carbon was found in the organic phase and 25% was present as water-soluble metabolites in the aqueous phase. *Cis*- 2-hydroxy-3-(perinaphthalene-9-yl)-propenoic acid and 2-hydroxy-2-(phenanthren-5-one-4-enyl)-acetic acid, which do not have a free existence, were proposed as intermediates in the degradation of pyrene (Walter *et al.*, 1991). Heitkamp *et al.* (1988), identified pyrene-4,5-dihydrodiol as an initial ring oxidation product and 4-phenanthroic acid as the major metabolite of the degradation of pyrene by a *Mycobacterium* sp. In addition, 4-hydroxyperinaphthanone was found as a ring fission product (Walter *et al.*, 1991). High pressure liquid chromatography (HPLC) analysis of an ethyl acetate extract from a 36-hour incubation of [$^{14}$C]pyrene with *Penicillium janthinellium* (No.403) revealed the presence of five major metabolites: 1-pyrenol, 1,6-pyrenediol, 1,8-pyrenediol, 1,6-pyrenequinone and 1,8-pyrenequinone (Launen *et al.*, 1995).

The other PAHs such as chrysene, fluoranthene, pyrrole and fluorene, which are present in substantial quantities in creosote, have also been found to be susceptible to microbial degradation to varying extents. However, the media in which they were studied varied from one investigator to another

(Gibson and Subramanian, 1984; Mueller *et al.*, 1991; Field, *et al.*, 1992; Sutherland *et al.*, 1995).

### 2.2.2 BIOREMEDIATION OF CREOSOTE-CONTAMINATED SOIL

Contamination of soil and ground water by creosote hydrocarbons and other contaminants is a widespread problem brought about by accidental spillage of chemicals on land used for chemical production and refining processes. Several workers have reviewed the possible alternatives for treating creosote-contaminated soils. Some of the alternatives considered include: excavation and replacement of contaminated soil, air stripping, encapsulation, chemical treatment, soil washing, incineration and bioremediation (Ellis, 1994). Bioremediation has continued to be favoured among these treatment options because of its naturalness, cost-effectiveness, ease of application and ecological friendliness. Thus, many of the successful cases of clean-up of creosote contaminated soils have been based on bioremediation techniques.

Ellis (1994) reported the bioremediation of the Blekholmstorget, in central Stockholm, in 1992. The creosote concentrations at two separate sites (60 $m^2$ and 30 $m^2$) and at depths varying between 0.9 and 4.5 m within the experimental area, exceeded 32 000 mg $kg^{-1}$. Both *in situ* and *ex situ* bioremediation techniques were employed in the reclamation project. The *in situ* treatment resulted in a reduction of more than 80 % in creosote-concentration while the *ex situ* treatment showed a reduction of about 68 %. Eriksson *et al.* (1999; 2000) treated creosote contaminated soil in an old gas works site in Stockholm using bioremediation methods and achieved a reduction in creosote concentration of about 80 %. Their approach involved mainly bioaugmentation with acclimated microorganisms isolated from the contaminated soil. Hughes *et al.* (1994) employed landfarming techniques in which indigenous species were used to boost the microbial population of the soil and reported a reduction of 99.8 % in the concentration of some of the creosote compounds thus monitored.

Landfarming has been reported by other workers to be effective in remediating creosote-contaminated soils to acceptable levels within a reasonable time. Sometimes intrinsic bioremediation is combined with landfarming to yield faster results (Connolly *et al.*, 1999; Winningham *et al.*, 1999). Commonly, the addition of organic and inorganic manures, tilling, irrigation, control of pH and moisture constitute some of the major activities in such operations.

Other treatment methods that have proved successful in the remediation of creosote-contaminated sites include biosparging, bioventing and composting. O'Melia *et al.* (1999) employed biosparging and bioventing to remediate creosote contaminated soils in the U.S.A. The combination of physical and biological techniques for treating waste components *in situ* is particularly useful for sites containing mobile recalcitrant constituents. Eggen *et al.* (1999) composted aged creosote-contaminated soils and successfully reduced the concentration of the creosote components by between 44 % and 91 % over a period of 7 weeks.

## 2.3 PRELIMINARY SITE INVESTIGATION

### 2.3.1 History

The experimental site is located in the KwaZulu-Natal midlands a few kilometres from the Albert Falls Dam, about 20 km north-east of Pietermaritzburg, KwaZulu-Natal, South africa. The facility, originally built in 1987, commenced operation in 1988 and, apart from a short shutdown period (1994-1998), it operated at full capacity, treating large quantities of timber products on a daily basis.

### 2.3.2 Materials and Methods

Twenty-one soil sample cores, to a depth of 35 cm, were collected from three different locations ( 7 cores in each location) at the site. Sub-samples from each location were mixed thoroughly to make a composite sample. The samples were placed in plastic bags and transported to the laboratory, where they were stored at 4°C. The samples were later sent to the KwaZulu-Natal Department of Agriculture and Environmental Affairs, at Cedara, the University of Natal Chemistry Department and a private laboratory for analysis.

#### 2.3.2.1 Characterization

No hydrological tests was carried out at the site before this study commenced. Contamination levels of both surface water and groundwater were not determined. All studies were carried out on soil samples selected solely on the basis of visual estimation of the levels of contaminants present. The depth of topsoil ranged from 30cm to 45cm. Beneath this was a continuous layer of shale that was found to impede the downward flow of fluid. The top layers of the shale were found to be impregnated with creosote in some areas. The site was predominantly flat, with very gentle gradients at the northern end of the location. Creosote contamination was detected at depths between 20cm and 35cm below the soil surface. The topsoil was characterised as a sandy loam with more than 60% sand and coarse silt and about 20% clay and fine silt each. It was classified as a Mispah form (Mispah series) (Lithosol; FAO). Soil density and pH were 0.93 g/mL and 5.45, respectively. Total extractable organic carbon content was >5.3% (w/w). Carbon , nitrogen and hydrogen (CNH) analyses showed that the soil had a CNH ratio of 80 : 0.08 : 8.14. The phosphorus content was determined as 4.75 mg $L^{-1}$. The water holding capacity was 63.5g/100g.

At the site various timber products are pressure treated with creosote and then stacked outside in open lots to dry. The major source of soil contamination is the creosote dripping from these wood stacks (Plate 2.1 & 2.2). In such areas, contamination levels between 180 000mg $kg^{-1}$ (18%) and 250 000mg $kg^{-1}$ (25%) (w/w) creosote were found. Other areas of very high contamination were the points of discharge of creosote from road tankers to the storage facilities. In these areas, contamination levels >250 000mg $kg^{-1}$ (>25%) (w/w) creosote were recorded. The highest level of contamination detected [>380 000mg $kg^{-1}$ (38%)] (w/w) creosote, most of which was unweathered, was in the vicinity of a disused creosote storage tank. Other characteristics of the contaminated soil are shown in Chapter 6 (Table 6.1).

Although the immediate intention is to use the plant site as a research facility to develop affordable remediation technology for the treatment of similar wood treatment operation sites, elsewhere attention was given to the situation at hand, to curb future hazards in the immediate vicinity of the plant. This was considered important because, although the nearest bodies of water, the Umgeni River and the Albert Falls Dam, were some distance (about five kilometres) from the contaminated site, many sugarcane and vegetable farms are immediately downslope from the facility.

Plate 2.1 Wood treated with creosote laid out to dry at factory site

Plate 2.2 Stack of wood treated with creosote at factory site

## 2.4. OBJECTIVES OF THE STUDY

The main objective of this research is to develop a cost-effective methodology for the remediation of creosote-contaminated soils in a typical sub-tropical environment, with particular reference to the KwaZulu-Natal province of South Africa. It is also aimed at providing basic information on creosote contamination and remediation in South Africa.

## 2.5 CONCLUSION

It is evident from the literature that compounds of creosote origin can be biodegraded to innocuous compounds in the soil. It is also apparent that the degradation of creosote in soil can be mediated through different metabolic pathways, depending on the organisms involved and, possibly, the nature of the compounds concerned and the environment in which they are present. Many microorganisms are known to have the capabilities of oxidizing creosote in the soil environment. The pathways through which the compounds are degraded depend on the organisms involved and the stereo-chemistry of the compound.

Considering the volume of soil involved, the physical and chemical characteristics of the soil, the extent of contamination, the objectives of the study and the amount of money available for the study, it was decided that landfarming and co-composting would be appropriate bioremediation techniques to investigate in this project. These two techniques have proved to be cost-effective and amenable to modifications to suit the demands of the present study.

# CHAPTER 3

# BATCH CULTURE ENRICHMENT OF INDIGENOUS SOIL MICROORGANISMS CAPABLE OF CATABOLIZING CREOSOTE COMPONENTS

## 3.1 INTRODUCTION

The study of microbial associations in an environment is usually aimed at understanding the interrelationships between members of these associations and between the associations and the environment in nature. It has become increasingly commonplace for studies involving microbiological activities in nature to start with some form of laboratory enrichment for organisms potentially suitable for the target environment (Parkes, 1982; Alexander, 1999). There are a wide range of variables that can be controlled under laboratory conditions and these may be selected and kept constant throughout the experiment, or they may be altered in an ordered and controlled manner as the need arises. This advantage makes laboratory studies extremely flexible and often easier to interpret than field studies.

The inapplicability of laboratory results to the natural environment have often been attributed to inappropriate conditions in the laboratory systems, e.g. the use of unrealistic temperatures and concentrations of carbon substrates (Parkes, 1982; Alexander, 1999). These problems can be minimized by an experimental design that takes into consideration conditions prevalent in the target ecosystem. It is now apparent that most microbial activities in the environment are a result

of the action of mixtures of different microorganisms (Bungay and Bungay,1968; Slater,1978) and that pure culture activity is the exception rather than the rule (Parkes,1982 ). The pure culture approach may have contributed, to some extent, to poor extrapolations of laboratory results to the field. This is particularly important in biodegradative studies, since it is apparent that mineralization of most xenobiotics is the result of community activity (Bull and Slater,1976). Researchers concerned with studying the microorganisms themselves and the effect of their metabolic activity in the environment should benefit from the use of more realistic laboratory systems for both the initial enrichment and the subsequent study of the microorganisms (Parkes, 1982; Diehl and Borazjani; 1998).

Most natural environments contain a large variety of microbes in numbers which reflect the habitat and the relative abilities of the individual species to compete for the available nutrients. Enrichment techniques are designed to change the environment in such a way that the organism or organisms of interest will successfully compete against all other organisms and hence become the dominant populations. The process is highly selective and many species initially present will be lost due to their inability to compete under the prevailing environmental conditions. There is a large number of enrichment techniques that can be employed (Veldkamp and Jannasch, 1972). It is, however, obvious that there is no single technique suitable to all environmental situations. Thus the relative advantages and disadvantages of particular techniques must be considered when choosing an effective enrichment method.

A closed (enrichment system) or batch enrichment system takes place initially under defined

conditions with no further input of growth substrates or removal of metabolic end-products or cells. Plate cultures, serial dilution techniques, shake flask systems and soil slurries are some classical examples. Control of the enrichment process is achieved through manipulation and the use of a large number of selective media for the enrichment of nutritionally distinct organisms. Although the initial enrichment conditions can often be defined, selection conditions are constantly changing due to the metabolic activity of the enriched organisms and the environmental conditions in the system. This can result in poor reproducibility in the enrichment procedure and even failure to enrich the desired organisms (Parkes, 1982). If, however, the microorganisms being enriched are nutritionally distinct, the enrichment can be reproducible in spite of these problems (Parkes, 1982). The constantly changing conditions during the enrichment process also mean that the conditions that actually apply during the selection process are often unknown. This situation tends to hinder development of new selective media (Parkes, 1982; Yateem *et al.*, 2002).

The initial concentrations of limiting nutrients need to be high if the enrichment is to be continued for any length of time. This is an essential consideration in the selection process. It is common practice in batch cultures to use media containing 1 to 2 % (w/v) substrate (Andrew, 1968) and this obviously causes problems in the enrichment of microorganisms capable of degrading toxic compounds. The use of a fed-batch reactor system will alleviate the problem of toxic substrates (Pirt, 1975).

In the presence of excess levels of substrates, as in batch cultures, selection of microbes resulting

from competition is based on maximum specific growth rate ($\mu max$). As a result, batch system organisms are characteristically high in growth efficiency at high substrate concentrations and low substrate specificity (Jannasch,1967), making the organisms behave like zymogenous or opportunistic organisms. Autochthonous organisms, i.e. those that utilize low substrate concentrations and have low maximum specific growth rates, are out-competed in the nutrient-rich batch system due to their low $\mu max$ values (Jannasch,1967; Parkes,1982). Batch systems may also encourage the growth of organisms which require specific growth factors (autotrophs). Batch systems thus enrich for microorganisms with characteristic growth parameters (zymogenous organisms) and those with specific nutritional requirements (autotrophs). However, these represent only two of the many different types of microorganisms present in the environment (Parkes,1982).

In spite of these problems, batch culture techniques have their own attributes apart from being simple to run. Enrichments that are substrate-limited may have their zymogenous community dormant but such organisms may become active and grow rapidly following nutrient additions and compete successfully in the environment(Parkes, 1982). Sometimes the accumulation of toxic substances may help to maintain conditions required in certain growth systems such as the maintenance of anaerobic conditions on the surface of a detritus particle by the accumulation of $H_2S$ produced during metabolism (Jørgenson,1977). It is therefore reasonable to study microbial growth with batch systems.

The isolation of microorganisms which degrade xenobiotic substances is routinely carried out by

this classical batch enrichment culture technique. However, because such microorganisms are usually present in small numbers in environmental samples, their numbers require amplification before isolation by selectively encouraging their growth over that of other more abundant species (Slater and Bull,1982 ). Non-selective growth requirements such as temperature, moisture, aeration and pH are always kept close to their known/presumed optima in batch enrichment culture systems (Bartha,1986). Usually, soils with a long contamination history provide a potential source of degradative species and, as such, act as starter inocula (Bartha,1986; Alexander,1999). Thus, for a proper understanding of the microbial activity of the soil under survey, with respect to the utilization of creosote hydrocarbons, the batch culture system was chosen for its simplicity, ease of maintenance and ability to meet the needs of the study at hand.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Sampling

Three 1kg soil samples contaminated with creosote, each representing light (<5 000 mg $kg^{-1}$), medium (5 000 - 25 000 mg $kg^{-1}$) and heavy (>25 000 mg $kg^{-1}$) contaminations, were collected with a spade to the depth of 30 cm from three different locations on the site. In each area, seven cores were collected and mixed thoroughly before placing them in a black polythene bag and transporting them to the laboratory in an ice box. The bags were heat-sealed and stored at 4°C until required.

### 3.2.2 Media

**(i) Mineral Salts Medium (MSM) (composition per litre)**

$K_2HPO_4$, 1.5g; $MgCl_2.6H_2O$, 0.2g; $NaH_2PO_4.2H_2O$, 0.85g; $Na_2SO_4$, 1.4g; $NH_4Cl$, 0.9g; $NaHCO_3$, 0.5g; $Na_2CO_3$, 0.2g; 1 mM solution of $NiCl_2.6H_2O$, 1 ml; vitamin solution, 1 ml; Trace Elements Solution A, 1 ml; Trace Elements Solution B, 1 ml. (Coutts, Senior and Balba, 1987).

**(ii) Vitamins (mg $L^{-1}$ distilled water)**

Biotin, 10; p-aminobenzoic acid, 10; folic acid, 10; pyroxidine HCl, 20; thiamine, 20; riboflavin, 30; nicotinic acid, 50.

**(iii) Trace Elements Solution A (mg $L^{-1}$ distilled water)**

$FeCl_2.H_2O$, 1500; NaCl, 9000; $MnCl_2.4H_2O$, 197; $CaCl_2$, 900; $CoCl_2.H_2O$, 238; $CuCl_2.H_2O$, 17; $ZnSO_4$, 287; $AlCl_3$, 50; $H_3BO_3$, 62; $NiCl_2.6H_2O$, 24; Conc. HCl, 10 ml.

**(iv) Trace Elements Solution B (mg $L^{-1}$ distilled water)**

$Na_2MoO_4.2H_2O$, 48.4; $NaSeO_3.xH_2O$ (31%Se), 2.55; $Na_2WO_4.2H_2O$, 3.3.

### 3.2.2.1 Preparation of media

#### (i) Mineral Salts Medium (MSM)

The medium was prepared in three stages.

**Stage 1:** All components of the medium except $Na_2CO_3$, $NaHCO_3$, trace element solutions A and B, and vitamins were dissolved in distilled water and diluted to 900 ml. This solution was dispensed into flasks and stoppered with cotton wool bungs wrapped in aluminum foil and autoclaved at 121°C for 15 minutes.

**Stage 2:** $NaHCO_3$ and $Na_2CO_3$ were dissolved in 97ml of distilled water and autoclaved as in stage 1 above.

**Stage 3:** Trace element solutions A and B and vitamins were filter sterilized through 0.2μm millipore membrane filters and 1ml of each was added to the medium before use (Lees,1996). All solutions were stored at 4°C before being dispensed under aseptic conditions using sterile glassware on a laminar flow bench.

#### (ii) Soil Extract Agar

Five hundred grams of soil from a grass field were suspended in 1000ml of distilled water in a 2 litre Erlenmeyer flask (flask1) and autoclaved at 121°C for 30 minutes. To a second flask (1litre) 1.5g $K_2HPO_4$, 0.5g $KH_2PO_4$ and 20g agar were added and made up to 500ml with distilled water and autoclaved at 121°C for 30 minutes. In a third flask (1litre) 0.5g $(NH_4)SO_4$ and 0.2g

$MgSO_4.7H_2O$ were added to a mixture of 400ml of distilled water and 100ml of autoclaved soil extract withdrawn from flask 1. The contents of flasks 2 and 3 were mixed aseptically after sterilization. The medium was cooled and poured into standard 100ml Petri dishes. Fifty micro-litres (50μL) of selected creosote components were dispensed on the surface of the solidified medium and spread with a sterile bent glass rod spreader.

**(ii) Mineral Salts Agar (MSA)**

To 900ml of mineral salts medium 20g agar were added and the mixture autoclaved at $121^{\circ}C$ for 15 min. The medium was cooled to about $50^{\circ}$ C and trace element solution A and B, and vitamins, previously filtered through 0.4μm membrane (millipore), were added and mixed before dispensing in petri dishes under aseptic conditions. The plates were stored at $4^{\circ}C$. To the (MSA) plates 50μL of filter sterilized selected creosote components were added by means of a syringe fitted with a 0.2μm disposable membrane filter and spread with a sterile glass rod spreader.

**(iii) Chemicals**

The chemicals used were: phenol, o-cresol, p-cresol, m-cresol, naphthalene, pyrrole, phenanthrene, anthracene, fluorene, fluoranthene, pyrene, chrysene, benzo(a)pyrene and creosote.

### 3.2.3 Enrichment of Creosote Catabolizing Microorganisms from Contaminated Soil

To 100ml sterile MSM in each of 270 250ml Erlenmeyer flasks, 15g of the light contaminated soil was added. The flasks were spiked with 50, 100, 500, 1000, 5000, 10 000, 15 000, 20 000, 25 000, and 30 000 mg $L^{-1}$ of phenol, naphthalene,o-cresol, m-cresol, p-cresol, pyrrole, phenanthrene, anthracene, fluorene, pyrene, chrysene, fluoranthene, benzo(a)pyrene and creosote. The flasks were stoppered with cotton wool bungs and incubated in the dark at 30° ± 2°C on a rotary shaker at 150 rpm for 21 days. Controls containing the same hydrocarbons in the same concentrations and 15g creosote contaminated soil but in distilled water instead of MSM was set up. The experiment was duplicated. Following incubation, 1ml from each flask was aseptically subcultured into another set of 270 flasks containing 100ml MSM each and spiked with aromatic hydrocarbons as described above, and incubated for a second 21 days at 30° ± 2°C in a rotary shaker in the dark. The subculturing was repeated 5 times. The final cultures (the sixth set of cultures) were used as inocula for growth curve studies and plate counts. At the end of each incubation period, samples were withdrawn from each flask for determination of concentrations of the spiking hydrocarbon by gas chromatography and for plate counts of total heterotrophs and hydrocarbon degraders.

**(i) Isolation of Creosote Degraders from Enrichment Culture**

Enrichment cultures were serially diluted to$10^{-8}$. To soil extract agar plates were added 0.1ml of serial dilution and each plate was overlayed with 50μL of one of the above aromatic

hydrocarbons. The experiment was duplicated. The plates were sealed in plastic bags and the bags were bound with adhesive tapes and incubated for 21-28 days at 30°C ± 2°C and checked daily for growth.

**(ii) Isolation of Total Heterotrophic Microorganisms**

To nutrient agar plates were added 0.1ml of serially diluted cultures and incubated at 30°C ± 2°C and checked daily for growth.

**(iii) Isolation of Catabolic Strains**

Swabs taken from each plate above were used to inoculate nutrient-rich agar (MSM) plates, which were then overlaid with the corresponding hydrocarbon compounds. Plates were checked daily for growth and counted before they became overcrowded. Distinct colonies were picked off the plates and used to produce pure cultures on nutrient agar plates.

**(iv) Scanning Electron Microscopy**

Scanning electron microscopy was carried out on all isolates.

### (v) Determination of hydrocarbon removal from liquid culture

Gas chromatography with a flame ionization detector (GC/FID) was used to estimate the reduction in the concentration of hydrocarbons in the culture media. The GC was a Varian-3800 model. Argon was the carrier gas. A 30 m capillary column with 0.25 mm internal diameter and 0.25 μm film thickness was used. Two temperature programmes were run. The first was: 60°C, 4 min., ramped at 10°C/min up to 200°C, and kept at this temperature for 40 min.; injector temperature 220°C. This programme was used for the higher molecular mass compounds. The second temperature programme was used for analysis of the lower molecular mass and more volatile compounds. This was 20°C 1 min: 40°C, 1 min., ramped at 10°C/min up to 200°C, and kept at this temperature for 20 min; injector temperature 220°C (Eriksson *et al.*, 1999).

## 3.3 RESULTS AND DISCUSSION

### 3.3.1 Enrichment of creosote catabolizing microorganisms from contaminated soils

Microbial activity occurred in most of the enrichment flasks spiked with the lower concentrations (50-500 mg $L^{-1}$) of the hydrocarbons including creosote, as indicated by the substantial increase in turbidity within 48 hours. With some of the higher molecular mass compounds like pyrene, chrysene, fluoranthene and benzo(a)pyrene no microbial activity was detected at these concentrations until the end of the third day. The appearance of growth in the flask spiked with creosote, despite the presence of the above higher molecular mass compounds in it, was

attributed to heterogeneous composition of creosote. Some of the components may possibly have supported the growth of some soil microorganisms. By the end of the first week of incubation, microbial activity was evident in all concentrations of all hydrocarbons (Fig.3.1). Total number of heterotrophic microorganisms continued to increase in most concentrations of all the hydrocarbons tested through the third week of incubation. The highest values recorded were in the cultures spiked with phenol at 5000mg $L^{-1}$ ($8 \times 10^5$), naphthalene at 5000mg $L^{-1}$ ($5 \times 10^5$), fluorene at 5000mg $L^{-1}$ ($6.57 \times 10^5$), phenanthrene at 10 000 mg $L^{-1}$ ($4.96 \times 10^5$) and chrysene at 500 mg $L^{-1}$ ( $6.5 \times 10^5$). At concentrations between 15 000 and 30 000mg $L^{-1}$ microbial counts, were lower, with populations decreasing as the substrate concentration increased. Counts in the cultures spiked with creosote were generally lower than in the other cultures (Fig. 3.1G). In this regard, the complexity of creosote may have imparted some inhibitory effects possibly due to the presence of many higher molecular mass components and the interaction between the components. However, at these higher concentrations a gradual but continuous increase in microbial activity did occur during the period of incubation. This can be attributed to slower acclimation of the organisms to high concentrations of these intrinsically toxic compounds (Otte *et al.*, 1994; Alexander, 1999; Renoux *et al.*, 1999) . This can be further explained by the fewer types of organisms constituting the associations isolated from these high concentrations. At such high concentrations the medium becomes highly restrictive to the type and number of organisms that can adapt to it. Thus only the organisms that have the capability of utilizing the substrate will become established in the medium. Even though a few bacteria (Plate 3.1A-F) were isolated from these high concentrations, the predominant organisms were moulds and yeasts (Plate 3.2A-C). The counts of hydrocarbon degraders showed a similar pattern to those of the heterotrophic

organisms. Counts were generally lower (between115 and 360cfu $ml^{-1}$) in the control which contained distilled water instead of MSM (Fig.3.2). These low counts were expected since there was no supportive growth medium and the cultures were spiked with different concentrations of hydrocarbons. However, the low numbers of organisms present is an indication suggests that the maintenance of a large population necessary to effect degradation in the contaminated soil would required biostimulation and possibly bioaugmentation.

The growth of microorganisms at hydrocarbon concentrations between 25 000 and 30 000mg $L^{-1}$ in liquid culture within 21 days of incubation was phenomenal. Such high concentrations of hydrocarbons have been previously reported to inhibit microbial growth (Jannasch, 1967; Baker and Herson, 1994; Yerushalmi and Guiot, 1998; Vipulanandan and Krishnan, 1993; Alexander, 1999). The growth observed in the prersent study at these high concentrations is believed to be due to the presence of adapted microorganisms in the contaminated soil used as inoculum. The long period of exposure of the contaminated soil may have allowed some microorganisms to adapt to the high concentration of creosote in the soil. These microorganisms may have been present in the soil in very limited populations as a result of the toxicity of the creosote. The nutrient present in the culture medium possibly afforded the microorganisms the opportunity to increase in population and adapt to the spiking molecule.

Although some fungi have been reported to grow in hydrocarbon-contaminated media (Bogan and Lamar, 1999; Eggen *et al.*, 1999; Kotterman *et al.*, 1999), the predominance of some fungi at concentrations between 15 000 and 30 000mg $L^{-1}$ over bacterial species was a significant

development. This suggests that these fungi were better adapted to the hydrocarbons tested at higher concentrations than the bacterial species present.

Fig. 3.1. Changes in counts of heterotrophic microorganisms in cultures spiked with different concentrations of creosote components. Values are means of duplicate cultures ± 1 Standard Error.

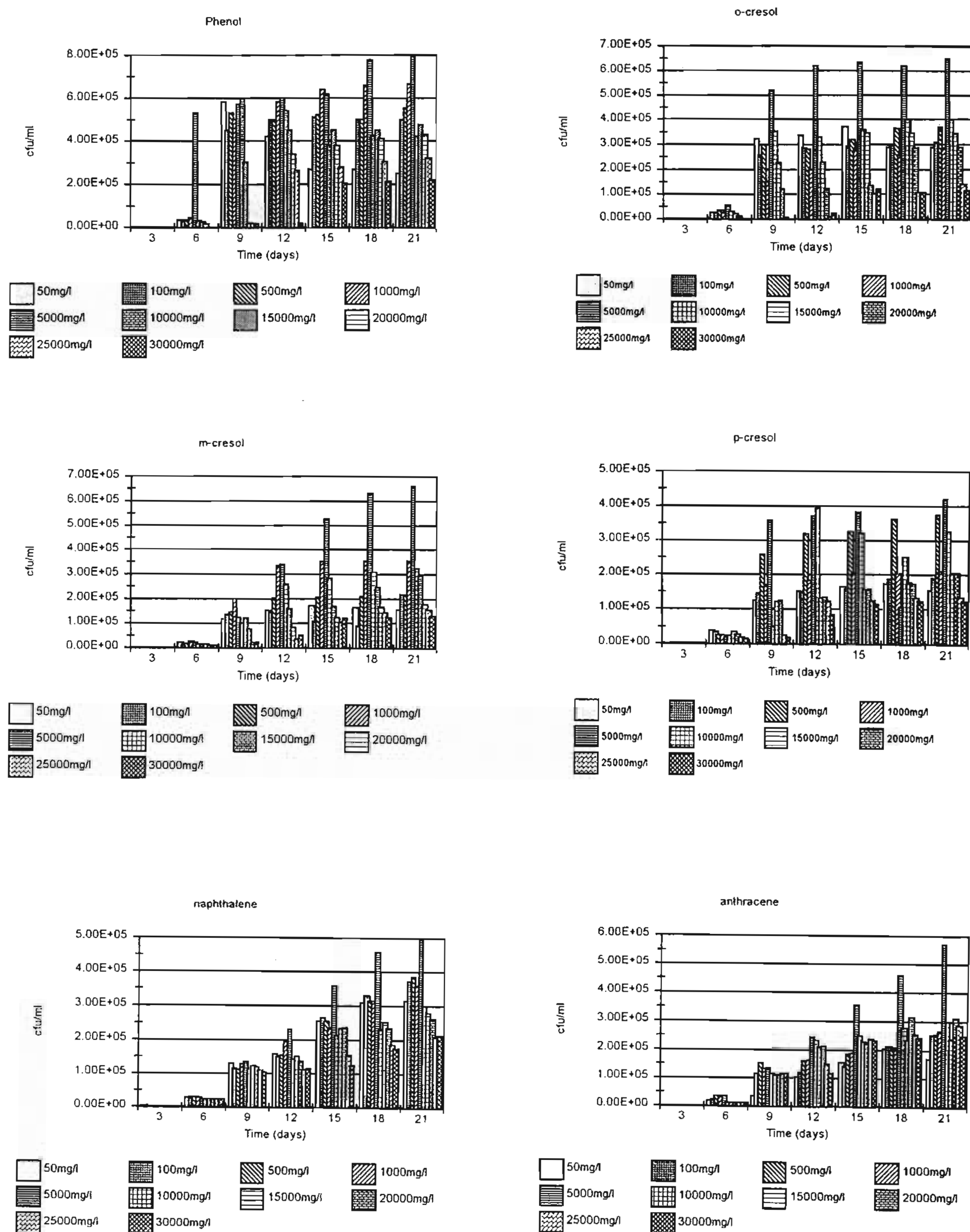


Fig. 3.1. (Continued).

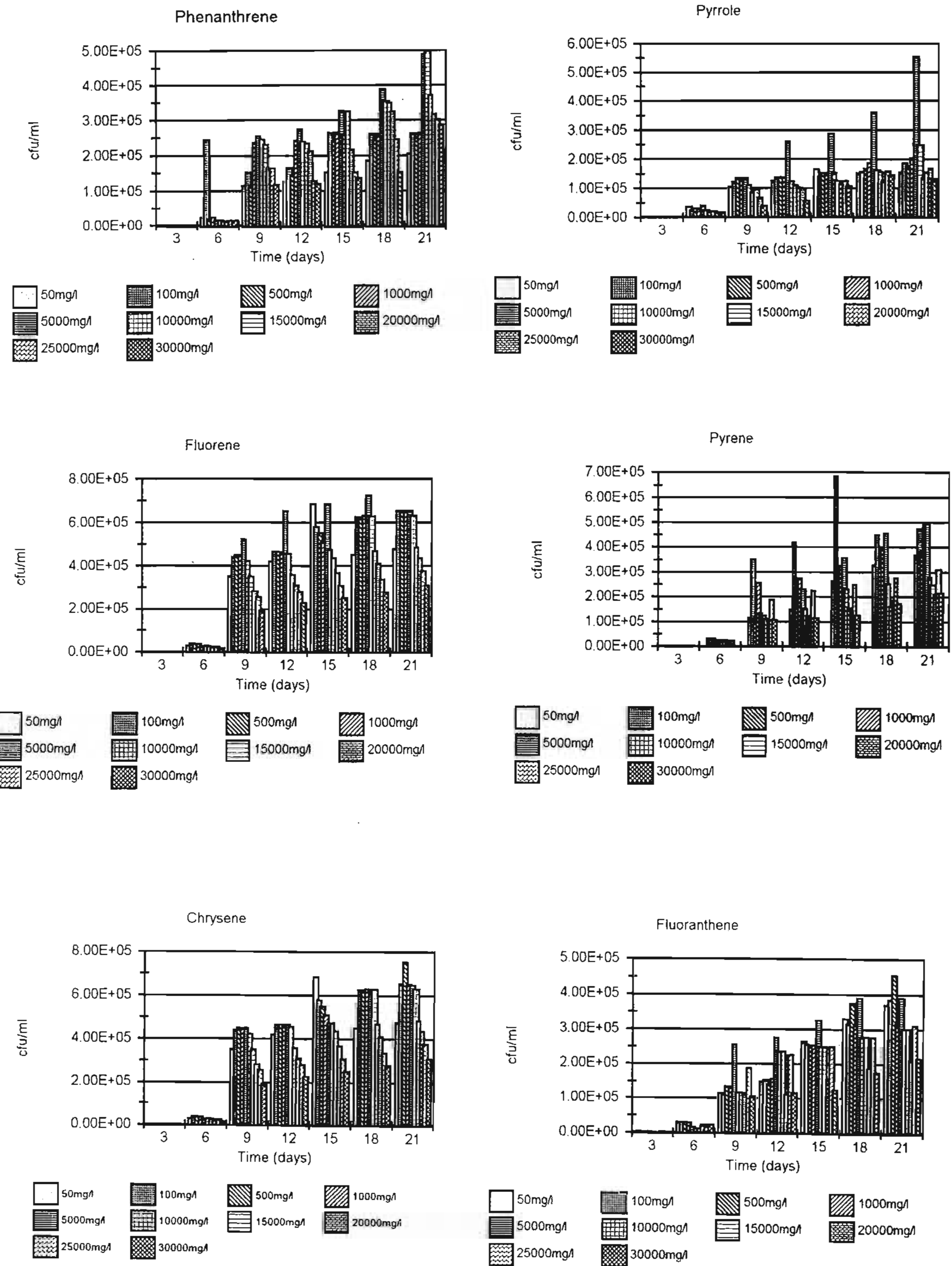
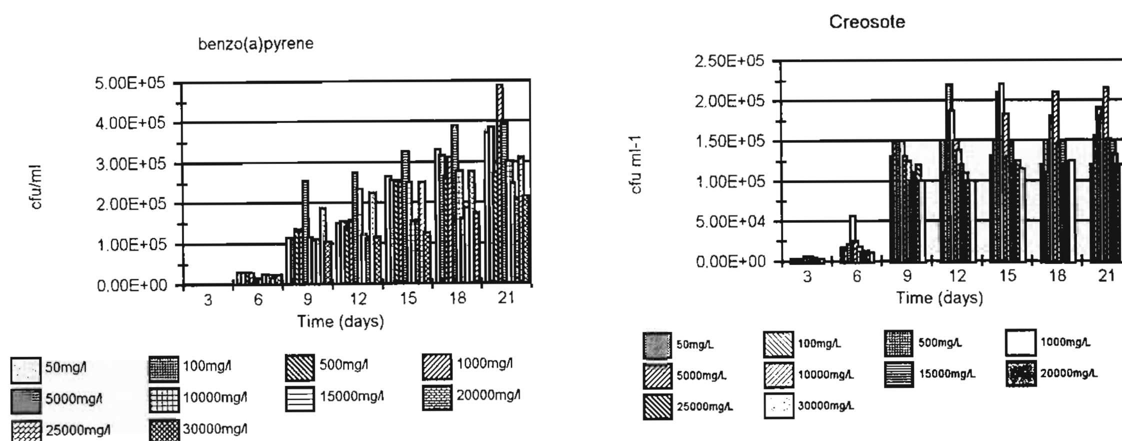


Fig. 3.1. (Continued).




At 50 and 100 mg $L^{-1}$, microbial activity in the presence of phenol, o-cresol, m-cresol, p-cresol, naphthalene, phenanthrene and pyrene tended to diminish towards the end of the third week (Fig. 3.2). This reduction in microbial activity was possibly due, at least partly, to the decrease in pH of the medium on extended incubation (Fig.3.3) and also to the diminution of the available hydrocarbon source, as reported by Weissenfels *et al.*(1990). For the phenolics, naphthalene, phenanthrene, anthracene, pyrrole, pyrene and creosote microbial counts were generally highest at 5 000mg $L^{-1}$. Even though peak microbial activity on the other higher molecular mass compounds like fluorene and benzo(a)pyrene was observed at 1 000mg $L^{-1}$, the cell counts were similar to those at 5 000mg $L^{-1}$. However, for chrysene and fluoranthene highest cell counts were recorded at 500 mg $L^{-1}$. At the time of the present study it was not obvious whether any concentration optima for microbial growth had been recorded for any of the hydrocarbons tested in liquid culture. Although different workers have previously reported growth in these compounds at relatively lower concentrations (Yang and Humphrey,1975; Weissenfels *et al.*,1990; Trzesicka-Mlynarz and Ward,1994; Bouchez *et al.*,1995; Annweiler *et al.*,2000), results

from this study have shown that most of the creosote components tested best supported microbial growth at a concentration of 5 000mg $L^{-1}$ . A few higher molecular mass components like chrysene and fluoranthene were most effective at 500 mg $L^{-1}$ and benzo(a)pyrene at 1000 mg $L^{-1}$. These results were phenomenal in the sense that a concentration optima had been observed at the set condition for the compounds tested. It has also shown that at the set condition most of the components tested could readily be degraded at 5 000mg $L^{-1}$. Even though the pH did not change significantly during the incubation period, it does seem that the conditions prevailing at the 5 000mg $L^{-1}$ concentration were relatively more conducive to microbial growth than at the other concentrations. It can further be argued that at this concentration there was an adequate supply of carbon at a non-toxic level.

Fig. 3.2. Changes in counts of creosote degrading microorganisms in cultures spiked with different concentrations of selected creosote components. Values are means of duplicate cultures ± 1 Standard Error.

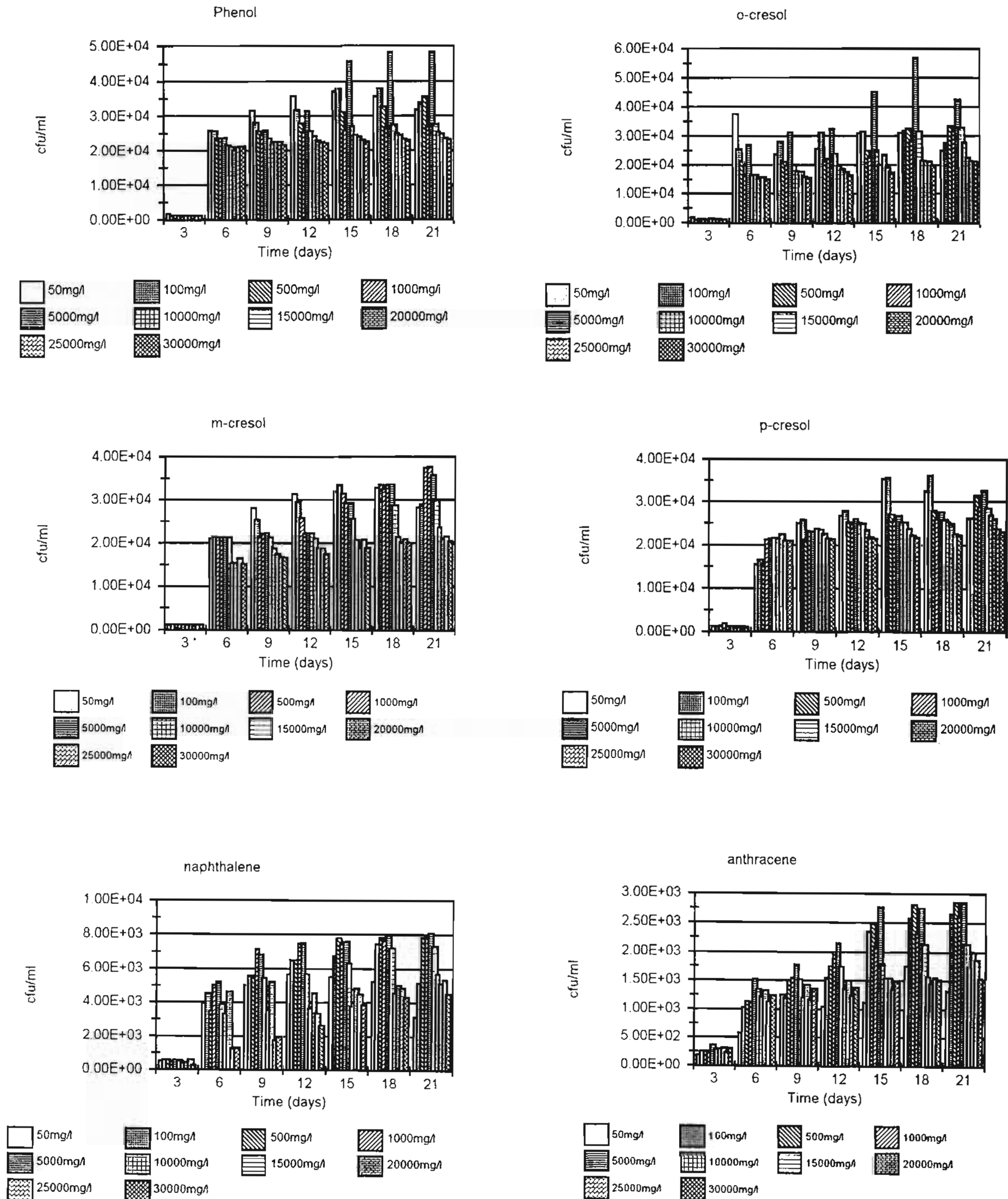
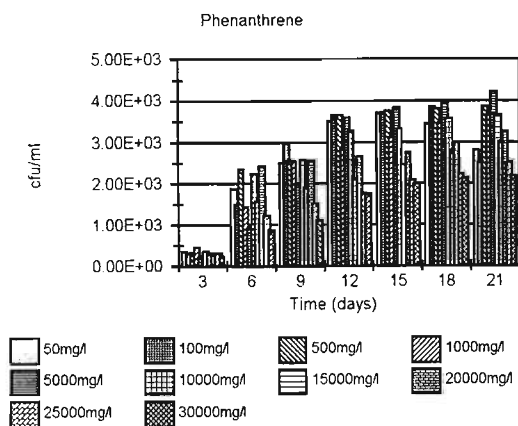
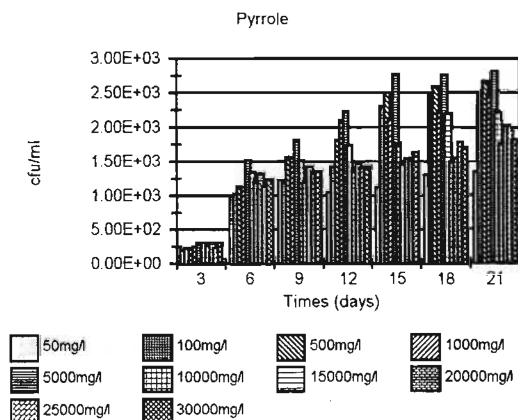
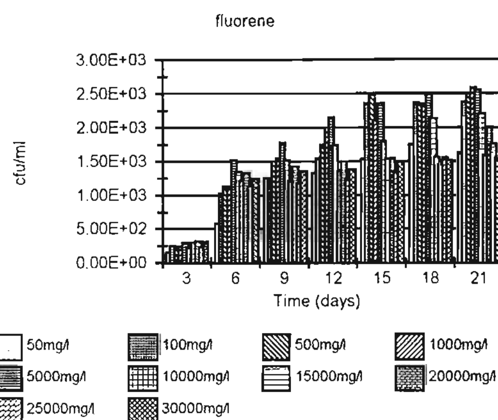
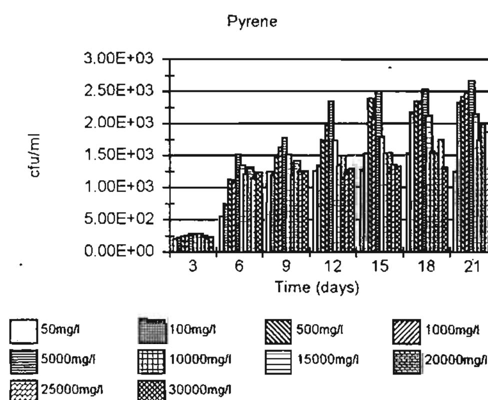


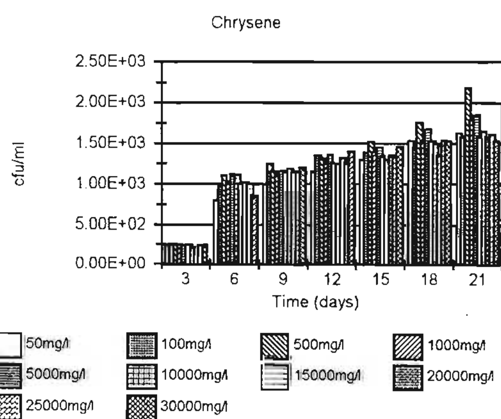
Fig. 3.2. ( Continued).



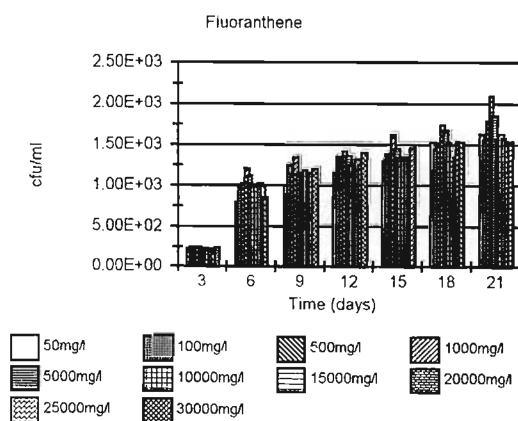
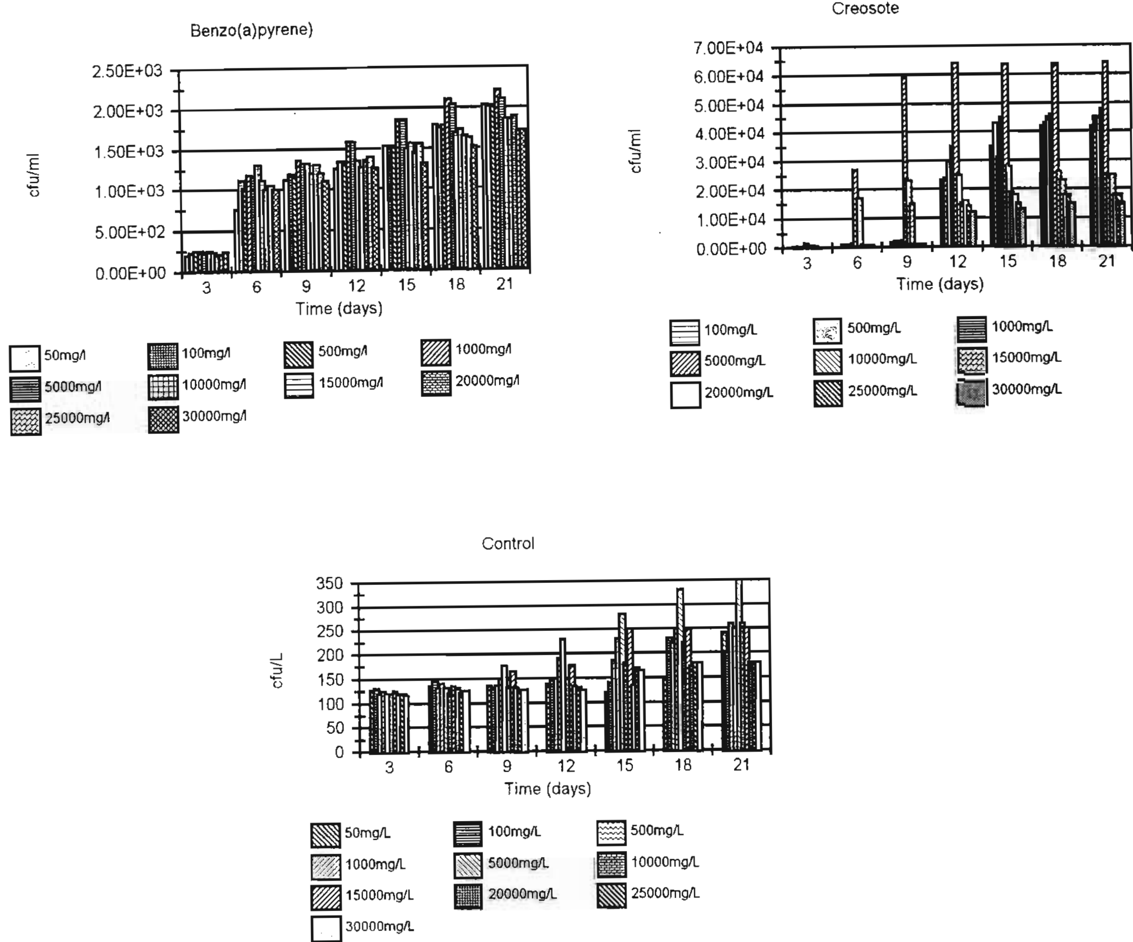



Fig. 3.2. (Continued)




In all the cultures, the pH ranged between a high of 8.59 (as recorded for anthracene 30 000 mg $L^{-1}$ in the second week) and a low of 5.33 (phenanthrene 100 mg $L^{-1}$ in the third week), respectively (Fig.3.3). A pH range of 7-7.5 has been reported (Weissenfels *et al.*, 1990) as optimal for the growth of bacteria on hydrocarbons at 30 $^0$C. Although there were fluctuations in pH in and outside this range in all the cultures (Fig.3.3), this did not affect the growth of the organisms very significantly, as shown by the cell count recorded (Fig. 3.1). The decrease observed in pH was possibly due to the production of acidic metabolic wastes by the organisms

as previously suggested by Veldkamp and Jannasch (1972). The influence of such acid production on microbial growth is unknown, but certainly increases in numbers were recorded during this period, as shown in Fig.3.1 and 3.2. On the other hand, the initial increases in pH recorded at some concentrations of hydrocarbons were attributed to relative inactivity of the microbial population at the early stages of incubation. Colony counts of the hydrocarbon degraders were lower than those of the total heterotrophic populations, but the pattern of population increase was similar in both groups of organisms, with most of the tested compounds supporting highest cell numbers at 5 000mg $L^{-1}$, while for a few, e.g. m-cresol, chrysene and fluoranthene, highest counts were recorded at 1000 and 500mg $L^{-1}$ (Fig.3.2). Concentration has been reported to play a significant role in the degradation of hydrocarbons (Dibble and Bartha,1979; Bartha and Bossert,1984; Sims *et al.*,1986). From the results in Table 3.1, it can be argued that the compound became toxic to the degrading consortia as the concentration increased. Lajoie and Strom (1994) had earlier suggested that the practical loading rate of the compound being degraded would be limited by its toxicity to the degrading organisms. Another possible explanation is the solubility of the compound, which may decrease with increase in concentration, thereby rendering the compound less available for microbial attack.

Gas chromatographic analysis of extracts from the batch cultures showed that the reduction in concentration of the spiking hydrocarbons depended on the specific compound and on its initial concentration in the medium (Table 3.1). Reduction in concentration in the creosote culture was higher than in the higher molecular mass compounds up to 10 000mg $L^{-1}$. The level of reduction started to decrease from 15 000mg $L^{-1}$. However, reduction in hydrocarbon concentration was

greater in each successive culture following transfer from the original enrichment cultures (Table 3.2). For the lower molecular mass compounds phenol, o-cresol, p-cresol, m-cresol, naphthalene, phenanthrene, anthracene and pyrrole, degradation levels ranged between 43.4 % (anthracene at an initial concentration of 30 000 mg $L^{-1}$) and 100 % in the case of phenolics and naphthalene at various concentrations (50, 100, 500, 1 000 and 5 000 mg $L^{-1}$) in liquid culture during the period of incubation. The higher molecular mass compounds pyrene, fluorene, chrysene, fluoranthene and benzo(a)pyrene, were degraded by between 45.3 % for chrysene at 30 000 mg $L^{-1}$ and 83.5 % for fluorene at 50 mg $L^{-1}$, leaving a significant amount of the compounds remaining at the end of the incubation period (Table 3.1). However, the degradative capabilities of the organisms increased markedly following repeated subculturing, so that by the sixth transfer most of the higher molecular mass compounds were removed from the culture media (Table 3.2). Acclimation of microorganisms to xenobiotic substances in the environment over a prolonged period of time has been reported in the literature. The organisms become physiologically adapted to the substance after a long period of exposure, either as a result of genetic changes in the organisms ( Harker *et al.*, 1994; Sanseverino *et al.*, 1994) or as a result of cometabolic actions that results from the ability to produce enzymes that may facilitate the breakdown of the substrate (Carberry, 1994; Santas and Santas, 1994) and thus become capable of utilizing/or oxidizing the substrate. Seeding of the cultures with organisms that have been previously adapted to the substrate also enhanced the growth and subsequent degradation of the substrate. This phenomenon has been reported widely in the literature (Baker and Herson, 1994; Alexander, 1999) . It was observed, however, that fewer types of organisms were involved in the degradation of these higher molecular mass compounds, with more fungal species being encountered compared to the associations which actively degraded the lower molecular mass

Fig. 3.3. Changes in pH in cultures spiked with different concentrations of creosote components. Values are means of duplicate cultures ± 1 Standard Error.

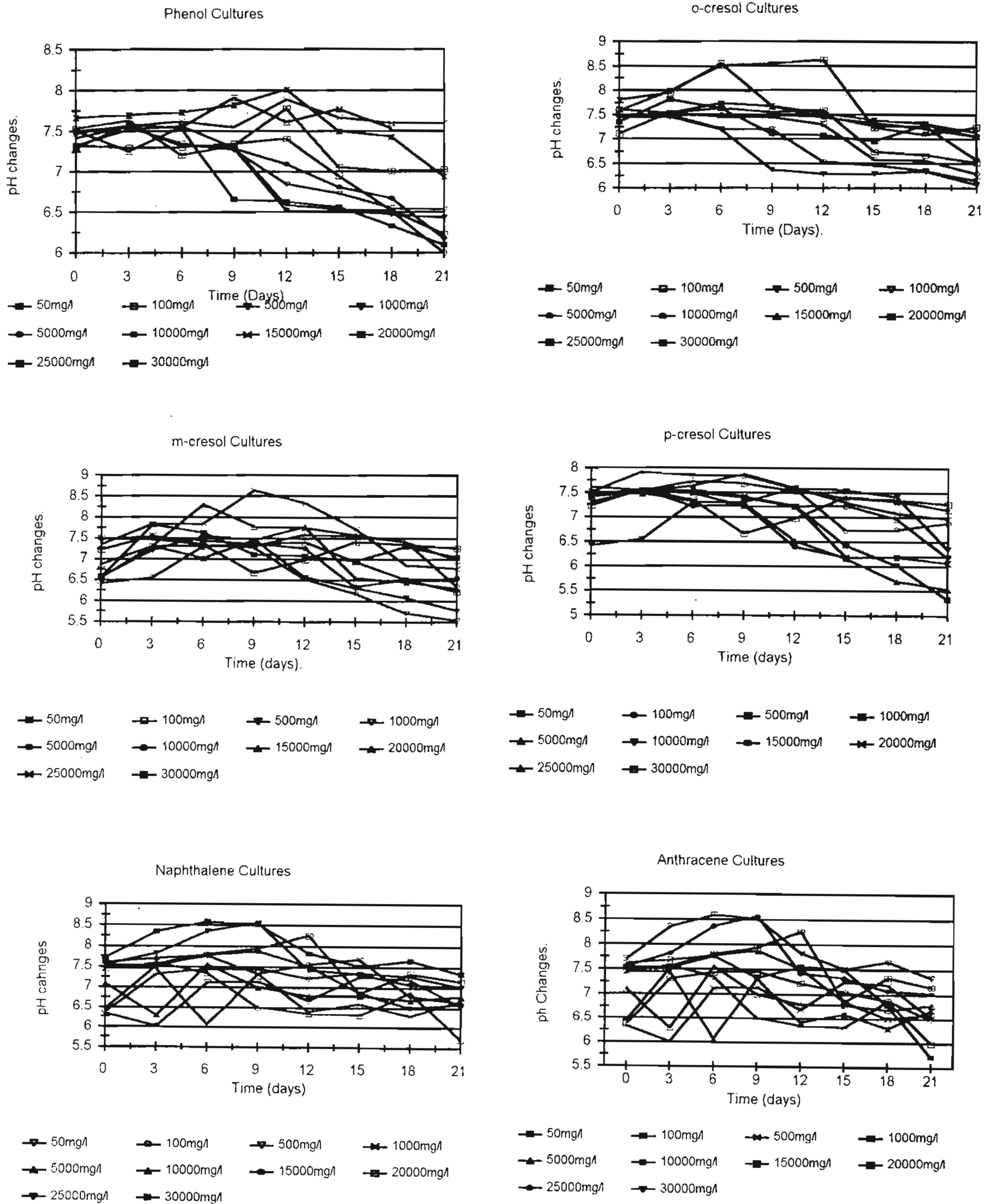
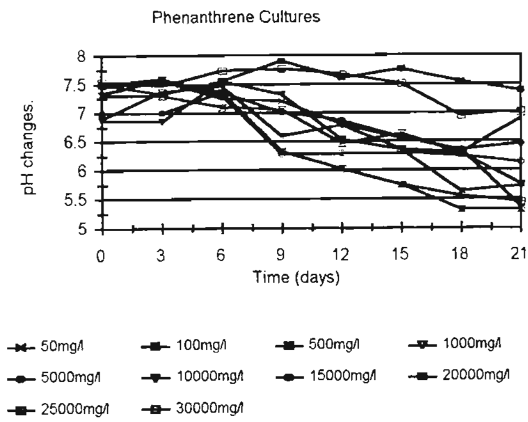
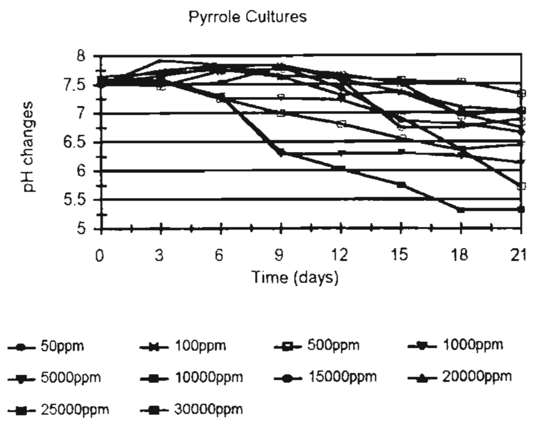
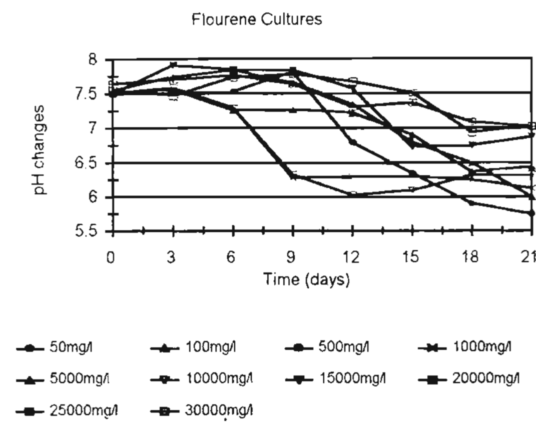
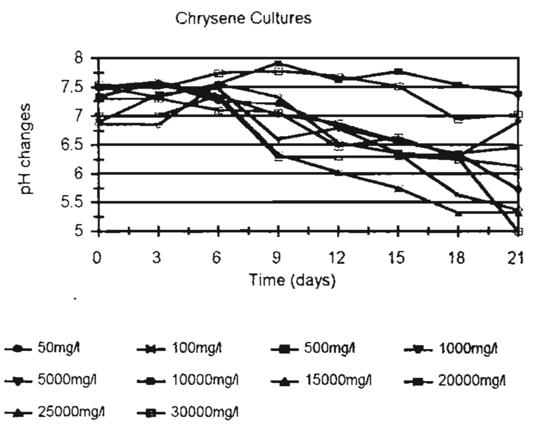


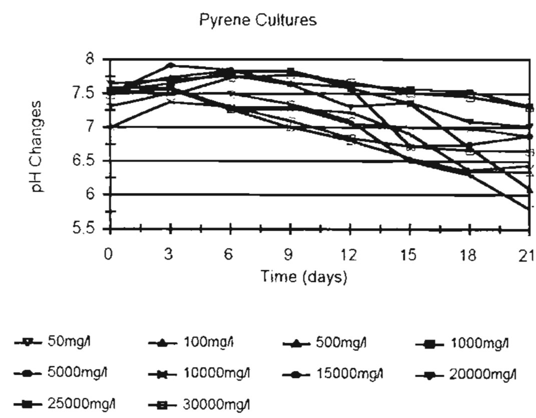
Fig.3.3. (Continued).



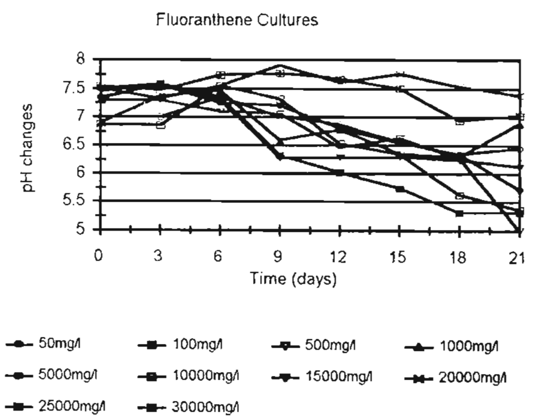
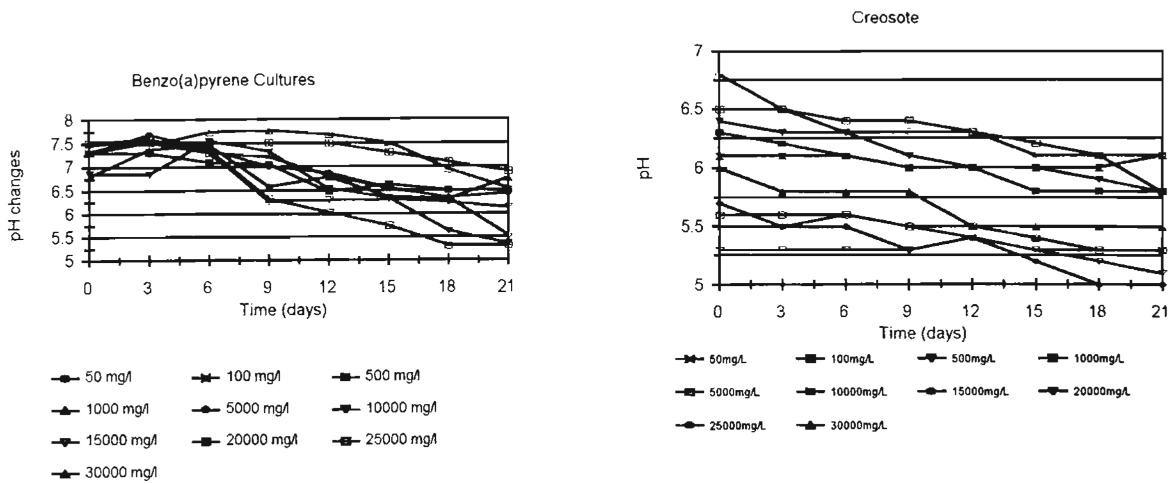



Fig. 3.3 (continued)




compounds. The relative susceptibilities of this class of compounds to degradation on the basis of their molecular mass has been widely reported in the literature (Wodzinski and Johnson,1968; Gibson and Subramanian,1984; Bossert and Bartha,1986; Trzesicka-Mlynarz and Ward,1995). For most of the compounds tested the highest level of biodegradation was observed at a concentration of 5 000mg $L^{-1}$ ,with most of the phenolics and naphthalene being completely degraded. The lowest level of removal observed at this initial concentration was 50.4 % in the culture spiked with chrysene.

Table 3.1 Percentage reduction in concentration of selected creosote components in the initial enrichment cultures after 21 days incubation. Values are means of two ± 1 Standard Deviation.

| mg $L^{-1}$ | phenol | o-cresol | m-cresol | p-cresol | naphthalene | phenanthrene | anthracene | pyrrole | pyrene | fluorene | chrysene | fluoranthene | benzo(a)pyrene | creosote |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 | 100 | 100 | 100 | 93.58 | 100 | 87.5 | 75 | 85 | 71.4 | 83.5 | 53.6 | 65.5 | 67.7 | 78 |
| 100 | 100 | 100 | 88 | 87.65 | 100 | 88 | 75 | 85 | 73.6 | 79 | 50.7 | 65.3 | 65 | 82 |
| 500 | 98 | 100 | 88 | 88.91 | 100 | 85 | 73.8 | 83.8 | 75 | 68.5 | 50 | 66.7 | 65 | 80 |
| 1000 | 98.7 | 95.2 | 85.4 | 83 | 88 | 85 | 79 | 83 | 68.2 | 68 | 50 | 67.3 | 63.5 | 80 |
| 5000 | 100 | 100 | 100 | 98.4 | 100 | 93.7 | 83.5 | 85 | 65.7 | 73.7 | 50.4 | 69.7 | 66.5 | 84 |
| 10000 | 86 | 67 | 80.1 | 68.4 | 82.4 | 77.6 | 48 | 65 | 66.3 | 71.6 | 50.5 | 65 | 63.6 | 80 |
| 15000 | 83 | 67 | 71 | 66.3 | 76.5 | 56 | 45 | 62 | 59.1 | 65.5 | 47.5 | 63 | 60.4 | 55 |
| 20000 | 66 | 55 | 56 | 52.3 | 73 | 53.8 | 45 | 56 | 62.5 | 63.5 | 47.5 | 58.9 | 55.3 | 38 |
| 25000 | 51 | 53.7 | 54.3 | 51.41 | 69.8 | 53.5 | 44.1 | 50.5 | 55.8 | 61 | 45.4 | 54.8 | 51.3 | 38 |
| 30000 | 50.47 | 50.6 | 53.2 | 51.37 | 55.21 | 51.45 | 43.4 | 49.7 | 55.5 | 53.6 | 45.3 | 55.5 | 50.7 | 33 |

As was expected, the solubility of the compounds studied affected their rate of degradation. For example, anthracene ( 0.05 mg $L^{-1}$) was more slowly degraded than phenanthrene (1.1 mg $L^{-1}$) (Tables 3.1 and 3.2). This was attributed to the generally accepted positive correlation that exists between solubility and bioavailabilty (Wodzinski and Bertolini,1972 and Wodzinski and Coyle,1974). Generally, the higher molecular mass compounds were more recalcitrant than the lower molecular mass substances. This was attributed to their low solubility in the aqueous phase, which is a function of their molecular mass and the number of rings found in their structure (Cerniglia and Heitkamp,1989; Weissenfel,1992; Cerniglia,1993; Sutherland *et al.*,1995).

Table 3.2 Percentage reduction in concentration of selected creosote components in the enrichment cultures after 21 days incubation with cultures from the 6th subculture. Values are means of two ± 1 Standard Deviation.

| mg $L^{-1}$ | phenol | o-cresol | m-cresol | p-cresol | naphthalene | phenanthrene | anthracene | pyrrole | pyrene | fluorene | chrysene | fluoranthene | benzo(a)pyrene | creosote |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 98.7 | 100 | 100 | 89 |
| 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 98.5 | 95.5 | 100 | 91 |
| 500 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 98 | 95.2 | 100 | 88 |
| 1000 | 100 | 100 | 100 | 98.7 | 100 | 100 | 100 | 98.5 | 100 | 100 | 98 | 97 | 100 | 93 |
| 5000 | 100 | 100 | 100 | 98.4 | 100 | 100 | 98.4 | 98.7 | 93 | 100 | 85.7 | 97 | 99.2 | 96 |
| 10000 | 100 | 100 | 100 | 98.1 | 100 | 97.6 | 91.5 | 90.5 | 95 | 99.1 | 85 | 95.6 | 89.4 | 93 |
| 15000 | 100 | 95.8 | 93.6 | 98.1 | 100 | 95.8 | 91.5 | 90 | 89 | 95.6 | 85 | 93 | 91.4 | 84 |
| 20000 | 97 | 95.5 | 93.5 | 96.2 | 95.5 | 95.5 | 91.3 | 90 | 85 | 95 | 75 | 93 | 95.6 | 61 |
| 25000 | 95.3 | 95.6 | 91.8 | 95.7 | 95.7 | 91.4 | 90.4 | 90 | 85 | 95 | 73 | 87.5 | 89.8 | 45 |
| 30000 | 93.1 | 95.3 | 91.5 | 95.3 | 95 | 91.5 | 89.7 | 88.2 | 85 | 88 | 73.1 | 88.2 | 88.7 | 45 |

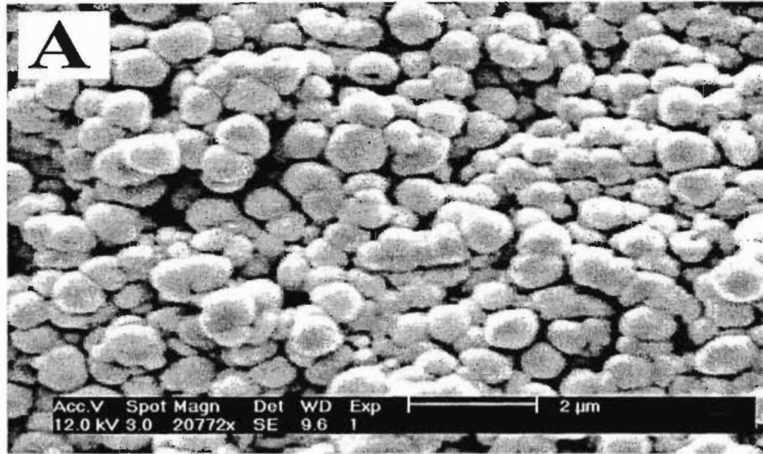
### 3.3.2 Isolation of microorganisms capable of degrading creosote hydrocarbons

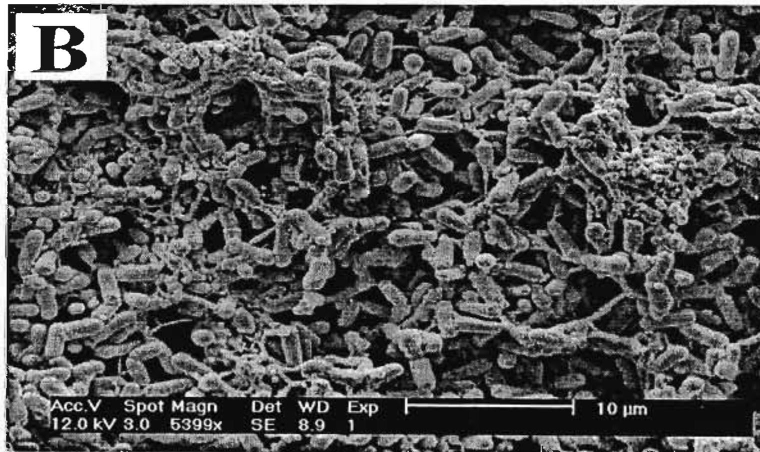
The microbial populations isolated from the liquid enrichment cultures comprised a wide range of different organisms adapted to growing on each of the hydrocarbon compounds tested (Plates 3.1 and 3.2). However, many of the morphotypes were common to most of the cultures (Plates 3.1A-F & 3.2A-C). At higher concentrations, the higher molecular mass compounds supported a relatively smaller number and variety of organisms (Plate 3.1E&F). This may be due to reduced bioavailability because of low water solubility and/or to toxicity at high concentrations and also to substituent groups attached to the molecules which cause growth inhibition at high concentration of the compounds. The mixed culture situation is well documented in the literature and such associations have been found to be more effective in

degradation of hydrocarbons than pure cultures, in many cases (Lynch and Poole,1979; Slater and Lovatt,1984; Roberts and Fedorak,1987; Mueller *et al.*,1989; Otte *et al.*,1994; Trzesicka-Mlynarz and Ward,1995). The isolates were predominantly bacterial rods and cocci, yeasts, actinomycetes and some filamentous fungi (Plates 3.1 A-F and 3.2 A-C). The rod-shaped bacteria were found to be mainly Gram negative types, as previously reported by Trzesicka-Mlynarz and Ward (1995).

Plates 3.1 shows electron micrographs of isolates from some of the enrichment cultures. The populations were predominantly of one morphotype, mainly cocci or short rods at the end of one week of incubation (Plates 3.1 A & B). The microorganisms are believed to be those that have successfully adapted to creosote in the contaminated soil (Tate, 1995). However, older cultures showed mixed populations of cocci, rods and some filamentous forms (Plates 3.1 C & D). The isolates from cultures spiked with creosote were mostly of mixed population.

Plate 3.1 Electron micrographs of isolates from liquid cultures spiked with creosote hydrocarbons.



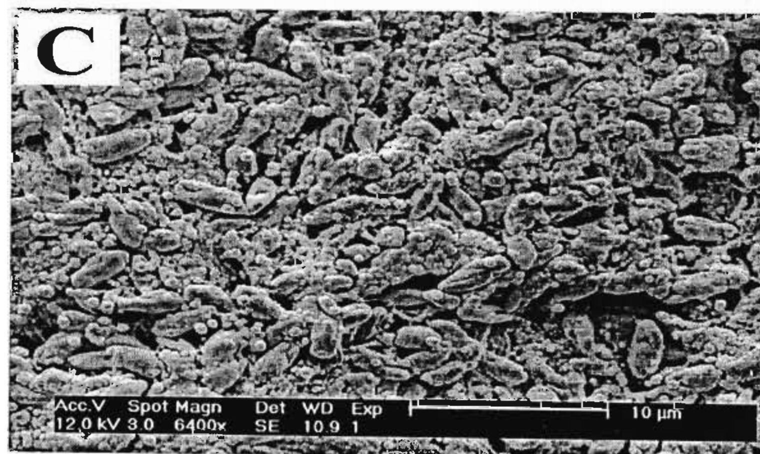
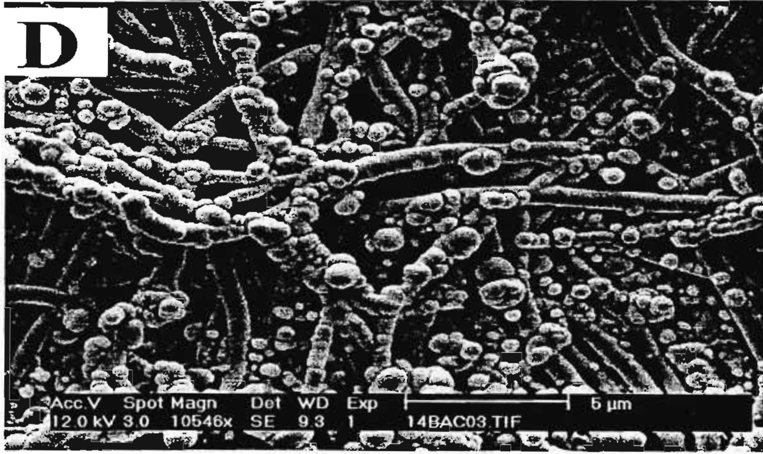
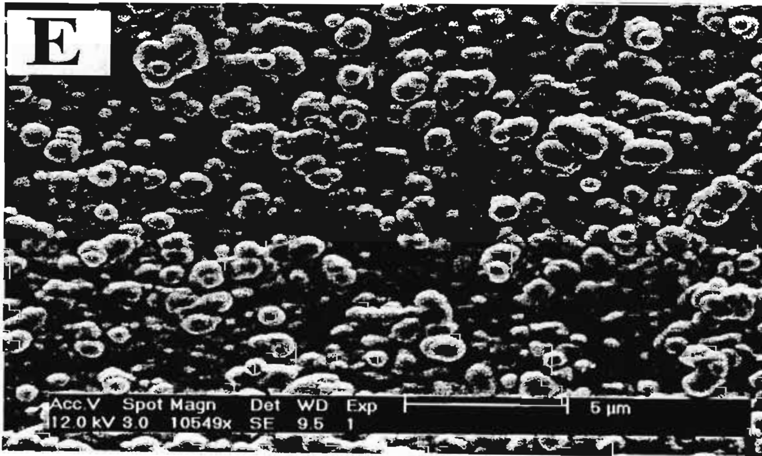



Plate 3.1 (continued).



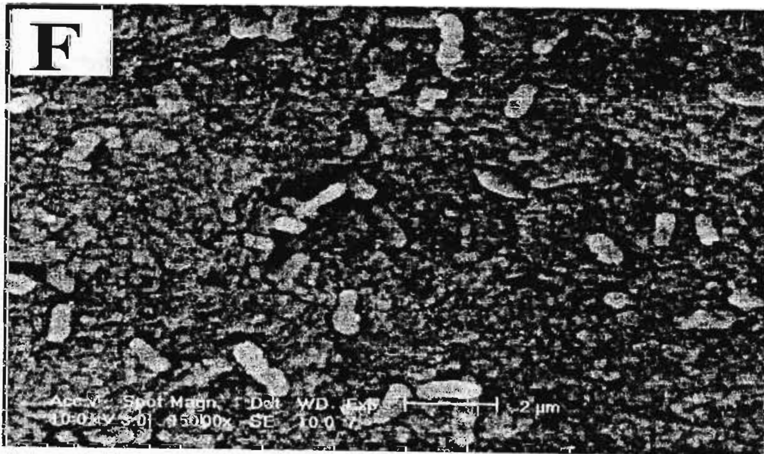
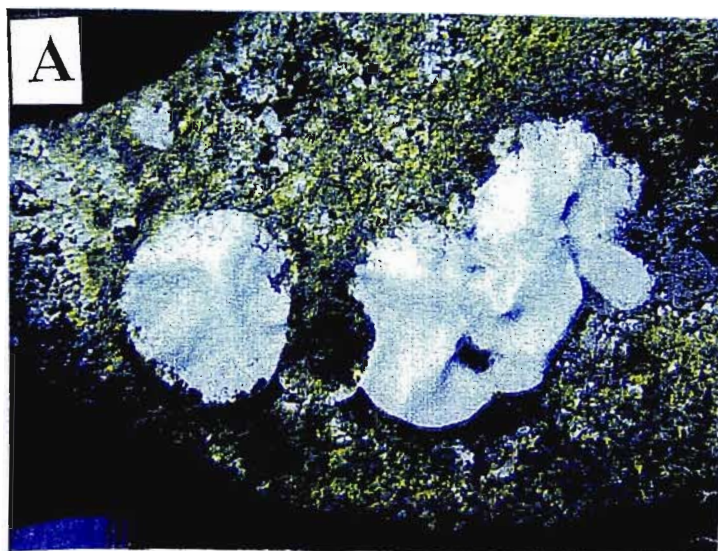
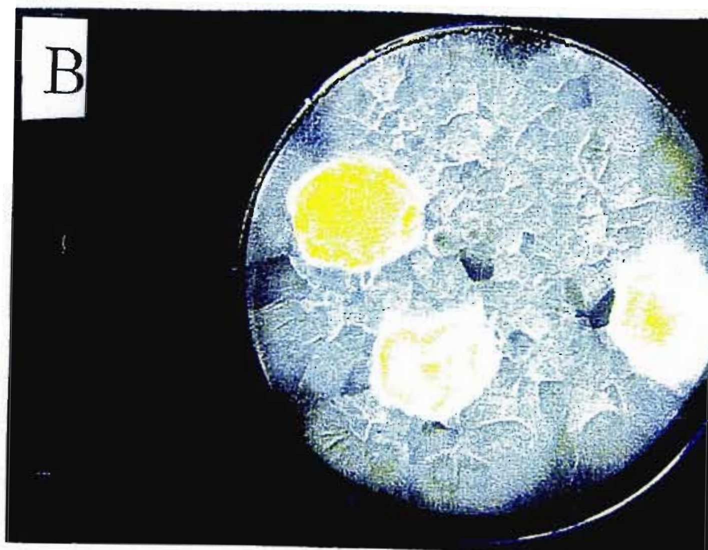
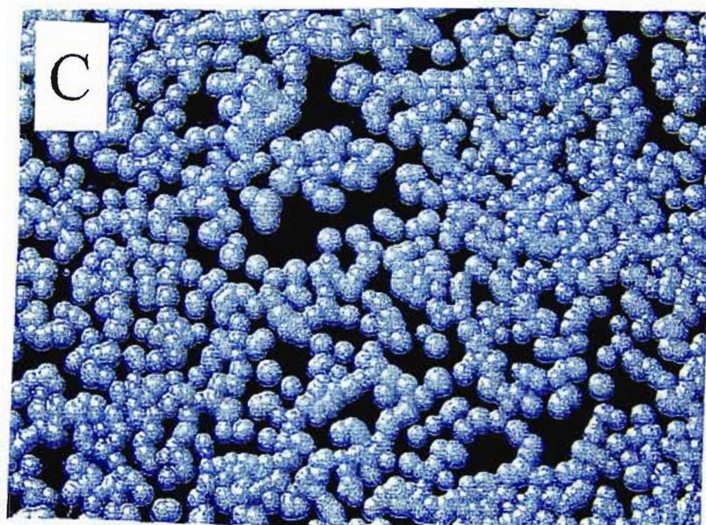



Plate 3.2. Fungal isolates from liquid cultures spiked with creosote hydrocarbons.



The appearance of a mixed population after a week of incubation can be attributed to spores of microorganisms that could not grow in the contaminated soil but become activated as a result of the culture medium (mineral salts medium) (Alexander, 1999). At higher concentrations in benzo(a)pyrene and chrysene, the populations still remained predominantly of one morphotype at the end of incubation period ( Plates 3.1 E & F). This is attributed to the toxicity of the hydrocarbons tested at very high concentrations to some of the organisms present. This toxicity allowed only a few of the microorganisms present to grow in such culture medium (Vipulanandan *et al.*,1994; Alexander, 1999).

The fungal isolates were predominantly filamentous growths ( Plates 3.2 A & B). However, yeasts were also isolated in some of the cultures (Plate 3.2 C). Fungi have been reported to constitute an important part of soil microflora and may be five to ten times more than bacteria in biomass (Fritsche, 1992). They are also known to break down xenobiotics by extracellular enzyme activity (Sanseverino *et al.*, 1994). However, their activity is generally lower than those of bacteria in the soil (Fritsche, 1992). Fungal spores are resistant to a wide range of adverse conditions and are ubiquitous because of their lightness. It is therefore no surprise to isolate fungi from such a contaminated soil.

The presence of this heterogeneous population in the enrichment culture is an indication that, given the right conditions, microbiological treatment of the contaminated soil will be possible. Although no major taxonomic characterisation was intended, the majority of the bacterial isolates were found to be Gram-negative, and some of the isolates could possibly belong to the genera *Arthrobacter, Bacillus, Pseudomonas, Mycobacterium, Alcaligenes, Corynebacterium, Rhodococcus, Proteus, Norcadia, Acinetobacter* or *Flavobacterium*, as these organisms have been previously isolated from different hydrocarbon-contaminated media in the past (Cerniglia,1979, Baker and Herson,1994). Even though there seemed to be

one or two dominant species in a number of cases, it was obvious that the isolated species showed individual, and possibly collective, capability to utilize the hydrocarbons tested. The mouldy associations were seen to have fewer bacterial colonies in them and were mostly isolated from the higher molecular mass cultures at higher concentrations. However, the yeast colonies were found to occur in both the associations with high bacterial density and those with high mould density.

## 3.4 CONCLUSIONS

From these results it is apparent that microbial utilization of creosote, phenolic compounds and polycyclic aromatic hydrocarbons (PAHs) from soils contaminated with creosote is closely related to the molecular mass and concentration of the compounds in liquid culture. Most of the creosote components tested supported better microbial growth at a concentration of 5 000mg $L^{-1}$.

Even though optimal pH for the biodegradation of organic compounds has been shown to be between 7 and 7.5 at $30^{O}$ C, a wider range of pH (between 5 and 8) can still support microbial activity to the extent that biodegradation can take place.

More microbial species can acclimatise to media containing lower molecular mass compounds than those containing higher molecular mass compounds. Higher molecular mass compounds are found to support fewer types of organisms. This was found to be more obvious at higher concentrations (20 000mg $L^{-1}$ and above).The lower numbers of microorganisms at higher concentrations was an indication that some form of biostimulation and possibly bioaugmentation would be necessary during the bioremediation operation.

There were more fungal species metabolising higher molecular mass hydrocarbons at concentrations above 20 000mg $L^{-1}$ than bacterial species.

Hydrocarbon catabolizing microorganisms become better adapted to utilizing particular hydrocarbons with repeated subculturing in media containing such hydrocarbons. Thus the time of exposure of the organisms to the substrate is important in the acclimation of the organisms to the polluting compound. The microbial utilization of creosote and the tested hydrocarbons in liquid culture is similar. This is an indication that all components of the contaminant creosote can be treated biologically.

### 3.5 Recommendations

The intimate relationship between microorganisms and contaminants in liquid culture is a function of the solubility of the contaminant in aqueous solution. This relationship enhances the utilization of the contaminant. However, this situation may be different in soil conditions, as the contaminant may sorb to varying degrees to soil particles and reduce the availability of the contaminants to microbial attack. From the above results, concentration has been observed to play a role in the degradation of the compounds studied. The next two chapters will be devoted to studying the effect of some locally available surfactants on the degradation of creosote and the reaction-kinetics of creosote degradation in liquid culture. It is hoped that these studies will enhance further understanding of the interaction between microorganisms and creosote in the environment.

# CHAPTER 4

## SURFACTANTS AS POSSIBLE ENHANCERS IN BIOREMEDIATION OF CREOSOTE-CONTAMINATED SOIL

### 4.1 INTRODUCTION

The biodegradation and mineralization of compounds present in creosote in soil and water have been well studied in the past (Eriksson *et al.*, 2000; Breedveld and Sparrevik, 2000; Broholm and Arvin, 2001). The activities of aerobic and anaerobic microorganisms in degrading the extensive number of compounds found in creosote is well documented (Annweiler *et al.*, 2001; Sullivan *et al.*, 2001). Studies have shown that these compounds can be degraded by different microorganisms under very diverse environmental conditions. Degradation of compounds typically found in creosote have been shown to occur at concentrations up to 50 000ppm as reported by Lajoie and Strom (1994). Although the concentration of the contaminating compound may play a role in the acclimation of the degrading organisms to the contaminated soils, organisms are known that have the capability to adapt to relatively high concentrations of these hydrocarbons (Lajoie and Strom, 1994).

The behaviour of organic compounds in water plays a very significant role in their availability for microbial utilization in the environment. Similarly, the sorption of hydrocarbon molecules to soil particles is an important factor in reducing the availability of the compound for microbial attack. Adsorption is the process of concentrating materials at the surface of organic and inorganic soil colloids and is attributed to the colloids' highly reactive surfaces and large surface

areas (Tan, 1998). The partitioning of PAHs to sediments strongly correlates with the organic carbon content of the sediment and the contaminant's relative hydrophobicity. This is typically described by its octanol/water partition coefficient ($K_{ow}$) (Karickhoff, 1980). Environmental parameters influence this equilibrium partitioning of PAHs between soil solution and the organic fraction. Temperature has a major effect (Platt *et al.*, 1996). PAHs exhibit a wide range of octanol/water partition coefficients. For example, naphthalene's log $K_{ow}$ (3.37) (Hansch and Fujita, 1964) is almost 5400 times less than that of benzo(g,h,i)terylene (log $K_{ow}$ = 7.10) (Yalkowsky and Valvani,1979), as reported by Hughes *et al.* (1997).

The hydrophobicity of many hydrocarbons, including the PAHs, have been found to be directly related to the number of rings in the compound's structure ( Carriere and Mesania, 1995). Thus, the aqueous solubility and volatility of such compounds, which decreases with increasing molecular mass (Carriere and Mesania, 1995), determines how much is available for microbial utilization. The aqueous phase concentration of individual contaminants in sediments or water systems, and the mass of an individual contaminant available for bioremediation, can vary greatly (Hughes *et al.*, 1997). The low solubility and high hydrophobicity of these compounds makes them less susceptible to bacterial attack (Sanseverino *et al.*, 1994; Yateem *et al.*, 2002).

Nonaqueous phase liquids (NAPLs) are low-solubility, hydrophobic chemicals that have a wide variety of uses and applications and are commonly known to contaminate soil and ground water (Kimball,1994). Their profuse usage has led to widespread and long-standing accumulations that have constituted disposal problems and consequent environmental concern. Nonaqueous phase liquids (NAPLs) of environmental concern include chlorinated organic compounds, polycyclic aromatic hydrocarbons of petroleum and creosote origin and other long-chain hydrocarbons.

Nonaqueous phase liquids may exist in two forms and/or four phases (Kimball,1994). The light nonaqueous phase liquids (LNAPL) include compounds that are lighter than water, whereas the dense nonaqueous phase liquids (DNAPL) consist of compounds that are heavier than water. Four NAPL physical states or phases can be found in the subsurface: air or vapour phase, phase adsorbed to soil solids, water phase (i.e. contaminants solubilized in water), and immiscible liquid phase (i.e. pure compounds pooled or trapped in soil interstitial pores) (Kimball, 1994).

Bioavailability, defined as the ability of a compound to be freely transported across the cell membrane for intracellular metabolism or available for extracellular metabolism, may be the most important factor limiting bioremediation of PAHs. For the metabolism of a compound to proceed at an appreciable rate, the compound has to be dissolved in a medium (Mihecic and Luthy, 1991; Guerin and Boyd, 1992). PAHs in soil are seen to partition to soil particles, sediments, humic material and other organic matter and biomass ( Gjessing and Berglind, 1981; Mihelcic and Luthy, 1991; Miller and Alexander, 1991).

Bioremediation is a water-based process influenced by sorption (adsorption/desorption), diffusion and dissolution mechanisms which determine the accessibility of the organic molecules to the bacteria that are present in the water. In most cases sorption is the rate-limiting step controlling both the rate and extent of biodegradation (Carriere and Mesania,1995). If bioremediation is to proceed at meaningful rates in the soil, desorption of the target molecules will have to be enhanced to facilitate bioavailability and consequent biodegradation of the sorbed molecules. Attention has, therefore, been given to the possible use of surfactants for the removal of sorbed pollutants in contaminated aquifers (Lee *et al.*, 1988). Surface-active agents or surfactants are thought to improve the stability of the emulsion and thus act as an emulsifier, the surfactant

reducing the surface or interfacial tension of the emulsion. Most emulsifying agents are surfactants but not all surfactants are emulsifying agents (Becher, 1965). Surfactants have been suggested as possible enhancers of desorption and, possibly, biodegradation of PAHs and other organics in the soil environments (Sanseverino *et al.,* 1994). Surfactants have also been found, by Oberbremer *et al.*(1990), to stimulate the degradation of a defined mixture of aliphatic and aromatic hydrocarbons in a soil suspension. Thus, the process of bioremediation can be enhanced by using surfactants to increase the availability of the PAH compounds to the microorganisms (Carriere and Mesania, 1995). This is a debatable issue, however, since it has also been reported (Hughes *et al.,* 1997) that the presence of non-ionic surfactants may decrease the rate of PAH degradation in soil.

Surfactants desorb a number of hydrophobic compounds sorbed to soil, for example, anthracene, phenanthrene, pyrene (Liu *et al.*, 1991), and PCBs (McDermott *et al.*, 1989). However, high concentrations of most synthetic surfactants are needed both to desorb the hydrocarbons and to bring these compounds of low water solubility into aqueous solution (Alexander, 1999).

Earlier studies have shown that the use of several types of surfactants in bioremediation studies has increased the solubility of hydrophobic compounds such as the PAHs. By substituting water with 0.5% (w/w)of emulsion of the surfactant Ethylan BCP the percentage of creosote leached from a sandy clay soil increased from 9% to 25%. Ethylan CD916 was found to leach 49.8% creosote from soil, compared to 37.2% leached with water. Both surfactants were found to increase the solubilization of creosote, from 9mg/L (amount solubilized with water alone) to 2 169 mg/L (ethylan CD916) and 2 858 mg/L (ethylan BCP) (Ellis,1994). At low concentrations (below 0.5%), these two surfactants are not known to be inhibitory to microbial growth.

The growth of microorganisms on hydrocarbons in a culture medium is often accompanied by emulsification of the insoluble carbon source (Gutnik and Rosenberg, 1977). This is generally attributed to the production of an extracellular emulsifier during growth on the hydrocarbon (Singer and Finnerty, 1984). Hydrocarbon uptake is, thus, facilitated by hydrophobisation of the cell envelope, or an increase of the interfacial area by hydrocarbon emulsifying biosurfactants (Singer and Funnerty, 1984). Nakahara *et al.* (1981), suggested that surfactants may stimulate degradation of hydrocarbons by making more of the surface of the chemical available. Commercial and industrial surfactants can play the same role as biosurfactants, particularly in areas where biosurfactants may not be adequate to emulsify large amounts of hydrocarbons in soil. These chemical dispersants may have different effects on the colonizing organisms and may disperse the hydrocarbons differently. Some chemical dispersants may enhance the oxidation of a particular hydrocarbon, while others will inhibit it (Cooney, 1984). However, if there are no toxic effects on the degradative population, dispersion could accelerate microbial degradation (Atlas, 1981). The molecular structure of surfactants consists of a hydrophobic group which has little affinity for the aqueous phase and a hydrophilic group which is readily soluble in the aqueous phase. Surfactants are also grouped into ionic and non-ionic surfactants. Generally, the ionic surfactants are known to be more toxic to microorganisms than the non-ionic ones (Hughes *et al.*, 1997). The mechanisms of enhancement of aqueous solubility of xenophobic compounds sorbed to soil particles have been studied by some researchers. Yeom *et al.* (1996) proposed two mechanisms to explain the surfactant-mediated release of sorbed PAHs. These were an increase of the concentration gradient at the soil-water interface, and an increase in the diffusivity of PAHs due to swelling of the soil organic matrix. From their experimental and model results, they concluded that the penetration of surfactant molecules, followed by swelling of the soil-tar

matrix, were responsible for the increase in diffusivity and were the primary factors for the enhanced PAH release from soil. Surfactants can increase the detergency of aqueous solutions through a number of processes including: *preferential wetting*, which involves the decreasing of the interfacial tension between the aqueous phase and the solid phase, *solubilization*, which results from the interaction of the surfactant molecules with molecules of the organic fraction and *emulsification*, which involves the dispersion of an insoluble organic phase within the aqueous phase (Sanseverino *et al.*, 1994).

The objective of the present study is, therefore, is to investigate the effect of different locally available commercial nonionic surfactants on the desorption and subsequent degradation of creosote hydrocarbons from contaminated soils and the concentrations at which they are most effective.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Batch culture treatment with surfactants

Into each of 50 250ml Erlenmeyer flasks were put 10g of a Mispah type (Lithosol; FAO) soil contaminated with industrial grade creosote (>250,000mg $kg^{-1}$). To each flask was added 100ml of sterile mineral salts medium. Sterile emulsions of five chemical dispersants, Arkopal-N-060, Arkopal-N-080, Arkopal-N-100, Hosaf-541-KS and Tween-80, were individually added to the flasks to give final concentrations of 0.01%, 0.1%, 0.35%, 0.5% and 1.0% (v/v). The suspension was thoroughly mixed in a rotary shaker at 150rpm for 1 hour and allowed to stand for another hour. The emulsifying capacity of the dispersants was estimated by measuring the turbidity of

the supernatant by spectrophotometry at a wavelength of 545nm (Mulkins-Phillips and Stewart, 1974). Control experiments without surfactants were used to compare the difference in turbidity. After 24 hours the mixture was used to determine the extent of hydrocarbon desorption, using infrared (IR) spectrophotometry.

The supernatant was decanted from the mixture and centrifuged before placing it in IR sample cells. The sample cells were then transferred into the spectrophotometer and scanned at wave numbers of between 400 and 4000 $cm^{-1}$ (Lees, 1996). The percent desorption was calculated from the difference from the initial concentration.

### 4.2.2 Enumeration of microorganisms

To determine whether the surfactants enhanced microbial growth, solutions were aseptically withdrawn from flasks containing similar preparations as above but incubated in a rotary shaker at 15rpm for three days. The solutions were filtered through 0.4μm membrane filters, and the filters were subjected to critical point drying prior to light microscope and scanning electron microscope examinations. The results from the surfactant solutions were compared with the results from the control experiments in which cultures with the different concentrations of the contaminated soil were set up without surfactants.

### 4.2.3 Batch culture determination of enhancement of degradation by surfactants

Into each of 50 250ml Erlenmeyer flasks were put 100ml each of mineral salts medium and 15g of soil contaminated with >250 000$mgkg^{-1}$ creosote. The five different surfactants were then

added separately to duplicate flasks in concentrations of 0.01%, 0.1%, 0.35%, 0.5% and 1.0%. A control which did not receive any surfactant was set up in duplicate. The flasks were incubated in the dark in a rotary shaker at 50 rpm for 21 days at $30^{O} \pm 2^{O}$ C. A 10 ml sample was taken from each flask and centrifuged for 1 min. at 500 rpm before transferring to an infrared spectrophotometer (IRS) cell. The samples were scanned at wave numbers of between 400 and 4000 $cm^{-1}$ to determine the changes in creosote concentration by comparing the peak areas. The extent of removal of selected creosote components in the soil was also determined by gas chromatography. The details of the GC/FID programme used are described in Chapter 3.

### 4.2.4 Determination of inhibitory effect of the surfactants

Nutrient agar plates were used to determine whether the surfactants inhibited or enhanced microbial growth. Double-layer agar plates, made by overlaying a support layer of mineral salts agar (MSA) containing 0.1% (v/v) soxhlet extracted creosote with a layer of molten agar containing 1 000, 5 000 or 10 000 mg $L^{-1}$ of the different surfactants, were used to determine carbon source utilization by inoculating the plates with 50μL of creosote + surfactant suspension from enrichment cultures, prepared as described above. The plates were incubated for 4 weeks in the dark. The method was adapted from Lees (1996).

## 4.3 RESULTS AND DISCUSSION

### 4.3.1 Emulsification properties of surfactants

The surfactants studied were nonionic surfactants. The Arkopal-N grades have ethylene oxide

forming the hydrophilic polyglycolether chains. The three types of Arkopal-N grade used are viscous, slightly yellow liquids. They have a pH of 7 in water. This was found to be ideal for microbial growth to facilitate creosote biodegradation. Arkopal-N-060 formed a cloudy solution in water. Arkopal-N-080 and Arkopal-N-100 were clear solutions at room temperature. Typically, the first two digits indicate the approximate number of molecules of ethylene oxide present. The Arkopal-N grade surfactants are used in the detergent and technochemical industries (Clariant Surfactant, Durban, South Africa, 2000).

Hosaf-541 K-S, a sorbitan oleate-type surfactant is a clear, amber, viscous liquid that is not very soluble in water. It has less than 1% (m/m) water content, a density of 0.99 g/cm$^3$ at 25$^{\circ}$C, an acid value of <6.0mg KOH/g, a saponification value of 130-150mg KOH/g, a flashpoint of 145$^{\circ}$C and its viscosity is 950-1350cp. Hosaf-541 K-S is commonly used as an emulsifier for explosives and ammonium nitrate fuel oil (ANFO)(Clariant Surfactants, Durban, South Africa, 2000).

Tween-80 is a viscous, colourless liquid that forms a slightly cloudy solution in water and is commonly used in the detergent industry (Hoechst S.A. (Pty) Ltd).

The Arkopal-N grade surfactants were generally found to greatly enhance desorption of creosote from soil (Fig. 4.1). The degree of desorption, however, varied according to the level of ethoxylation and the concentration of the surfactant used. Enhancement of creosote desorption increased with decreasing level of ethoxylation (Fig. 4.1). Arkopal-N-060 was found to be the most effective surfactant tested in desorbing creosote from contaminated soil with well over 80% of the contaminant creosote desorbed within 24 hours. These results support those of Lees (1996), who demonstrated that Arkopal-N-60 was effective in enhancing emulsification of oil

in soil.

Hosaf-541 K-S and Tween-80 were also effective in desorping creosote from contaminated soil. There was, however, no significant difference between the two compounds. Tween-80 was slightly more effective than Hosaf-541 K-S (Fig.4.1). The performance of Tween-80 is not surprising since a related compound (Tween-85) is documented to have an enhancing effect on the solubilization of oil (Sanseverino,1994).

The concentration of the surfactant was found to significantly affect desorption of the creosote from the soil. Measurements of percentage transmittance (which was used to determine the extent of desorption) at different concentrations showed that the higher concentrations had lower transmittance values for all the surfactants studied (Table 4.1), but GC results revealed that there is an optimal concentration range above and below which desorption begins to decrease. Fig. 4.1 shows that the effectiveness of all grades of Arkopal-N tested decreased slightly at concentrations above 0.5% and below 0.35%. Depending on the grade, they were found to function optimally at 0.35% and 0.5% in creosote-contaminated soil at room temperature. This discrepancy can be attributed to the initial cloudy suspension produced by the surfactants on dissolution in water. It can also be ascribed to the difference in standing time before the samples for GC analysis were taken. This difference in contact time could possibly have allowed for more chemical interaction between the sorbed creosote molecules and the surfactants, thereby improving the reduction of the surface tension and improving the wetting and solubilization capabilities of the surfactants. This concentration range of surfactants has been reported previously to be effective for desorption of creosote (Carriere and Mesania,1995) and petroleum (Lees,1996) in soil. The optimal concentration for Arkopal-N-060 and Arkopal-N-100 was 0.5%, whereas for Arkopal-N-

080 it was 0.35%, but the difference in the efficacy of the two concentrations was not statistically significant. Although slightly less effective than Arkopal-N-60, Tween-80 exhibited a concentration pattern similar to that of the Arkopal-N grade compounds. However, Hosaf-541 K-S showed increasing desorption activity with increasing concentration over the entire range tested (Fig.4.1). Surfactant concentrations up to 0.75% have been successfully used in the desorption of oil, as reported by Abdul *et al.*(1992). For all the surfactants tested, 0.1% was found to be the least effective concentration for desorption of creosote from the contaminated soil. However, irrespective of the concentration, all the surfactants showed enhanced desorption when compared with the control, which had no surfactant added. The concentration-limitation exhibited by these surfactants may be due to some of their physical and chemical characteristics, such as the octanol/water partition coefficients ($K_{ow}$) and the concentration at which they form micelles in aqueous solution, known as the critical micelle concentration (CMC)(Kimball, 1994). This may vary from one surfactant to another and may also be affected by other physical conditions and chemical characteristics of the target compound (Kimball, 1994).

### 4.3.2 Enhancement of creosote degradation by application of surfactants

The results of growth studies showed that none of the surfactants tested inhibited microbial growth at the concentrations used. Earlier reports have demonstrated the use of Tween-80 and Arkopal-N-grade surfactants in enhancing biodegradation of hydrocarbon compounds such as petroleum (Miller, 1995, Lees, 1996). Low hydrocarbon concentration, however, negatively affected the growth of microorganisms in all the surfactants tested ( Kimball, 1994). In the present investigation, combinations containing both higher creosote and surfactant concentrations were found to support more microbial growth (Table 4.2). Very low hydrocarbon concentrations

Table. 4.1. Percentage transmittance at 545nm of the supernatant of aqueous solutions containing different concentrations of selected commercial surfactants and 10 g of creosote-contaminated soil(250 000 mg $kg^{-1}$), after standing for one hour at 30 $^{O}C$

| Surfactants. | Concentration % (v/v) | % Transmittance (545nm) |
|---|---|---|
| Arkopal-N-060 | Control | 10.5 |
| | 0.01 | 10.2 |
| | 0.1 | 10.3 |
| | 0.35 | 10.5 |
| | 0.5 | 9.5 |
| | 1.0 | 5.0 |
| Arkopal-N-080 | Control | 13.8 |
| | 0.01 | 13.5 |
| | 0.1 | 12.3 |
| | 0.35 | 11.5 |
| | 0.5 | 11.1 |
| | 1.0 | 7.5 |
| Arkopal-N-100 | Control | 14.6 |
| | 0.01 | 13.8 |
| | 0.1 | 13.5 |
| | 0.35 | 11.5 |
| | 0.5 | 10.4 |
| | 1.0 | 8.7 |
| Hosaf.541-K-S | Control | 11.5 |
| | 0.01 | 10.7 |
| | 0.1 | 10.3 |
| | 0.35 | 8.6 |
| | 0.5 | 8.2 |
| | 1.0 | 5.9 |
| Tween-80 | Control | 11.8 |
| | 0.01 | 11.3 |
| | 0.1 | 10.5 |
| | 0.35 | 9.5 |
| | 0.5 | 9.2 |
| | 1.0 | 7.4 |

have been found not to effectively support growth of microorganisms( Baker and Herson, 1994). Dilution of the culture (inoculum source) did not seem to affect the creosote degradative activity of microorganisms very much. However, decreases in growth were occasionally noticed with an increase in dilution (Table 4.2). Generally, none of the surfactants was found to be inhibitory to microbial growth.

Infrared spectrophotometric results for all treatments showed that all the surfactants at all the concentrations tested, enhanced creosote degradation significantly, in some cases by more than 50% compared to the control (Fig. 4.2). Degradation at 0.5% surfactant concentration was significantly greater than at any other concentration for all the surfactants except Arkopal-N-100, where there was hardly any difference between the degradative activity at the concentrations 0.35% and 0.5%. Creosote degradation in the presence of 0.5% surfactant occurred in the order:

Arkopal-N-60 > Hosaf-541 K-S >Arkopal-N-80 > Tween-80 > Arkopal-N-100.

Enhancement of creosote degradation in soil by addition of surfactants has been reported by many workers (Carriere and Mesania, 1995; Diehl *et al.*, 1998; Shiohara *et al.*, 2001).

As far as the concentration was concerned, the effect of surfactant on creosote degradation was found to be in the following order:

0.5%>0.35%>0.1%>1.0%>0.01%>control (Fig. 4.2).

The results from gas chromatography analysis also showed that removal of the individual creosote compounds studied was reasonably enhanced in the presence of the surfactants (Fig. 4.3-4.8).

Table 4.2: Growth of indigenous microorganisms from enrichment cultures inoculated with contaminated soil containing low (<5 000mg kg$^{-1}$)(LC), medium (5 000-250 000mg kg$^{-1}$)(MC) and heavy (>250 000mg kg$^{-1}$)(HC) levels of creosote on MSA plates containing creosote and overlaid with NA containing single surfactants at different concentrations.

| | | | GROWTH | | | | | |
|---|---|---|---|---|---|---|---|---|
| Inoculum source. | Culture dilution. | Surfactant conc. mg $l^{-1}$ | Arkopal-N-060 | Arkopal-N-080 | Arkopal-N-100 | Hosaf 541-KS | Tween-80 | Control |
| LC | $10^{-3}$ | 1000 | + | - | - | + | - | - |
| LC | $10^{-5}$ | 1000 | - | - | - | - | - | - |
| LC | $10^{-7}$ | 1000 | - | - | - | - | - | - |
| LC | $10^{-3}$ | 5000 | + | + | - | + | + | - |
| LC | $10^{-5}$ | 5000 | + | + | - | + | - | - |
| LC | $10^{-7}$ | 5000 | - | - | - | + | - | - |
| LC | $10^{-3}$ | 10000 | + | ++ | + | + | + | - |
| LC | $10^{-5}$ | 10000 | + | + | + | - | + | - |
| LC | $10^{-7}$ | 10000 | + | - | + | + | + | - |
| MC | $10^{-3}$ | 1000 | + | ++ | + | ++ | ++ | - |
| MC | $10^{-5}$ | 1000 | + | + | + | + | + | + |
| MC | $10^{-7}$ | 1000 | + | - | + | + | + | + |
| MC | $10^{-3}$ | 5000 | ++ | ++ | +++ | +++ | ++ | + |
| MC | $10^{-5}$ | 5000 | ++ | ++ | + | ++ | ++ | + |
| MC | $10^{-7}$ | 5000 | ++ | + | ++ | ++ | ++ | + |
| MC | $10^{-3}$ | 10000 | +++ | +++ | +++ | +++ | +++ | - |
| MC | $10^{-5}$ | 10000 | +++ | +++ | ++ | +++ | ++ | - |
| MC | $10^{-7}$ | 10000 | ++ | ++ | ++ | +++ | ++ | - |
| HC | $10^{-3}$ | 1000 | +++ | +++ | +++ | +++ | +++ | + |
| HC | $10^{-5}$ | 1000 | ++ | +++ | ++ | +++ | ++ | + |
| HC | $10^{-7}$ | 1000 | ++ | ++ | ++ | +++ | ++ | + |
| HC | $10^{-3}$ | 5000 | +++ | +++ | ++ | +++ | +++ | + |
| HC | $10^{-5}$ | 5000 | +++ | +++ | ++ | +++ | +++ | + |
| HC | $10^{-7}$ | 5000 | +++ | +++ | ++ | +++ | ++ | + |
| HC | $10^{-3}$ | 10000 | +++ | +++ | +++ | +++ | +++ | + |
| HC | $10^{-5}$ | 10000 | +++ | +++ | +++ | +++ | +++ | + |
| HC | $10^{-7}$ | 10000 | ++ | +++ | ++ | +++ | +++ | + |

Note:+ = Sparse growth ++ = Moderate growth +++ = Profuse growth Low contamination (LC) Medium contamination (MC) Heavy contamination (HC)

Removal of most of the phenolics was above 60% within 21days. Phenol and naphthalene degradation were most affected by the action of the surfactants. The degradation of phenol in the presence of Arkopal-N-80 and Arkopal-N-100, was similar to that of the PAHs, which were poorly degraded in the presence of these two surfactants (Fig.4.5 and 4.6). This low level of degradation is believed to be due to the reduced ability of these surfactants to desorp the compounds as a result of their higher level of ethoxylation (manufacturer's information). Arkopal-N-60 and Hosaf-541 K-S showed relatively better enhancement when compared with the results from the control (Fig.4.4 and 4.7). The enhanced hydrocarbon degradation capacity is attributed to improved desorption of the compounds from the soil matrix.

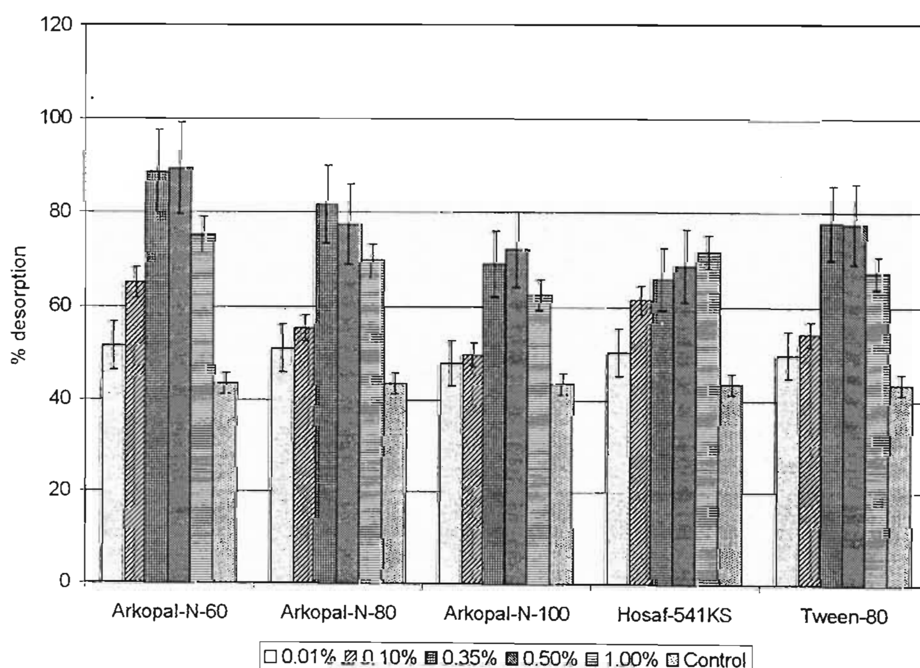



Fig. 4.1. Desorption of creosote after 120 hours contact time from contaminated soil treated with surfactants. Error bars represent ± 1 Standard Error.

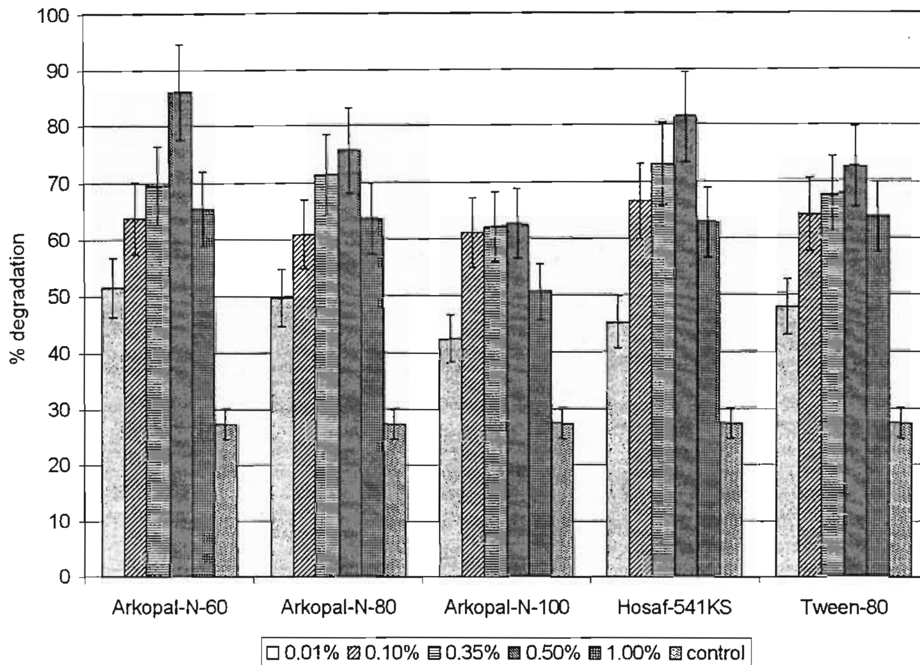



Fig. 4.2. Degradation of creosote after 21 days in contaminated soil treated with surfactants. Error bars represent ± 1 Standard Error.

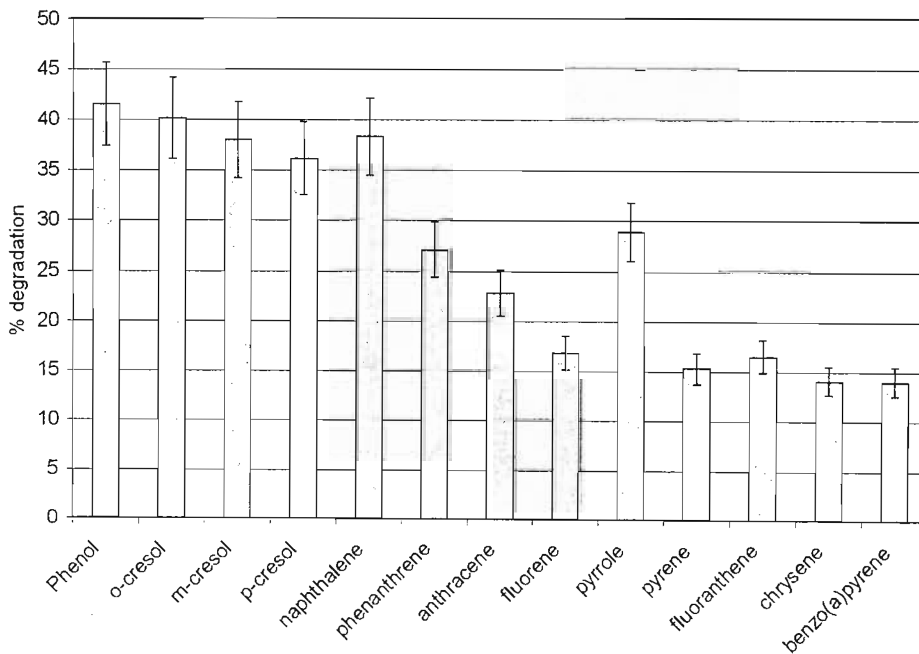



Fig. 4.3. Degradation of selected creosote components after 21 days in contaminated soil without surfactant. Error bars represent ± 1 Standard Error.

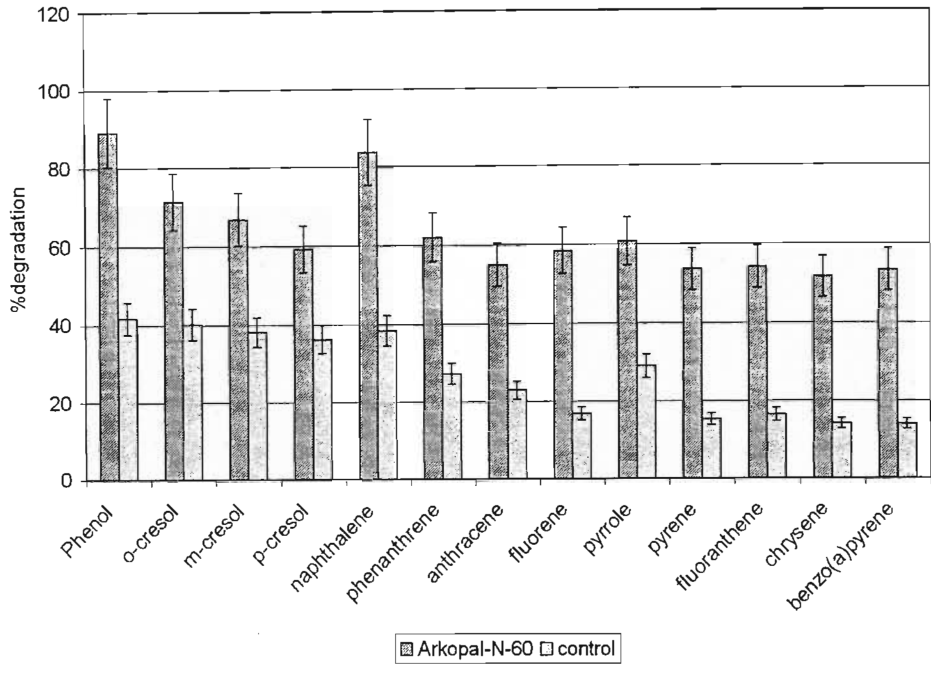



Fig. 4.4. Degradation of selected creosote components after 21 days in contaminated soil treated with 0.5%(v/v) Arkopal-N-060. Error bars represent ± 1 Standard Error.

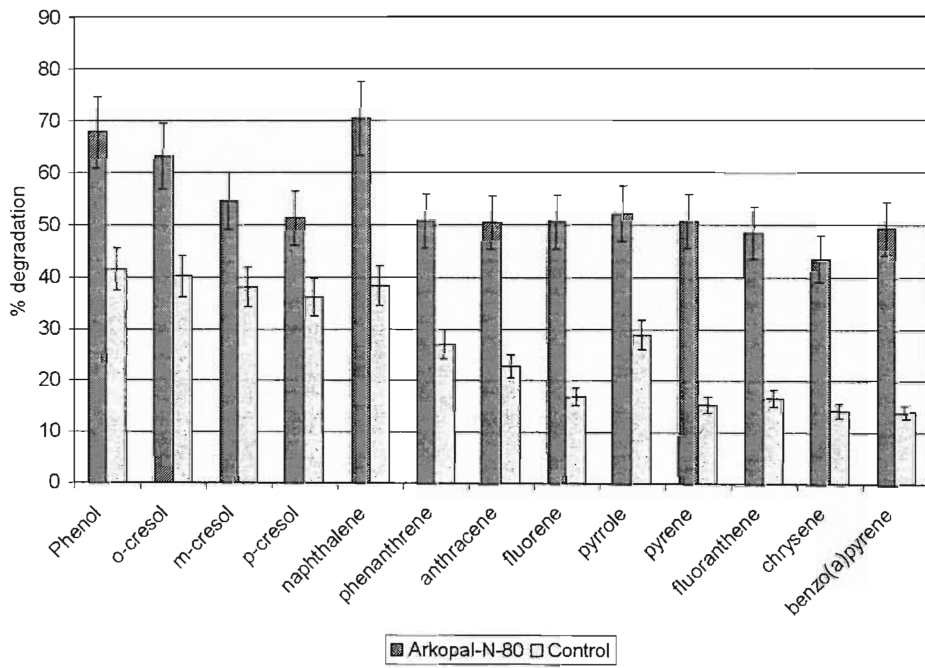



Fig. 4.5. Degradation of selected creosote components after 21 days in contaminated soil treated with 0.5% (v/v) Arkopal-N-080. Error bars represent ± 1 Standard Error.

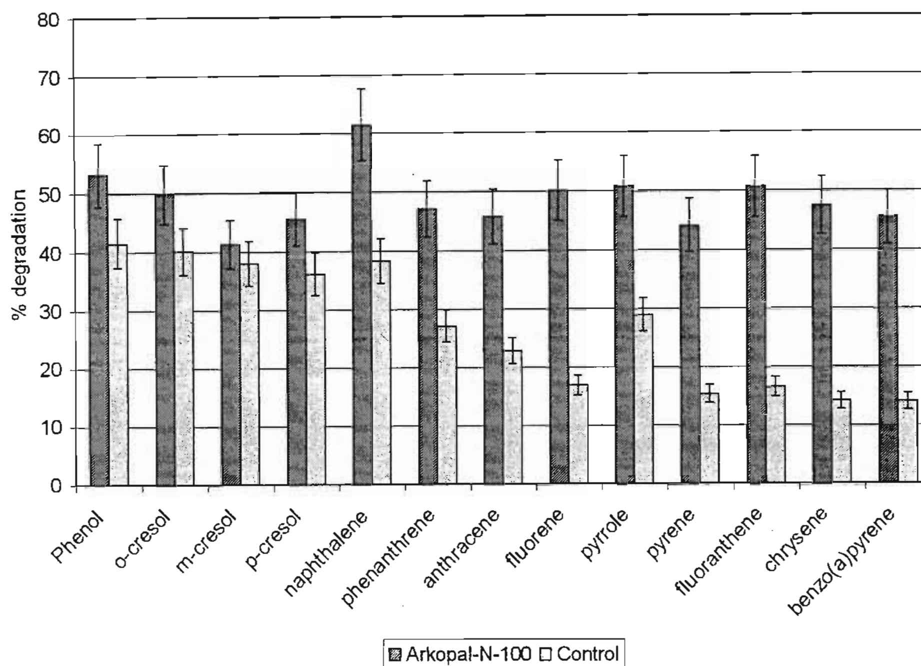



Fig.4.6. Degradation of selected creosote components after 21 days in contaminated soil treated with 0.5% (v/v) Arkopal-N-100. Error bars represent ± 1 Standard Error.

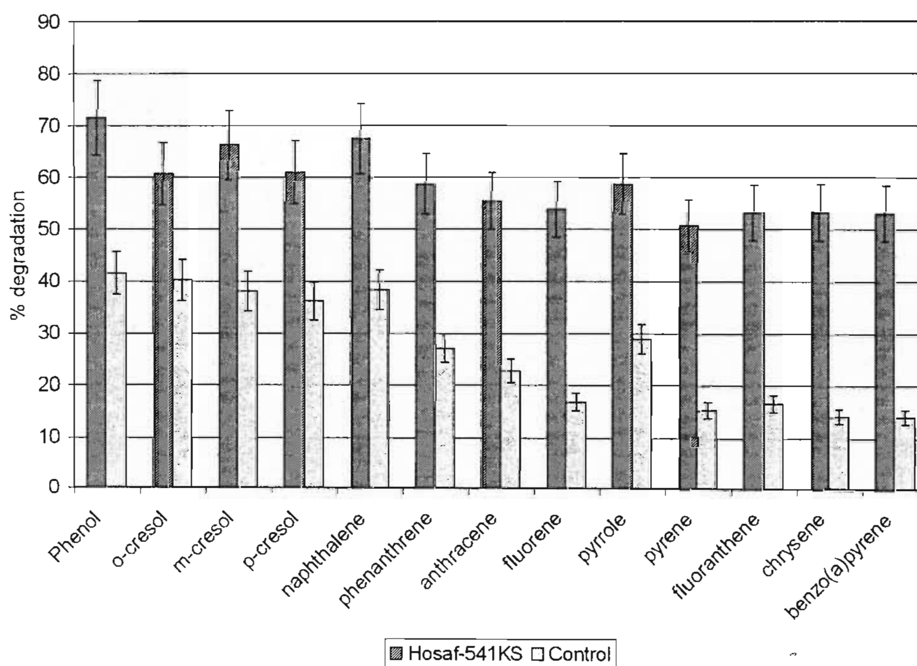



Fig. 4.7. Degradation of selected creosote components after 21 days in contaminated soil treated with 0.5% (v/v) Hosaf-541-KS. Error bars represent ± 1 Standard Error

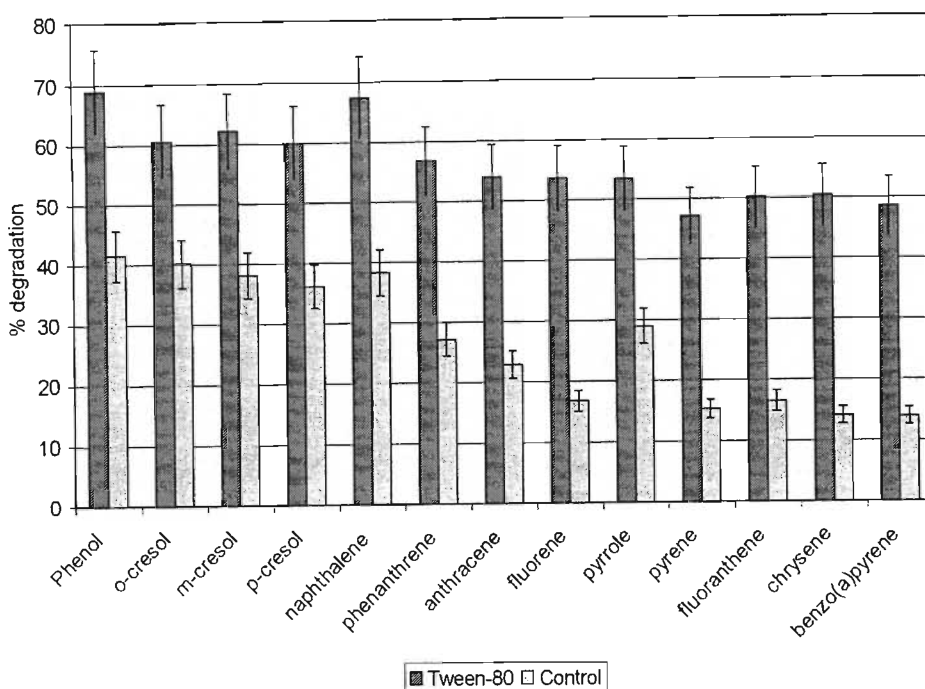



Fig.4.8. Degradation of selected creosote components after 21 days in contaminated soil treated with 0.5% (v/v) Tween-80. Error Bars represent ± 1 Standard Error.

## 4.4 CONCLUSIONS

The results obtained from the analyses show that surfactant application to soils contaminated with creosote hydrocarbons can greatly enhance the desorption of creosote from soil matrices. It is also apparent that the enhanced desorption facilitates solubilization which is necessary for effective degradation of the creosote compounds in soil. The concentration of surfactant was shown to play a significant role in both the desorption and degradation of creosote components in soil. None of the surfactants tested inhibited microbial degradation or desorption of creosote from soil. All the surfactants tested were therefore effective in enhancing removal of creosote from contaminated soils. Since the surfactants are biodegradable, the risks of seepage and groundwater contamination after the target molecules have been removed would not be very high.

However, a comparison of the results obtained from these experiments and those from the batch enrichment cultures in Chapter 3 shows that there were no significant differences in the degradation of creosote and of its components tested in the two set of experiments. This means that the application of surfactants to the creosote-contaminated soil would not, overall, significantly enhance the degradation of creosote. Although the surfactants are required in small concentrations to effectively desorb the contaminating creosote, their large-scale application was judged not to be cost effective on the size of land (125m X 75m X 0.4m) to be treated. This area of land represents a total volume of 4 219$m^3$ of soil. Based on these rationalizations, the further exploitation of the use of surfactants in the present study was discontinued.

# CHAPTER 5

# GROWTH KINETICS OF CREOSOTE-DEGRADING MICROORGANISMS IN DIFFERENT CONCENTRATIONS OF CREOSOTE AND SELECTED CREOSOTE COMPONENTS IN BATCH CULTURES

## 5.1 INTRODUCTION

Information on the reaction kinetics involved in biodegradation is essential for understanding the reaction processes, predicting the rate of reaction in response to changes in concentration of the substrate, temperature or pH, and optimizing the process variables involved. Knowledge of the kinetics of the biodegradation process is also vital for evaluating the persistence of organic pollutants in the environment and for assessing the levels of exposure of animals, plants and human beings to the substances. During degradation of a chemical pollutant, the amount of the substance degraded with time, and the shape of the disappearance curve, is a function of the compound in question, its concentration, the organisms responsible and a variety of environmental factors (Alexander, 1999). Thus, reaction kinetics allows for the quantification of the pollutant remaining in the environment at any given time and also permits prediction of the amount of the substance that may be present in the environment at some future time (Alexander, 1999). It is a measure of the microbial population's degradative efficiency (Limbert and Betts, 1995). Kinetic studies, therefore, will facilitate optimisation of the conditions governing maximum efficiency in a given system. Such information is useful for determining whether the pollutant will be degraded before it is transported to sites where it can constitute a hazard to plants, animals or human beings.

Kinetics parameters are constants specific for the particular system under study (Gaudy and Gaudy, 1980; Mulder *et al.*, 2001). Hence the biodegradative performance of different systems has been compared on the basis of kinetic features (Anselmo *et al.*,1989; Limbert and Betts,1995). Kinetic studies would also permit the detection and elucidation of possible interferences between various components in a multi-substrate medium (Limbert and Betts,1995).

Research on reaction kinetics has focussed on two topics: (1) assessment of the factors that affect the amounts of the compound transformed per unit time. In this area, much information is available on the influence of temperature, pH, soil moisture and other carbon sources on the rates of transformation; (2) determination of the shapes of the curves that depict the transformation and evaluating which of the patterns of decomposition best fit the metabolism of given compounds in a microbial culture (Alexander, 1999; Schirmer *et al.*, 2000).

Most often, the rate of loss of the parent molecule is used as the determining factor in kinetic studies. This is the case in situations where the inhibitory effect of the compound is completely destroyed upon degradation, or where toxic intermediates do not accumulate. In other cases, mineralization of the compounds, which signifies detoxification, is used as a source of information in kinetic studies.

The study of the kinetics of biodegradation in natural environments is often empirical, reflecting the rudimentary level of knowledge about microbial populations and activity in these environments. An example of the empirical approach is the power rate model (Hamaker, 1972)

$$-dC / dt = kC^n,$$

where $C$ is substrate concentration, $t$ is time, $k$ is the rate constant for chemical disappearance and

$n$ is a fitting parameter. This model can be applied to substrate disappearance curves by varying $n$ and $k$ until a good fit is achieved (Alexander, 1999). In this equation it is obvious that the degradation rate is proportional to a power of the substrate concentration. This model provides a basis for comparison of different curves, but does not give any explanation or reason for the shapes obtained. Hence, it is often not predictive of the reaction outcome. An appropriate introduction to the kinetics of biodegradation is to consider a pure culture of a specific bacterium growing on, and degrading, a single, soluble organic compound and to assume that no barriers exist between the substrate and the cells (Alexander,1999; Mulder *et al.*, 2001).

The biodegradation of a particular organic substrate may be carried out by microorganisms that are: (a) growing at the expense of that substrate and using it as a source of carbon and energy, or possibly as a source of another nutrient element needed for proliferation; (b) growing at the expense of another organic nutrient that is used as a source of carbon, energy, or both, but metabolizing the substrate of interest (although not using it to supply building blocks for cell synthesis); or (c) not growing as they metabolize the chemical of concern (Alexander, 1999). In the latter case the growth rate of the organism will continue to increase under ideal conditions, with increase in concentration of the substrate. However, above some moderately high substrate concentration, the growth rate does not rise markedly with increasing concentrations. At very high substrate levels, the growth rate may cease to increase with further increase in concentration (Alexander, 1999).

The earliest attempt at modelling the kinetics of bacterial growth was by Monod (1949) in Vipulanandan *et al.* (1994). In both batch and continuous culture systems the rate of growth of bacterial cells can be defined by the following relationship (Vipulanandan *et al.*, 1994):

$$r_g = \mu X \qquad (1)$$

where: $r_g$ = rate of bacterial growth ( mass/unit volume · time)

$\mu$ = specific growth rate ($time^{-1}$)

$X$ = concentration of microorganisms (mass/unit volume)

In general, $\mu$ will be a function of either the initial ($S_0$) or current concentration (S) of the substrate compound. Because dX/dt = $r_g$ for a batch culture, the following relationship is also valid for a batch culture:

$$dX / dt = \mu X \qquad (2)$$

Based on experimental data, Monod proposed that the effect of a limiting substrate or nutrient concentration can often be defined adequately by the following equation:

$$\mu = \mu_m \quad S / Ks+S \qquad (3)$$

Where $\mu_m$ = maximum specific growth rate ($time^{-1}$ )

S = concentration of growth-limiting substrate in solution (mass/unit volume)

Ks = half-saturation constant, substrate concentration at half the maximum growth rate (mass/unit volume)

If the value of $\mu$ from Eq. (3) is substituted in Eq. (1), the resulting expression for the rate of growth is:

$$r_g = \mu_m XS / Ks + S \qquad (4)$$

Several researchers have found that the Monod expression does not adequately describe the degradation of toxic organic molecules because the model is valid only if the substrate is not inhibitory to its own biodegradation. If the compound is inhibitory, the Haldane modification of the Monod expression better describes the process. The Haldane equation is:

$$r_g = \mu_m XS / Ks + S + S^2 / K_1 \qquad (5)$$

where $K_1$ = inhibition coefficient (mass/unit volume)(Vipulanandan *et al.*, 1994).

Different kinetic models have been suggested for substrates that are inhibitory to the growth of the degrading organisms. However, it is necessary to carry out a detailed study of the phenomena occurring in the system under investigation before any prediction is made, since models provide only reasonable approximations to the real process. In a mixed culture situation, a number of interactions such as competition, synergism and antagonism between the microbes are known to occur, kinetic model is thus only a phenomenological representation of the actual scenario (Vipulanandan *et al.*, 1994). However, any useable model must recognise the essential features such as peak specific growth rate, inhibitory substrate concentration, and degree of inhibition (Vipulanandan *et al.*, 1994; Shrout and Parkin, 2000).

Most kinetic studies on hydrocarbon substrates are based on individual components of the hydrocarbons and pure cultures of specific microorganisms. However, natural environmental conditions that prevail in contaminated soils often consist of a complex mixture of hydrocarbons such as creosote and crude oil and a mixed population of soil organisms whose individual and interactive actions jointly influence many growth parameters in the soil.

The aim of this chapter is to determine the growth rate ( $\mu$ ) of the consortia of degrading soil microorganisms in different concentrations of creosote in liquid culture, with a view to identifying the pollutant concentration(s) at which the population's growth rate is highest and the concentration(s) at which the substrate becomes inhibitory. It is also aimed at identifying those components in creosote which best support the growth of the degrading organisms and those which are inhibitory to their growth. Results obtained from the present study will be used to

determine the potentials of microorganisms present in the contaminated soil to degrade the contaminant creosote. The estimated period of the remediation programme will also be determined.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Chemicals

The hydrocarbon compounds used were creosote, phenol, o-cresol, m-cresol, p-cresol, naphthalene, anthracene, phenanthrene, pyrrole, fluorene, pyrene, chrysene, fluoranthene and benzo (a) pyrene. All chemicals were analytical grade except creosote, which was commercial grade.

### 5.2.2 Culture media

The vitamin supplemented mineral salts medium (MSM) contained ($L^{-1}$): $K_2HPO_4$ 1.5g; $MgCl_2.6H_2O$, 0.2g; $NaH_2PO_4.2H_2O$, 0.85g; $Na_2SO_4$,1.4g; $NH_4Cl$, 0.9g; $NaHCO_3$,0.5g; $NaCO_3$, 0.2g; 1ml (1mM solution of $NiCl_2.6H_2O$), 1ml vitamins (containing in mg $L^{-1}$ : biotin, 10; p-aminobenzoic acid,10; folic acid,10; pyroxidine HCl, 20; thiamine, 20; riboflavin, 30; nicotinic acid, 50) and 1ml trace elements solution which contained in mg $L^{-1}$ : $FeCl_2.2H_2O$, 1500; NaCl, 9000; $MnCl_2.4H_2O$,197; $CaCl_2$, 900; $CoCl_2.H_2O$, 238; $CuCl_2.H_2O$, 17; $ZnSO_4$, 287; $AlCl_3$, 50; $H_3BO_3$, 62; $NiCl_2.6H_2O$, 24; $Na_2MoO_4.2H_2O$, 48.4; $NaSeO_3.xH_2O$ (31% Se), 2.55; $Na_2WO_4.2H_2O$, 3.3; 10ml Conc. HCl.

### 5.2.3 Enrichment culture

Enrichment for soil-inhabiting microorganisms capable of utilizing creosote was carried out by using the mineral salts medium (MSM) described above and prepared as described in Chapter 3. Erlenmeyer flasks (250 ml)containing 100ml MSM, described in section 5.2.2, spiked separately with 50, 100, 500, 1000, 5000, 10 000, 15 000, 20 000, 25 000, and 30 000 mg $L^{-1}$ of creosote, phenol, o-cresol, m-cresol, p-cresol, naphthalene, anthracene, phenanthrene, pyrrole, fluorene, pyrene, chrysene, fluoranthene or benzo(a)pyrene, were prepared and inoculated as described in Chapter 3 and incubated for 21 days at $30^0 \pm 2^0$ C in a rotary shaker at 150 rpm in the dark. At 21-day intervals subcultures were made from these cultures, as described in section 3.2.3, Chapter 3. This was performed six times to enrich for cultures with improved substrate-degrading qualities.

### 5.2.4 Growth experiments in conical flasks

Growth of soil microbial populations enriched on whole creosote or one of the selected creosote components listed above was investigated in duplicate 250 ml conical flasks containing 100 ml MSM spiked with the appropriate compound at the concentrations specified above. A one ml sample of each culture was used to inoculate the corresponding spiked medium. Control flasks were identically prepared, except that they lacked the carbon substrate. All flasks were incubated in a rotary shaker at 100 rpm at $30^O$ C in the dark. This method was modified from Bouchez *et al.* (1995).

### 5.2.5 Analytical procedures

Changes in concentrations of residual creosote and the selected creosote components and the microbial biomass were measured over time. The concentrations of the residual hydrocarbons were measured by gas chromatography (GC) with a flame ionization detector, as described in Chapter 4 section 4.2.3 (v). Each sample was analysed in duplicate. The sample volume injected in each case was 4µ*l*. The GC was calibrated for each of the carbon sources. Changes in residual creosote concentration were determined by infrared spectrophotometer (IRS), as described in Chapter 4. Two methods were used for determining biomass. Firstly, the gravimetric method, in which a 25 ml sample of each culture was filtered, separately, through a 0.45 µm membrane filter. The filter was dried in an oven at 100$^{o}$ C for 6 hours and then weighed to one decimal place (0.1mg), using a Mettler AE240 analytical balance. The dry mass of the bacteria was then obtained from the difference between the mass of the dried filter and the tare. The tare was the mass of an identical membrane filter through which 25ml of fresh salt solution had been filtered before drying at 100$^{o}$ C for 6 hours. Ten tare measurements were made and the average of these was used in subsequent calculations. This method was adapted from Vipulanandan *et al.* (1994). Secondly, spectrophotometric analysis was used. This entailed measuring the absorption of light by the samples at a wavelength of 540 nm in a Milton Roy Spectronic 301 spectrophotometer, to determine microbial cell density in cultures spiked with creosote components.

## 5.3 RESULTS AND DISCUSSION

### 5.3.1 Biomass increase and substrate utilization

Figures 5.1 to 5.10 show the rates of substrate utilization and biomass increase in the enrichment medium spiked with increasing concentrations of creosote. The patterns show that growth of the microorganisms and the resultant decrease in concentration of the substrate is a function of the concentration (Tate, 1995; Alexander, 1999). Very low substrate concentrations are known not to support microbial growth and, as a result, limit biodegradation (Tate, 1995). Very high concentrations likewise inhibit microbial activity and biodegradation of the compound is limited (Alexander, 1999). Poeton *et al.* (1999) observed that the rate of PAH degradation increased with increase in concentration up to the solubility coefficient of the compound. Thus, above and below certain concentrations, microbial activity and substrate degradation becomes critical. Large molecular mass, increased complexity of the molecules and the positions of attachment of functional groups also affect the degradation of the carbon substrate (Sutherland, *et al.*, 1995).

Results from the gravimetric measurement of biomass in cultures spiked with creosote show that the lag phase varied from zero in the 50 and 100mg $L^{-1}$ cultures (Fig. 5.1 and 5.2) to about 72 hours in the 30 000mg $L^{-1}$ cultures (Fig.5.10). Initial slow increase in biomass is typical of microbial populations introduced into a medium containing chemical substances to which the organisms must adapt before they can effectively commence utilizing the substrate (Alexander, 1999). This usually occurs when potentially metabolizable substrates, such as many xenobiotic compounds, are added to

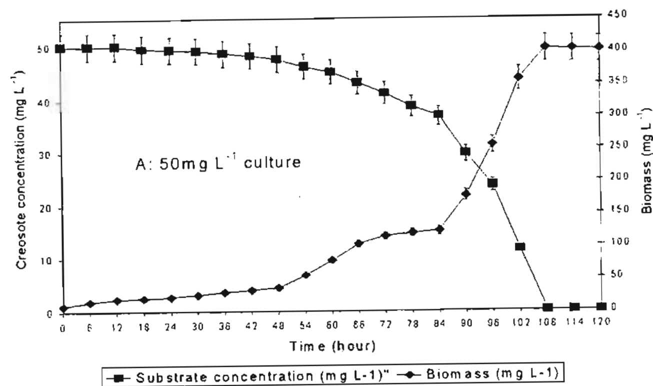



Fig.5.1. Changes in microbial biomass and substrate concentration with time in medium containing 50mg $L^{-1}$ creosote. Error bars indicate ± 1 Standard Error.

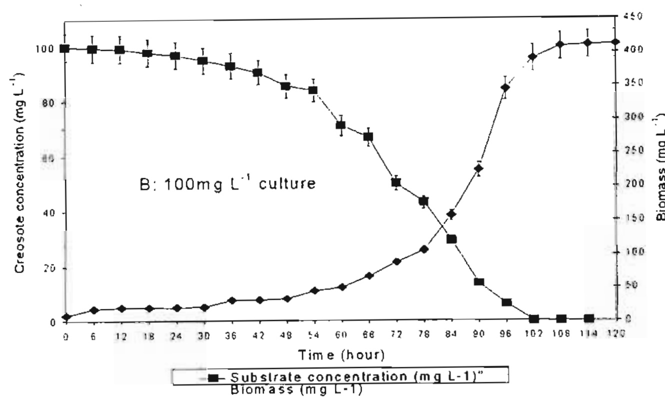



Fig.5.2. Changes in microbial biomass and substrate concentration with time in medium containing 100mg $L^{-1}$ creosote. Error bars indicate ± 1 Standard Error.

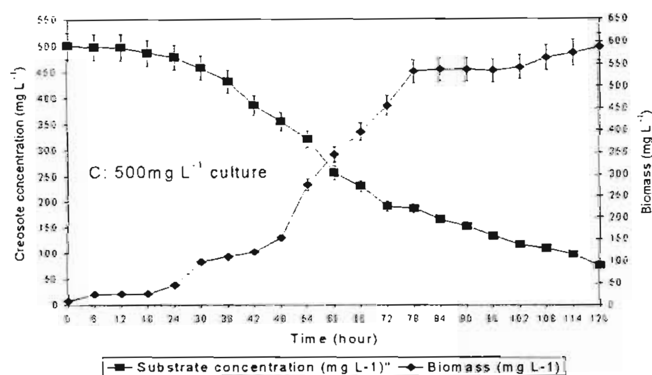



Fig.5.3. Changes in microbial biomass and substrate concentration with time in medium containing 500mg $L^{-1}$ creosote. Error bars indicate ± 1 Standard Error.

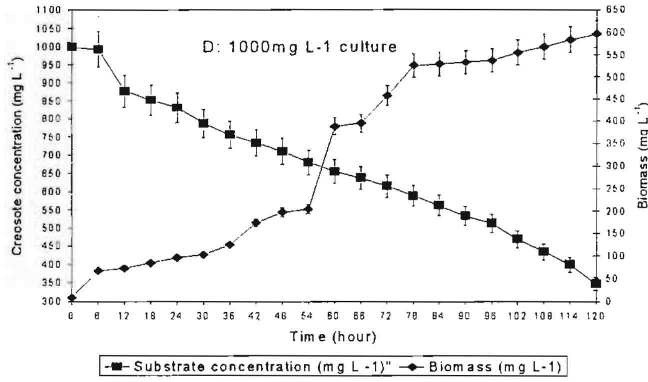



Fig.5.4. Changes in microbial biomass and substrate concentration with time in medium containing 1000mg $L^{-1}$ creosote. Error bars indicate ± 1 Standard Error.

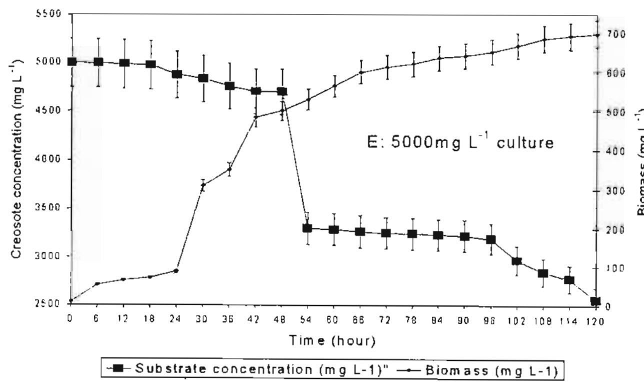



Fig.5.5. Changes in microbial biomass and substrate concentration with time in medium containing 5000mg $L^{-1}$ creosote. Error bars indicate ± 1 Standard Error.

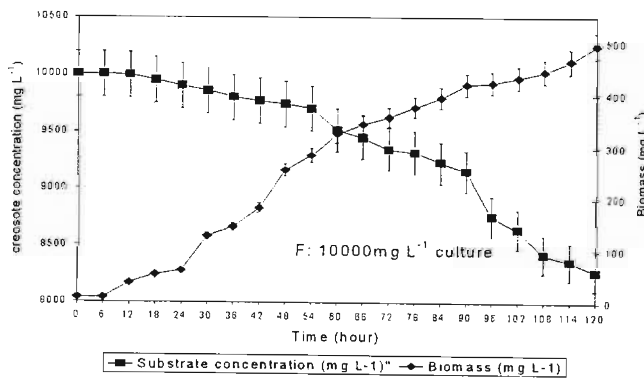



Fig.5.6. Changes in microbial biomass and substrate concentration with time in medium containing 10000mg $L^{-1}$ creosote. Error bars indicate ± 1 Standard Error.

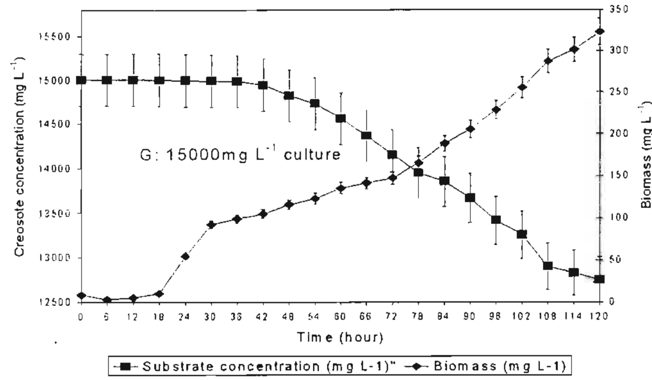



Fig.5.7. Changes in microbial biomass and substrate concentration with time in medium containing 15000mg L$^{-1}$ creosote. Error bars indicate ± 1 Standard Error.

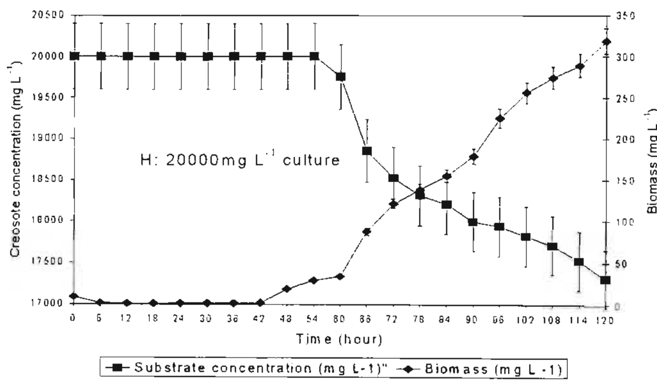



Fig.5.8. Changes in microbial biomass and substrate concentration with time in medium containing 20000mg L$^{-1}$ creosote. Error bars indicate ± 1 Standard Error.

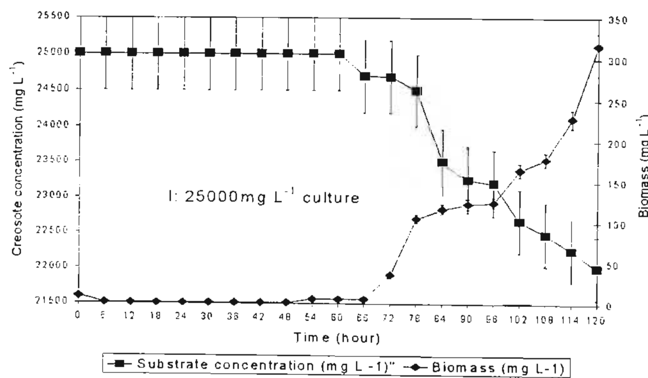



Fig.5.9. Changes in microbial biomass and substrate concentration with time in medium containing 25000mg L-1 creosote. Error bars indicate ± 1 Standard Error.

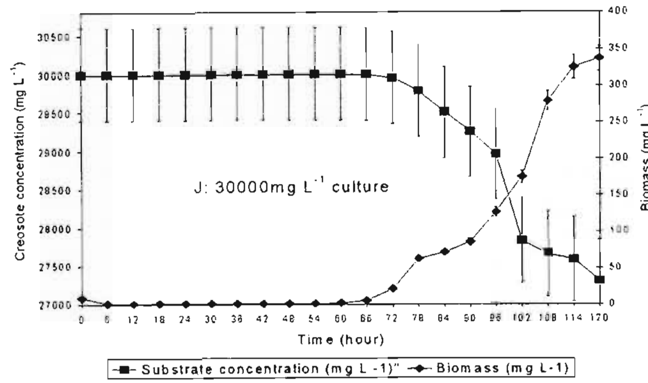



Fig.5.10. Changes in microbial biomass and substrate concentration with time in medium containing 30000mg $L^{-1}$ creosote. Error bars indicate ± 1 Standard Error.

a medium that did not previously contain them, or when a change in some physical parameters, such as pH, occurs (Tate, 1995). However, because of the long enrichment period employed in the present study, the microbial populations were found to increase rapidly after relatively short or no lag phases, particularly at low initial substrate concentrations such as 50 and 100mg $L^{-1}$ (Fig. 5.1 and 5.2). The lag phase of between 48 hours and 72 hours observed at concentrations between 20 000 and 30 000mg $L^{-1}$, was regarded as short, considering the stresses imposed on the culture by the high concentrations of creosote involved. At such high concentrations, more time is required for the microbial population to become large enough to cause a detectable loss in the substrate concentration than will be required at lower concentrations (Alexander, 1999). Such high concentrations may be toxic or inhibitory to the organisms, so that only a small percentage of the population will be able to metabolize the substrate. In such a situation, biodegradation will not be detectable until these species become sufficiently numerous to cause appreciable chemical loss (Alexander, 1999). Sometimes, in a mixture of compounds present in an environment, one or more may be inhibitory to the organisms. With time such toxicants may disappear either by degradation, enzymatic destruction, sorption or volatilization. The loss of the substrate only becomes evident at such a time (Alexander, 1999).

When mutant strains occur, as a result of a chemical concentration factor, the time required for such strains that possesses degradative capabilities to multiply and cause a substantial change in concentration will be reflected as a typical lag. Organisms growing in low concentrations of carbon source show a long lag phase (Vipulanandan *et al.*, 1994). However, in the present study there was no lag phase observed in the cultures containing concentrations of between 50 and 5000 mg $L^{-1}$. What may have been seen as a lag phase in the 50 and 100 mg $L^{-1}$ cultures was a gradual but steady increase in biomass and cannot be described as a lag, i.e. no cell multiplication, because there was an increase in biomass and a corresponding decrease in concentration of creosote during the period of slow growth. The evidence of reduction in substrate concentration in these cultures confirms that there was an increase in microbial biomass at the time of measurement. This lack of lag phase at the lower concentrations can be attributed to the wide array of hydrocarbons present in the mixture many of which supported the growth of some of the organisms, perhaps because of their high solubility in water. The low concentrations may have also reduced the toxicity of many of the component of the creosote, thus allowing the organisms to readily attack some of the components within a very short time after incubation.

These results indicate that bioremediation of sites contaminated with such low concentrations of creosote can be achieved. Although the contaminated site being studied has creosote levels up to 250 000mg $L^{-1}$, this result suggests that small sections of the land with low concentrations of creosote may not experience any inhibition in microbial growth and may thus have faster degradation of the creosote. In the present study, lag phase only became evident at higher concentrations (10 000-30 000mg $L^{-1}$). Such lag phase observed at higher concentrations was reported by Straube *et al.* (1990) in cultures containing phenol. It is evident from the results obtained for biomass increase that the length of the lag phase, which characterises substrate

inhibition, increased with concentration. For example, 10 000mg $L^{-1}$ (6 hr), 20 000mg $L^{-1}$ (48 hr) and 30 000mg $L^{-1}$ (72 hr), as shown in Fig.5.6 to 5.10. Times required for acclimation have previously been observed to increase with concentration (Grover, 1967; Amrein *et al.*, 1981; Nyholm *et al.*, 1984). Although such lag phases were not observed at low concentrations, substantial increases in biomass in the first six hours were only observed from 1000 mg $L^{-1}$ and above. It is therefore possible to have some concentrations below which periods of acclimation may be very short or even unnoticed, as suggested by Spain and van Veld (1983). The report by Spain and van Veld, like most others, was based on single molecules such as phenol. The present study, however, has demonstrated that complex organic mixtures such as creosote can exhibit the same phenomenon at the concentrations studied. The duration of acclimation, even at a single concentration, is not fixed. It changes with site and sometimes with the microbial community involved (Spain and van Veld, 1983; Hoover *et al.*, 1986).

### 5.3.2 Estimation of growth rate of microorganisms

The calculated growth rate ($\mu$) of microorganisms in the different concentrations of creosote were seen to show patterns similar to each other. Growth rate was seen to increase from the lowest concentration of 50mg $L^{-1}$ ( 0.1686 $h^{-1}$) to a peak at 5 000mg $L^{-1}$ ( 0.2049 $h^{-1}$), before declining steadily to the lowest of 0.0777 $h^{-1}$ at 30 000mg $L^{-1}$ (Table 5.1). This decline in growth rate has been reported for some organic compounds ( Straube *et al.*, 1990). It is thus obvious that above 5 000mg $L^{-1}$ concentration began to inhibit microbial growth and the extent of inhibition increases with increase in concentration. The growth remained similar in all the concentrations: showing an initial lag phase, where there was one, during which period the organisms adapted to the substrate medium; an exponential phase during which the highest growth rate was

attained; and a stationary phase during which the organisms developed on the available substrate. The stationary phase was not very evident between 15 000 and 30 000mg $L^{-1}$ as the biomass was seen to continue to increase. This continued growth is believed to be due to abundant substrate availability for microbial utilization ( Poeton *et al.*, 1999). In many cases this represents the complete depletion of the substrate, as shown in 50mg $L^{-1}$ and 100 mg $L^{-1}$ ( Fig. 5.1 & 5.2). The highest substrate removal rates in creosote cultures were observed in 5 000mg $L^{-1}$ (0.0989 $h^{-1}$) and 10 000mg $L^{-1}$ (0.0974 $h^{-1}$), as shown in Table 5.1.

Table 5.1. Mean growth rate(μ) and substrate utilization (S) in cultures growing in different creosote concentrations. Values are means of two ± 1Standard Deviation.

| Concentration (mg $L^{-1}$) | Growth rate (μ) $h^{-1}$ | Substance utilization rate (mg $L^{-1}$ $h^{-1}$) |
|---|---|---|
| 50 | 0.1686 | 0.0551 |
| 100 | 0.1941 | 0.0567 |
| 500 | 0.1611 | 0.0776 |
| 1000 | 0.1864 | 0.0783 |
| 5000 | 0.2049 | 0.0989 |
| 10000 | 0.1532 | 0.0974 |
| 15000 | 0.1514 | 0.0845 |
| 20000 | 0.1484 | 0.0828 |
| 25000 | 0.1078 | 0.0717 |
| 30000 | 0.0777 | 0.0734 |

The decrease in concentration of creosote was observed to inversely correlate with biomass

increase (Fig. 5.1-5.10). The substrate utilization rate, however, was observed to increase with concentration increase up to 5 000mg $L^{-1}$, before it began to decline slowly (Table 5.1). Concentration of creosote was observed to start decreasing during the exponential phase of the biomass increase. This suggested that the utilization of creosote became evident only after the organisms had adapted to creosote and multiplied sufficiently to start causing substantial decreases in creosote concentration (Alexander, 1999). Where biomass increased very rapidly, substrate depletion was also rapid. Thus decrease in concentration of creosote followed the pattern of biomass increase in an inverse relation. At higher concentrations (15 000-30 000mg$L^{-1}$), the substrate level continued to remain unchanged for long periods, compared to the lower concentrations (50-10 000mg $L^{-1}$). This phenomenon is attributed to explanations given earlier in this chapter with respect to the occurrence of lag phase in the growth of the degrading organisms.

Increase in numbers of microbial cells in cultures growing on 5 000mg $L^{-1}$ of the different creosote components, measured by spectrophotometer, shows the same pattern of increase in microbial biomass observed in the cultures with creosote, viz. an initial prolonged lag phase preceding the exponential phase ( Fig. 5.11-5.23). The stationary phase was, however, short with naphthalene and flourene (Fig. 5.15 & 5.19). All the curves are of the typical sigmoid type, corresponding to the Monod's relationship. The highest microbial growth rates were recorded in the phenol(between 0.04269 ) and naphthalene (0.04269 $h^{-1}$) cultures (Fig. 5.24). The other compounds with high microbial growth rate include o- and m-cresol, pyrene, phenanthrene and fluorene (see Table 5.2). The lowest microbial growth rate were recorded on the higher molecular mass compounds benzo(a)pyrene and fluoranthene (0.00301 $h^{-1}$ and 0.00892 $h^{-1}$ ), respectively (Table 5.2).

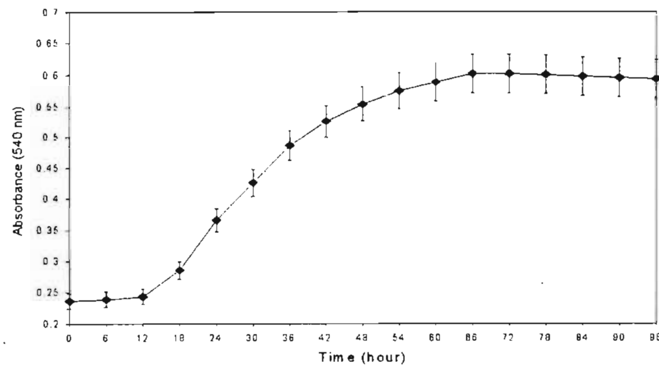



Fig.5.11. Changes in microbial population size in liquid culture containing 5000mg $L^{-1}$ phenol. Error bars indicate ± 1 Standard Error.

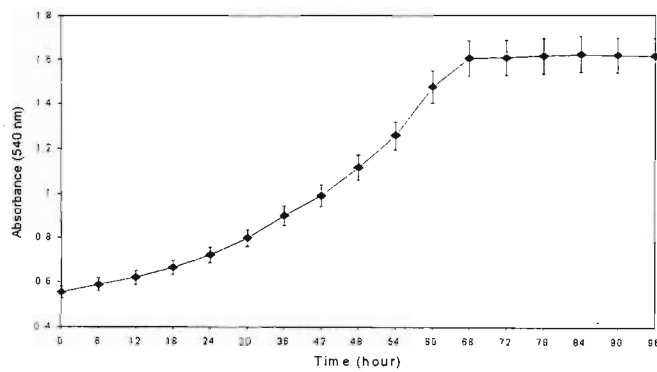



Fig.5.12.Changes in microbial population size in liquid culture containing 5000mg $L^{-1}$ o-cresol. Error bars indicate ± 1 Standard Error.

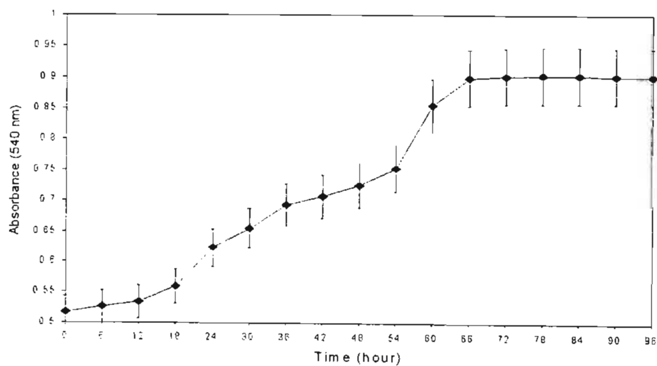



Fig.5.13. Changes in microbial population size in liquid culture containing 5000mg $L^{-1}$ m-cresol. Error bars indicate ± 1 Standard Error.

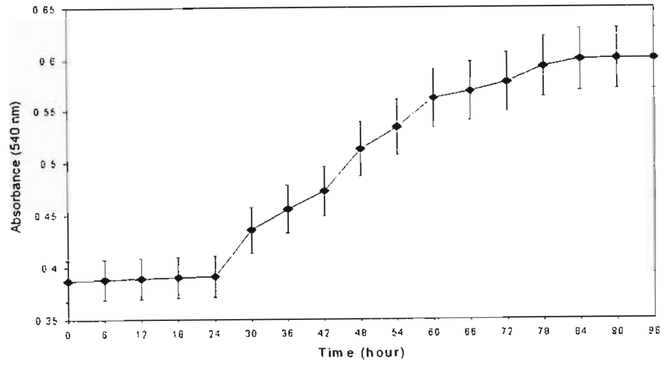



Fig.5.14. Changes in microbial population size in liquid culture containing 5000mg $L^{-1}$ p-cresol. Error bars indicate ± 1 Standard Error.

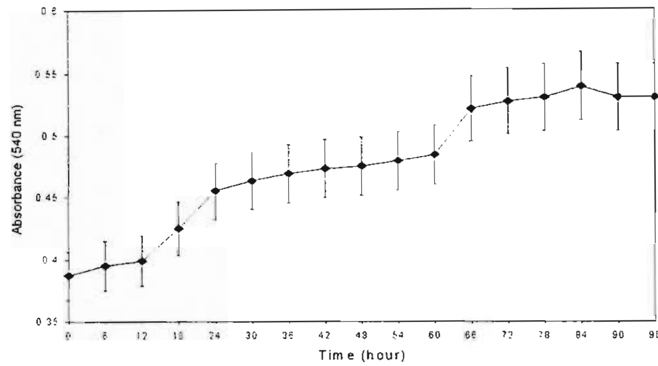



Fig.5.15. Changes in microbial population size in liquid culture containing 5000mg $L^{-1}$ naphthalene. Error bars indicate ± 1 Standard Error.

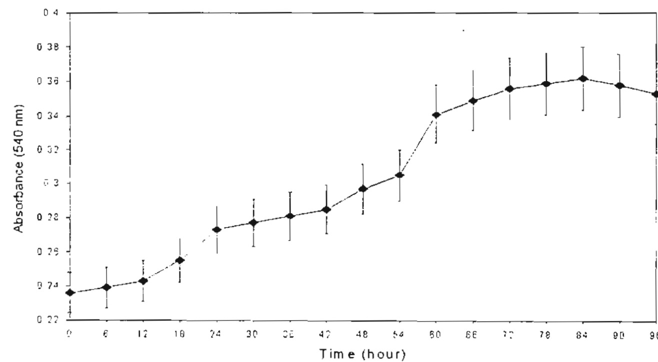



Fig.5.16. Changes in microbial population size in liquid culture containing 5000mg L-1 anthracene. Error bars indicate ± 1 Standard Error.

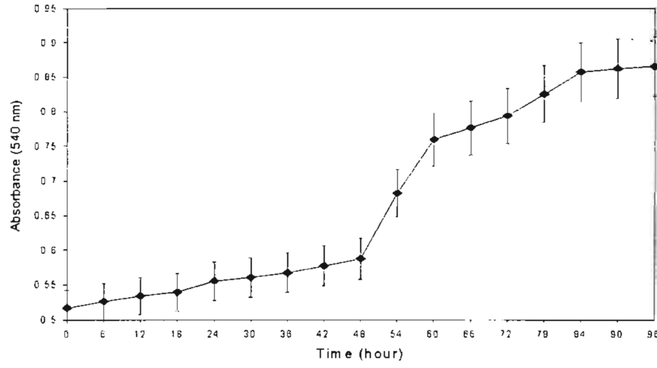



Fig. 5.17. Changes in microbial population size in liquid culture containing 5000mg L-1 phenanthrene. Error bars indicate ± 1 Standard Error.

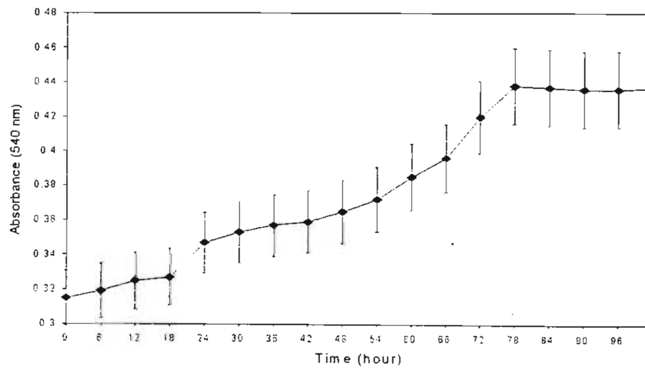



Fig.5.18. Changes in microbial population size in liquid culture containing 5000mg L-1 pyrrole. Error bars indicate ± 1 Standard Error.

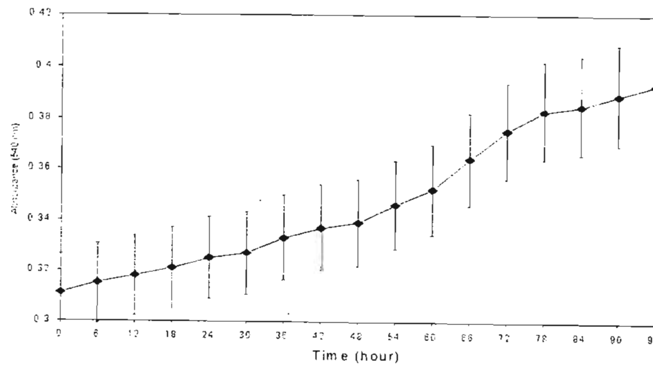



Fig.5.19. Changes in microbial population size in liquid culture containing 5000mg L-1 fluorene. Error bars indicate ± 1 Standard Error.

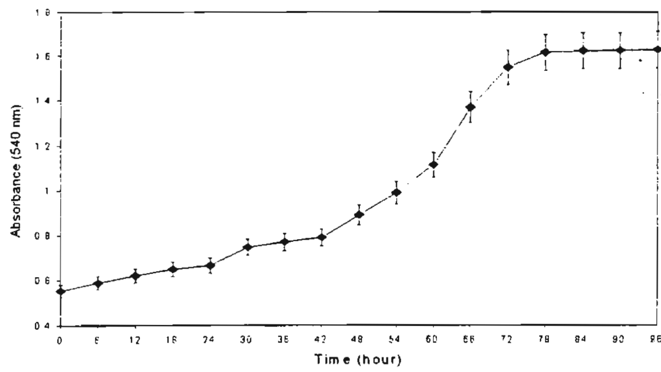



Fig.5.20. Changes in microbial population size in liquid culture containing 5000mg L-1 pyrene. Error bars indicate ± 1 Standard Error.

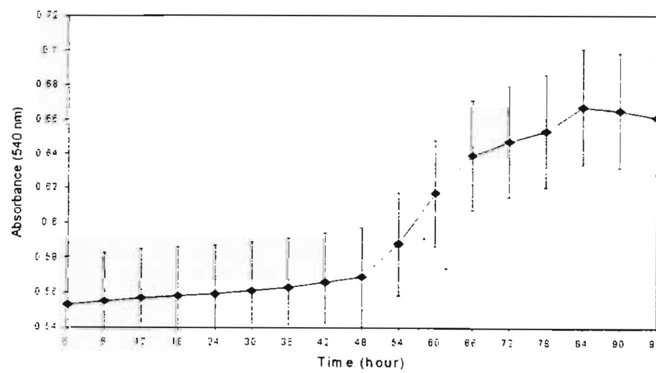



Fig.5.21. Changes in microbial population size in liquid culture containing 5000mg L-1 chrysene. Error bars indicate ± 1 Standard Error.

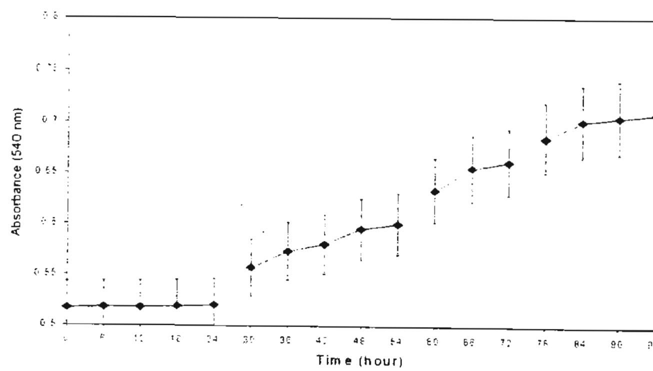



Fig.5.22. Changes in microbial population size in liquid culture containing 5000mg L-1 fluoranthene. Error bars indicate ± 1 Standard Error.

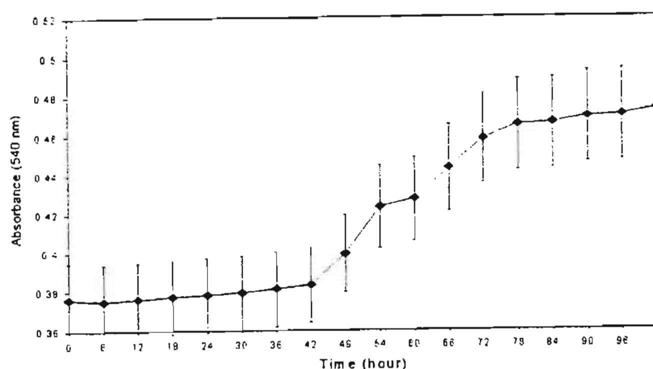



Fig.5.23. Changes in microbial population size in liquid culture containing 5000mg L-1 benzo(a)pyrene Error bars indicate ± 1 Standard Error.

These results confirm earlier findings that the rate of utilization of the substrate depends on the molecular mass of the compounds involved (Gibson and Subramanian, 1984; Tate, 1995; Alexander, 1999).

Even though microorganisms were seen to be well adapted to the different concentrations of creosote, higher concentrations proved to be initially toxic and inhibitory. Initial decreases in the microbial biomass in cultures spiked with 30 000, 25 000, 20 000, 15 000mg $L^{-1}$ ( Fig. 5.7-5.10) indicate a reasonable amount of cell mortality at these concentration. Growth was observed to be more rapid in the cultures spiked with 5 000mg $L^{-1}$ of creosote than those spiked with other concentrations of creosote. Preliminary tests with the selected creosote compounds also showed that the organisms grew faster in the 5 000mg $L^{-1}$ cultures than in other concentrations of the compounds tested. The substrate utilization pattern was, however, not very obvious. The growth of the organisms in the culture media containing 5 000mg $L^{-1}$ of the selected creosote compounds showed an initial lag phase. The acclimation period in these cultures was directly related to molecular mass and number of rings in the compounds; the larger the molecular mass and the greater the number of rings in the structure the larger the acclimation period required. Thus

chrysene and benzo(a)pyrene showed the longest lag phases, viz. 40 and 50 hours, respectively.

Table 5.2: Mean growth rates($\mu$) of microorganisms in selected creosote components at 5 000mg $L^{-1}$ measured by absorbence at 540 nm. Values are means of two ± 1 Standard Error.

| Creosote component (5000mg $L^{-1}$) | Calculated growth rate ($\mu$) ($h^{-1}$) |
|---|---|
| phenol | 0.04269 |
| o-cresol | 0.04083 |
| m-cresol | 0.03502 |
| p-cresol | 0.02154 |
| naphthalene | 0.04269 |
| pyrrole | 0.01735 |
| anthracene | 0.01860 |
| phenanthrene | 0.02500 |
| fluorene | 0.02804 |
| pyrene | 0.01480 |
| chrysene | 0.00915 |
| flouranthene | 0.00892 |
| benzo(a)pyrene | 0.00301 |

Notwithstanding the growth constraints imposed by the concentration and molecular mass of the compounds, significant growth occurred in all concentrations of all the compounds studied. Similarly, substrate depletion was significant even at very high concentrations of the higher molecular mass compounds studied. Growth of microorganisms has been reported in concentrations of up to 50 000mg $L^{-1}$ of creosote by Ellis (1994) and several workers have also

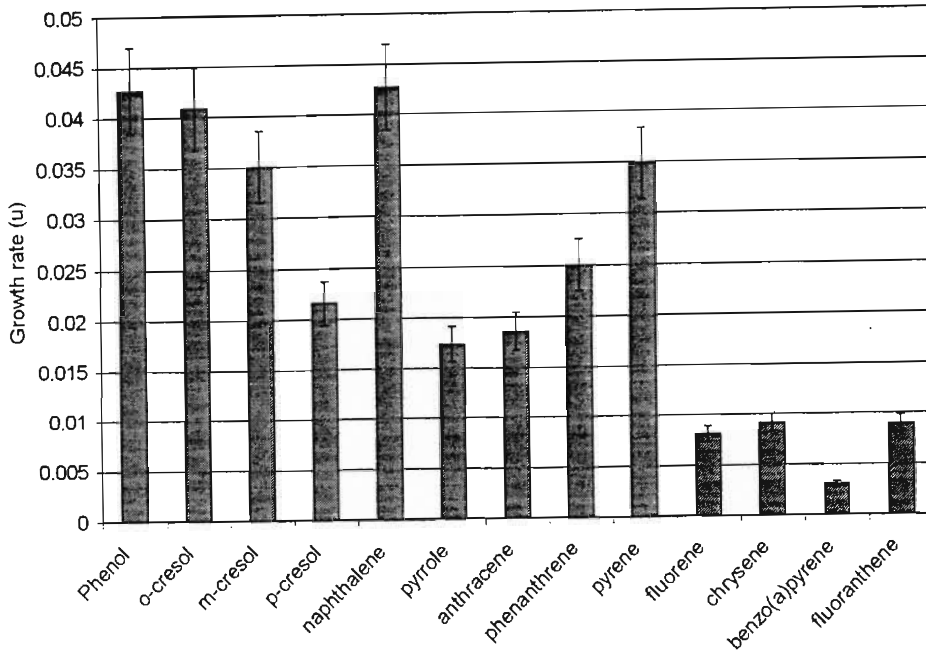



Fig.5.24. Comparison of growth rates of microorganisms on selected creosote components (5000mg $L^{-1}$) Error bars ± 1 Standard Error.

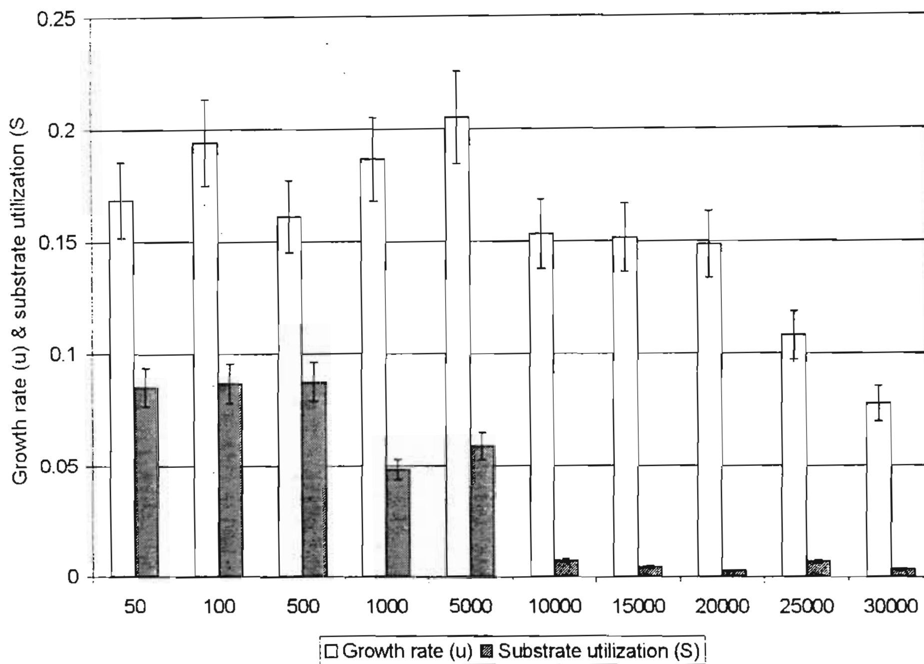



Fig.5.25. Comparison of microbial growth rate and rate of substrate utilization in different concentrations of creosote. Error bars indicate ± 1 Standard Error.

reported growth of different organisms on 3, 4, and 5 ring PAHs ( Walter *et al.*, 1991; Mueller *et al.*, 1994; Sanseverino *et al.*, 1994; Trzesicka-Mlynarz and Ward, 1995; Hughes *et al.*, 1997; Atagana *et al.*, 2000). However, many of the reports are inconclusive and leave room for misunderstanding and misrepresenting the results. It is therefore important to continue to explore all possible avenues to properly understand the behaviour of these compounds in nature and their corresponding degrading microbial consortia for the effective harnessing of technologies based on the results of such studies and the management of their applications.

## 5.4 CONCLUSIONS

From the results and discussion presented in this chapter, the following conclusions can be drawn:

- the growth rate (μ) of the degrading microorganisms in liquid culture increases with increase in concentration of creosote up to 10 000mg $L^{-1}$, where the rate begins to decline, marking the beginning of substrate toxicity and inhibition to the microorganisms.
- the rate of biomass increase correlates with the rate of substrate utilization, i.e. biomass increases with increase in substrate utilization.
- microbial growth rate, biomass increase and substrate utilization were highest at a concentration of 5 000mg $L^{-1}$ of creosote and all creosote components tested.
- microbial growth rates were highest on the lower molecular mass components of creosote and lowest on the higher molecular mass components.
- growth of degrading microorganisms can continue in concentrations of up to 30 000mg $L^{-1}$ of the various creosote components tested.
- acclimation periods for organisms growing in creosote media varies with concentration,

molecular mass and the stereo chemistry of the compounds.

- even high concentrations (up to 30 000 mg $L^{-1}$) of the large molecular mass and multi-ringed compounds were not completely inhibitory to growth of the microbial consortia investigated.

Although these results show that degradation of creosote and several of its components *does* occur in liquid media under controlled conditions, natural soil conditions may present new challenges as the physical and chemical conditions prevailing in soils vary greatly from those in liquid media. Because of this, further experiments were designed to study specifically the effects of certain physical and chemical parameters in soil on the degradation of creosote, with a view to optimizing the process of creosote biodegradation under natural soil conditions.

# CHAPTER 6

# OPTIMIZATION OF SOIL PHYSICAL AND CHEMICAL CONDITIONS FOR THE BIOREMEDIATION OF CREOSOTE-CONTAMINATED SOIL

## 6.1 INTRODUCTION

The growth of heterotrophic bacteria and fungi is dependent on the presence of a number of nutrient elements and an electron acceptor, as well as on the organic compound that serves as the source of carbon and energy. For aerobic organisms, the electron acceptor is oxygen. Some organisms have the capability of utilizing some inorganic compounds such as nitrates, sulphates, carbon dioxide, ferric iron, and some organic compounds, as electron acceptors for electrons released by the oxidation of the substrate carbon source. Some bacteria and fungi also require low concentrations of some amino acids, vitamins and/or other organic molecules, referred to as growth factors. The absence of any of these essential factors from an environment will prevent the growth and metabolism of soil microorganisms.

The supply of nutrient elements in the soil, apart from carbon, usually exceeds the needs of the resident microbial communities. Indeed, the growth-limiting nutrient in natural environments such as soil, sediments and water is commonly carbon (Alexander, 1999; Bredveld and Sparrevik, 2000). In the event of the introduction of an organic pollutant that has the potential to be utilized by the resident microorganisms in the environment, this situation may change considerably. This will occur

where the concentration of the polluting substance is high enough to make one or more of the previously non-limiting nutrients become limiting. That is, the concentration of available soil nutrients becomes too low to meet the high demand caused by the introduction and consequent utilization of the pollutant carbon source. Most often, the nutrients that become limiting are nitrogen and phosphorus (Alexander, 1999). The increased microbial activity results in a simultaneous increase in the demand for, and consumption of, an electron acceptor, which in the case of introduced hydrocarbons is usually oxygen. Creosote oil consists of more than 200 hydrocarbons of various molecular masses. The introduction of creosote into the soil environment thus creates an imbalance in the nutrient ratio in the soil because of the high level of carbon supplied. The addition of N and P to soil contaminated with hydrocarbons is known to stimulate the biodegradation of such compounds and increase the abundance of microbial species (Baker and Herson, 1994; Baroon *et al.*, 2000). Sometimes the stimulation immediately becomes apparent, but in others it may take some time for the benefit of such nutrient addition to become evident (Bossert and Bartha, 1984). In other situations, the addition of nutrients may be without benefit, either because of existing high levels of N and P in the soil or low concentration of the polluting substance (Alexander,1999; Harris, 2000).

The amount of N and P needed for the biodegradation of hydrocarbons is usually assumed to reflect the amount that must be incorporated into the biomass that is formed as the microorganisms use the carbon source for growth. The C:N:P ratio in bacterial biomass is quoted by Zitrides (1983) to be 100:15:3 and by Alexander (1977) to be 120:10:1. Although both estimates vary widely, possibly due to the difference in the microbial population used in the estimation and the environment from which the organisms were isolated, it is possible to use these ratios to approximate the amount of

N and P that will be necessary to sustain bacterial growth if the amount of carbon from the pollutant that ends up as bacterial biomass can be calculated (Thibault and Elliot, 1980). To ensure that these nutrients do not limit microbial activity, sufficient nitrogen and phosphorus should be applied (Alexander, 1981).

Gaseous exchange takes place continuously between the soil and the air. It should be noted, however, that the amount of oxygen in the surface soil is about half that in the air (Riser-Robert, 1992). Conversely, the amount of carbon dioxide present in the air is many times less than that present in the soil (Riser-Roberts, 1992; Koenigsberg and Farone, 1999). Saturation of soil with water limits diffusion of gases and speeds up the consumption of oxygen. This can render the soil anaerobic, which not only affects the microflora of the soil but also affects its chemistry, as amines, hydrogen sulphide and other compounds are produced (Parr *et al.*, 1983; Larson and Voegeli; 2001). This may result in the soil becoming phytotoxic. However, if the oxygen balance is maintained, rapid aerobic decomposition will occur and the end products will be inorganic carbon, nitrogen and sulphur compounds (Riser-Roberts, 1992; Koenigsberg and Sandefur, 2001).

Nevertheless, for biodegradation to proceed effectively, soil water is essential. Mobilization of nutrients and incorporation of these nutrients into microbial biomass requires adequate quantities of water. The density and texture of the soil determines the water-holding capacity, which in turn affects the available oxygen, redox potential and microbial activity (Parr *et al.*, 1983, Zahiralislamzadeh and Bensch, 2001). The microbial species composition of a soil is often dependent upon water availability (Alexander, 1977; Baker and Herson, 1994; Tate, 1995) . Even

though the capacity of soils to hold water under field conditions varies from one soil to another, microbial activity has been reported to proceed optimally in conditions of between 50% and 70% field capacity (Alexander, 1999).

Soil pH has been widely reported to affect microbial growth and the degradation of hydrocarbons in soils (Smith and Mason, 1999; Baeseman and Novak, 2001). A pH of between 5 and 7 has been found to be optimal for bacterial growth (Alexander, 1977; Tate, 1995). However, organisms are known that grow in habitats with very low and very high pH. Soil pH thus partially determines the organisms that are present and the extent of bioremediation that can be carried out with a given compound.

The focus of the present study is therefore to optimize soil conditions such as nutrient status and aeration, moisture and pH levels for the degradation of creosote in soil.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Soil Samples

Seven sample cores constituting approximately 5kg of creosote-contaminated Mispah type (Lithosol: FAO) soil were taken up to depths of about 30 cm with a spade from each of three different locations, representing heavy (>250 000 mg $kg^{-1}$ ), medium (5 000 -250 000 mg $kg^{-1}$) and light (<5 000 mg $kg^{-1}$) contaminations at the experimental site. The samples were mixed in an electric concrete

mixer before use. A 1 kg sub-sample (field moist) of the heavily contaminated sample was sterilized by gamma irradiation at a dosage of 2.5 Mrad.

### 6.2.2 Carbon, Hydrogen and Nitrogen Analyses

Analyses of carbon and hydrogen content of soils from the three different locations, representing light, medium and heavy contamination, were carried out in a Perkin Elmer 2400 CHN Elemental Analyser. Tin boats were used for holding the samples, the combustant was oxygen, the carrier gas was helium and the furnace temperature ranged between 98°C and 150°C (University of Natal, Chemistry Department). The nitrogen in the sample was converted to nitrogen gas ($N_2$), the carbon was converted to carbon dioxide ($CO_2$) and the hydrogen was converted to water ($H_2O$). The gases produced were analysed using frontal gas chromatography.

### 6.2.3 Inorganic Nutrient Treatments

Based on the carbon and nitrogen content of the soil samples, five different ratios of C: N and one of C:N:P were established in the different soils in the jars. The C:N and C:N:P nutrient amendments were added as $NH_4NO_3$ and $K_2HPO_4$, respectively. The treatment combinations of C:N were: (A) 25:1; (B) 20:1; (C) 15:1; (D) 10:1; (E) 5:1, and the C:N:P was (F) 10:1:2. A sterile control (G) was set up for each of the treatments above, using the gamma( $\gamma$)-irradiated soil. One biological control (H), which received only water, was also set up. All treatments, including the controls, were duplicated.

### 6.2.4 Soil Moisture

Water-holding capacity of the soil was determined by placing duplicate 20g field-moist soil samples in a funnel fitted with filter paper and mounted on preweighed collecting flasks. Distilled water (100g) was added to the soil in small amounts, the flasks were covered with aluminum foil to prevent evaporation and allowed to stand overnight. A blank experiment as above was set up, but without soil. The collecting flasks were weighed with water. The soil was dried in an oven at 105 $^{O}$ C to constant mass, cooled in a desiccator and re-weighed. Percent water-holding capacity was calculated by:

$$[(100-Wp)+Wi]/dwt \times 100$$

where Wp is the mass of percolated water in grams, Wi is the initial amount of water in grams contained in the sample and dwt is the soil dry mass in grams (Forster, 1995).

### 6.2.5 Jar Microcosms

Shallow glass jars of about 100 ml in volume and a height of about 5cm were used to allow air to reach every part of the sample. Each 100ml glass jar was soaked in Extran™ for two hours rinsed with a 1M solution of $HNO_3$ and then rinsed twice with deionised water before drying overnight at 110$^{O}$C. The jars were then autoclaved at 121$^{O}$C for 15 minutes. Forty grams (fresh mass) of the heavily contaminated soil were placed in each of 216 sterilized glass jars. To duplicate jars containing the heavily contaminated soil were separately added each of the five C:N ratios and one of the C:N:P ratio described above. Each sterile control and biological (natural) control jar was also

duplicated. The duplicate jars of the biological control received only water. For each jar the nutrients were dissolved in 3.5 ml of deionised water, corresponding to approximately 80% of the field capacity of the same volume of soil packed at a bulk density of 1370 kg $m^{-3}$ . This was to ensure retention of the nutrients in the microcosms without water-limitation or water logging. The content of each jar was mixed thoroughly, weighed and sealed with parafilm to prevent water loss, but allow oxygen diffusion, before being incubated at 30°C ± 2°C in the dark. The jars were re-weighed on a weekly basis and replenished with deionised water when necessary. This method was adapted from Lees (1996). The duplicate jars were sampled destructively every seven days. The soils were sub-sampled for microbial counts, infrared analysis was carried out to determine change in creosote concentration and gas chromatography to determine changes in concentration of selected creosote components. The pH was also measured. The sub-samples for microbial counts were used immediately and the remainder was heat-sealed in a cellophane bag and stored in the freezer at -17 °C until required for analyses.

### 6.2.6 Media

#### 6.2.6.1 Mineral Salts Agar

Mineral salts agar was prepared, as described in Chapter 3 [section 3.2.2.1(iii)], but each plate was overlaid with 50 µl of creosote oil filtered through a 0.4 µm filter and spread with a bent sterile glass rod.

#### 6.2.6.2 Soil Extract Agar

Soil extract agar was prepared, as described in Chapter 3 [section 3.2.2.1 (ii)].

### 6.2.7 Plate counts of total heterotrophs and creosote-degrading species

A 1 g sample of soil was taken from each reactor on a weekly basis and analysed for total number of indigenous microorganisms present, using standard dilution plating techniques on nutrient agar. The soil was analysed for total number of creosote-degrading microorganisms by inoculating a modified version of the medium described by the Organisation for Economic Co-operation & Development (OECD) containing 30ml creosote oil as the sole carbon source. The OECD medium was prepared by adding 17g agar, 4ml $FeCl_3$ (0.25g $L^{-1}$), 1ml each of $MgSO_4.7H_2O$ (22.5g $L^{-1}$), $CaCl_2$ (27.5g $L^{-1}$) and $(NH_4)_2SO_4$ 40g $L^{-1}$ to 2ml of the following mixture: $KH_2PO_4$ (8.5g $L^{-1}$ ), $K_2HPO_4$ (21.75g $L^{-1}$), $NaH_2PO_4$ .$7H_2O$ (33.4g $L^{-1}$ ) and $NH_4Cl$ (1.7g $L^{-1}$ ) and diluting to one litre with distilled water. The mixture was autoclaved at 121$^{0}$ C for 20 minutes and cooled. Creosote oil was filtered through a hydrophilic membrane (0.4μm) pore filter and agar was added to it before the plates were poured.

### 6.2.8 Soil pH Measurement

Soil samples from the jars were air-dried for 72 hours in the dark at room temperature ( 25 $^{0}$ C ). The soil was then ground and passed through a 2mm sieve. Ten grams of each soil sample were put in

50 ml beakers into which 25 ml of sterile distilled water were added. The slurry was stirred for one minute and allowed to stand for 15 minutes. The pH of the supernatant was measured with a standard pH metre (Crison Micro pH 2000). Adjustment of soil pH in the jars was made by the addition of lime at the start of the experiment as the soil pH was found to be originally acid (<5.0).

#### 6.2.9 Chemical Analyses

##### 6.2.9.1 Infrared spectrophotometry (IRS) and gas chromatography(GC)

Total creosote concentration in the soil was analysed using the USEPA 4181.1 (1982) method as follows: creosote contaminated soil (2 g) and 2 g anhydrous $Na_2SO_4$ were placed in a 30 ml amber glass vial. Carbon tetrachloride (10 ml) was added before sealing the vial with a teflon-lined screw cap. The sealed vial was vortexed for 15 seconds and placed in a sonicating bath (Whaledent Biosonic) for 15 minutes before remixing on the vortex mixer for about 15 seconds . It was then placed in the sonicating bath for another 15 minutes. The solvent was transferred to a clean, dry vial containing 1g activated Florosil™ (Sigma) and 0.6 ml water [i.e. 6% water (w/w)]. The sealed vial was shaken for one minute and allowed to stand overnight at ambient temperature. This silica "clean-up" procedure was used to remove interfering humic materials (EPA, 1985). The extract was finally filtered through a Whatman GF/C glass fibre filter. The filtrate was made up to 10 ml in a volumetric flask and the absorbance determined with a Nicolet Avater 320 Infra-red Spectrophotometer at wave numbers between 400 and 4000 $cm^{-1}$ . Calibration of the reference creosote was done by diluting commercial grade creosote with carbon tetrachloride to a series of five working standards ( 10; 100;

250; 500; 1000 mg $L^{-1}$) and a calibration curve was derived by determining the absorbance of each standard. A calibration plot of the absorbance versus mg creosote (100 ml $L^{-1}$) solution was generated with Omnic™ software. The concentration of creosote in each extract was determined by comparing the response with the calibration plot. To calculate the results, a linear equation ( $y = 1.192\,x + 1.363$) was generated by Omnic™ software, where $x$ = creosote concentration of sample (kg $^{-1}$ soil) and $y$ = peak area. The actual hydrocarbon concentration in mg $kg^{-1}$ soil was then calculated by multiplying $x$ by a factor of 50 which compensates for the cell pathlength, sample size and dilution factor. This formula was then programmed into a spreadsheet which automatically calculated the creosote concentration in mg $kg^{-1}$ from the infra-red absorbance values as they were entered.

Determination of changes in the concentration of selected creosote hydrocarbons was done by Soxhlet extraction and GC/FID. The GC was a Varian-3800 with argon as the carrier gas and fitted with a 30 m capillary column with 0.25 mm internal diameter and 0.25 μm film thickness, and a flame ionization detector (FID). Two temperature programmes were run in order to obtain good separation and quantification of the more volatile compounds. The first temperature programme was: 60 $^{O}$ C, 4 min., followed by ramping at 10 $^{O}$ C/min. up to 235 $^{O}$ C, maintained for 40 min.; injector temperature 220 $^{O}$ C. The second temperature programme was used for the analysis of the more volatile compounds, viz. 20 $^{O}$ C; 1 min 40 $^{O}$ C, 1 min., 10 $^{O}$ C/min., ramping up to 200 $^{O}$ C, maintained for 20 min.; injector temperature 220 $^{O}$ C ( Eriksson *et al.*, 2000).

## 6.3 RESULTS AND DISCUSSION

### 6.3.1 Carbon content in the contaminated soil samples

The analyses of the soil samples collected from the contaminated sites showed that the mean carbon content of the samples is 13.02%, hydrogen 0.85% and nitrogen 0.084 (m/m) (Table 6.1). This percentage of carbon represents approximately 130 000 mg C $kg^{-1}$ soil. This amount of carbon greatly affects the carbon : nitrogen : phosphorus (C:N:P) ratio in the soil. Samples from creosote-contaminated soil had carbon contents an order of magnitude greater than those from uncontaminated sites.

Table 6.1: Percentage(m/m) of carbon, hydrogen and nitrogen in contaminated soil samples collected from experimental site. Values are means of two ± 1 Standard Deviation.

| SAMPLE SITE | % C (m/m) | % H (m/m) | %N (m/m) |
|---|---|---|---|
| Within 5m of discharge facility (a) | 24.73 | 0.93 | 0.081 |
| Within 5m of discharge facility (b) | 24.32 | 0.97 | 0.083 |
| Creosote-treated wood stacking lot (a) | 13.5 | 0.81 | 0.081 |
| Creosote-treated wood stacking lot (b) | 12.5 | 0.86 | 0.082 |
| Uncontaminated wood stacking lot (a) | 2.65 | 0.76 | 0.085 |
| Uncontaminated wood stacking lot (b) | 3.43 | 0.77 | 0.09 |
| Mean | 13.52 | 0.85 | 0.084 |

The carbon content of the different samples collected from the site was seen to vary greatly, ranging from above 24% in the areas within 5 metres of the creosote discharge facilities and storage tanks,

to about 13% in the treated wood stacking lots, and about 3% in the untreated wood stacking lots. This variation was used as the basis for classifying the samples into heavily, moderately and lightly contaminated soils.

### 6.3.2 The effect of soil nutrient ratio on hydrocarbon degradation

The imbalance in C:N ratio resulting from the polluting carbon source (Fig. 6.1) is expected to affect the metabolic activity of the soil microorganisms and consequently the rate at which the polluting hydrocarbon will be degraded. This possibly accounts for the reason why there still remained such a large amount of hydrocarbon (about 250 000 mg $kg^{-1}$) in the soil long after operations had ceased at the site. Nutrient imbalance in the soil between carbon, nitrogen and phosphorus, resulting from the excess carbon supplied by the pollutant hydrocarbon, is known to limit the extent of hydrocarbon degradation in contaminated soils. This phenomenon has been widely reported in the literature (Raiser-Roberts, 1992; Alexander, 1999; Breedveld and Sparrevik, 2000). Although a C:N:P ratio of 30:5:1 is needed for unrestricted growth of soil bacteria (Paul and Clark, 1989), a much lower ratio (20:1:1) has been found to be very effective in degradation of crude oil (Lees, 1996). In theory, approximately 150 mg of nitrogen and 30 mg of phosphorus are consumed in the conversion of 1 mg of hydrocarbon to cell material (Rosenberg *et al.*, 1983). This kind of complete assimilation of hydrocarbon into biomass is not achievable under natural conditions, given the fact that some of the compounds found in the hydrocarbon complex are recalcitrant or are only slowly metabolised over long periods and such components may constitute the major part of the carbon load (Lees, 1996). This situation can be expected in hydrocarbon complexes with a large percentage of high molecular mass fractions which will only slowly degrade, and under conditions where such hydrocarbons are strongly

sorbed to soil or sediment particles. Thus the conversion of substrate carbon into cellular biomass will only very rarely achieve 100% efficiency (Baker and Herson,1994; Alexander,1999).

### 6.3.3 Soil microbial population and hydrocarbon utilization

Microbial counts for all the treatments in mineral salts agar, soil extract agar and nutrient agar showed that the different treatments supported microbial activities in the following increasing order: sterile control < natural control < C:N 5:1 < C:N:P10:1:2 < C:N15:1 < C:N 20:1 < C:N 10:1 < C:N 25:1. Colony counts were found to be very low in the sterile control (Fig. 6.1). The few colonies observed may have resulted from contamination during the plating or incubation process, because the soil sample was stringently sterilized.

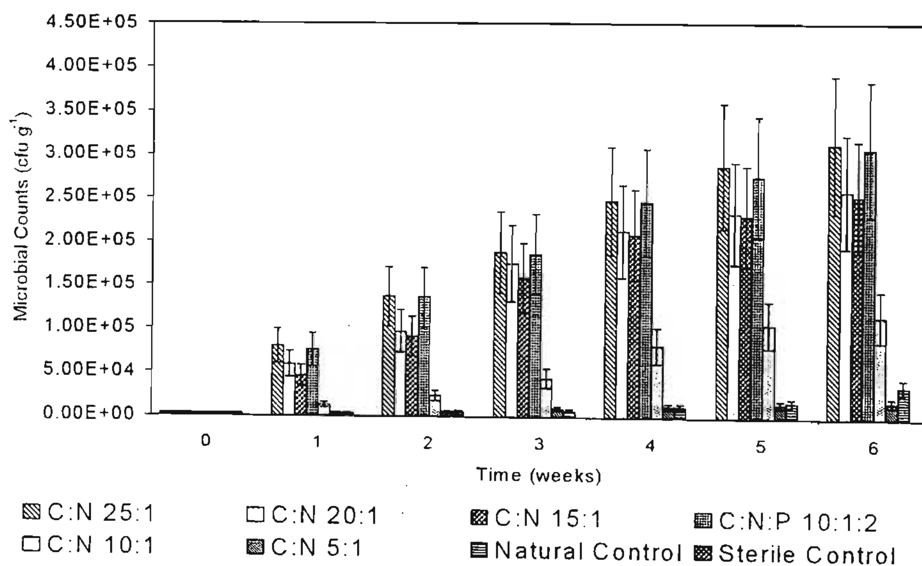



Fig. 6.1. Counts of creosote degraders (cfu $g^{-1}$) after 24 hr incubation at $30^{0}C$. Error bars indicate ± 1 Standard Error.

The results of these experiments showed that reductions in hydrocarbon concentrations are related to increases in microbial activity. Counts of the microbial populations showed that the increase in the numbers of hydrocarbon degraders at C:N ratios of 25:1; 20:1; 15:1 and 10:1 were positively corelated to corresponding decreases in creosote concentration of 68.7%, 61.1%, 56.3% and 63.8%, respectively (Table 6.2). Thus microbial activity and the corresponding creosote removal rate were more effective at lower nutrient supplementation, as was earlier reported ( Dibble and Bartha, 1979;

Table 6.2: Total creosote degradation of heavily contaminated soil incubated with different ratios over a six week period at $30^{O} \pm 2^{O}$ C. Values are means of two ± 1 Standard Error.

| TREATMENT | % CREOSOTE REMOVAL (MEAN) | MEAN % REMOVAL RATE WEEK$^{-1}$ |
|---|---|---|
| 25:1 | 68.7 | 11.45 |
| 20:1 | 61.1 | 10.18 |
| 15:1 | 56.3 | 9.38 |
| 10:1 | 63.8 | 10.63 |
| 10:1:2 | 50.2 | 8.36 |
| 5:1 | 33 | 5.50 |
| Sterile control | 18.2 | 3.03 |
| Natural control | 46.6 | 7.76 |

Brown *et al.*, 1991; Phaal, 1996). The highest nutrient supplementation used (C:N 5:1), was found to be the least effective in supporting growth of microorganisms and consequently resulted in only 33% hydrocarbon removal (Table 6.2). Thus high levels of N and P supplementation can be inhibitory to

microbial degradation of creosote in soil. Approximately 18% creosote removal was recorded for the sterile control. This must be attributed very largely to volatilization, since only small numbers of microorganisms were present. (Fig. 6.1 & Table 6.2).

In the natural control that received only water in place of the C:N supplementation treatment, a total creosote removal of 46.6% was recorded. This suggests that the high rates of removal of creosote at the lower C:N ratios was due to microbial metabolism rather than volatilization, because the microbial counts in the two controls were very low compared to the samples supplemented with N and P.

Results from the natural control are comparable with those from the treatment with C:N:P 10:1:2 in which it could be seen that the high phosphorus content was inhibitory to microbial growth and creosote removal. Another explanation for the poor removal of creosote in the natural control was the low pH (4.79). Such acid pHs have been reported to inhibit biodegradation of organic pollutants in the environment (Alexander, 1999). Because the hydrocarbon content of the soil was very high (>250 000mg $kg^{-1}$ soil), and the nitrogen and phosphorus levels were relatively lower than the required percentage, the N and P supplementation was necessary to boost the activity of degrading microorganisms to optimal levels.

The sterile control was not expected to support microbial growth. However, a few colonies did develop on the solid media used to enumerate the autochthonous microbial population. A population density of 75 cfu $ml^{-1}$,which is a very low count, was recorded at the end of the incubation period of 6 weeks (Table 6.3). Except in the C:N 5:1 and control jars microbial activity showed a rapid

increase after the first week of incubation. Over subsequent weeks a slight but steady decrease in the growth rate occurred. The natural control showed steady population increase from the first week up until the end of the incubation period. These measured growth rates were difficult to correlate with the rate of creosote removal in all the treatments. In many of the treatments, substrate utilization was found to increase progressively and then slowed down before rising again to relatively high levels. This irregularity in growth rate and substrate removal rate can be attributed to utilization of the hydrocarbon substrate at certain times during the incubation period without such utilization manifesting itself in the size of the microbial biomass. If the bacterial cell density is high relative to the substrate concentration, little or no increase in cell numbers is possible (Alexander,1999).

### 6.3.4 Soil pH and creosote degradation

Although pH in all the treatments showed slight decreases during the six weeks of incubation, microbial activity continued to increase during this period. The decrease in pH was found to be more evident in the C:N ratios 25:1, 20:1, 15:1. The highest nutrient supplementation, 5:1 showed an overall increase in pH from 6.48 to 6.57. The sterile control, natural control, 10:1:2 and 10: 1:1, showed various levels of decreases. The decrease in pH was observed to be directly related to decrease in substrate concentration (Fig. 6.2). This is believed to be due to the production of microbial metabolites which resulted in decrease in pH as the microorganisms utilize the substrate. Microbial degradation of xenobiotic compounds have been reported to result in alteration of pH in soil and aquatic media (Baker and Herson,1994; Bourquin *et al.*, 2001).

The general trend reflecting a decrease in pH as the concentration decreased was important in the

development of the methodology for the remediation of the contaminated soil. This phenomenon suggests that there was possibly an accumulation of acid metabolites/or by-products, and/or final products as the substrate was utilized. However, since the biomass continued to increase the acid pH attained was not sufficient to cause inhibition of microbial growth.

The results of the GC analyses for the removal of selected individual creosote hydrocarbons show that there was a general trend for a decrease in the concentration of all the creosote compounds studied. There were, however, some peculiarities in the behaviour of some of the compounds at certain concentrations. Phenol was observed to be completely removed by the end of the sixth week in all the treatments except the controls. The other phenolic compounds; o-cresol, m-cresol and p-cresol, were also completely degraded in all the treatment with the exception of m-cresol at C:N 5:1

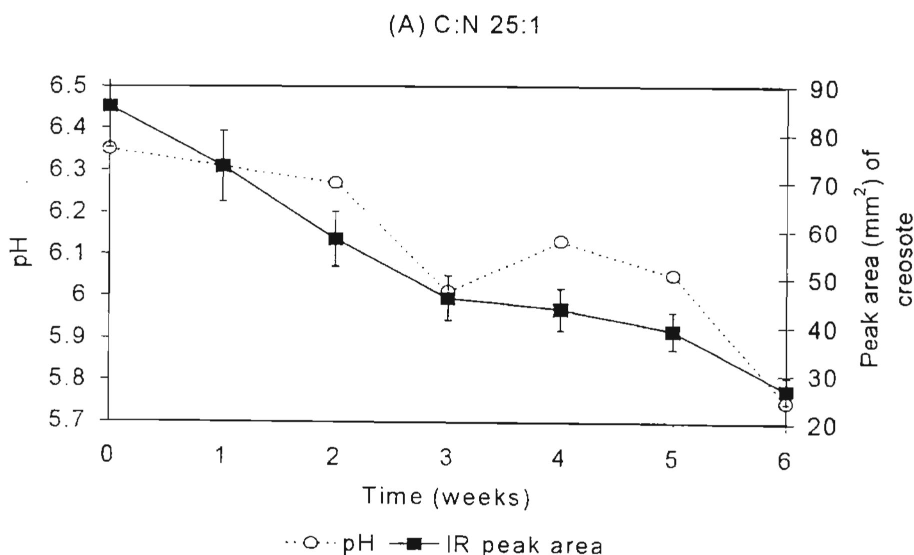



Fig.6.2(A). Changes in pH and peak area of creosote in the C:N ratio 25:1. Error bars indicate ± 1 Standard Error.

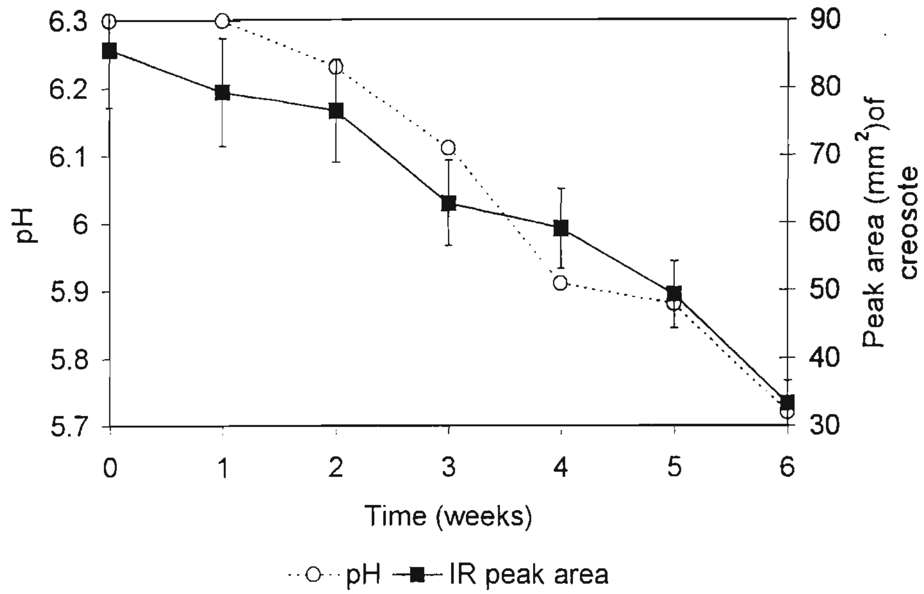



Fig.6.2 (B). Changes in pH and peak area of creosote in the C:N ratio 20:1. Error bars indicate ± 1 Standard Error.

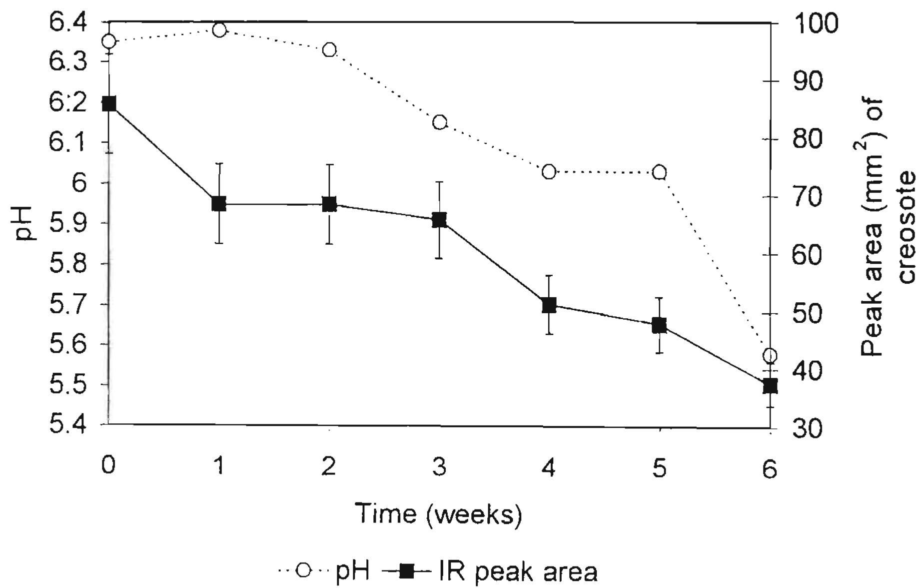



Fig. 6.2 (C) Changes in pH and peak area of creosote in the C:N ratio 15:1. Error bars indicate ± 1 Standard Error.

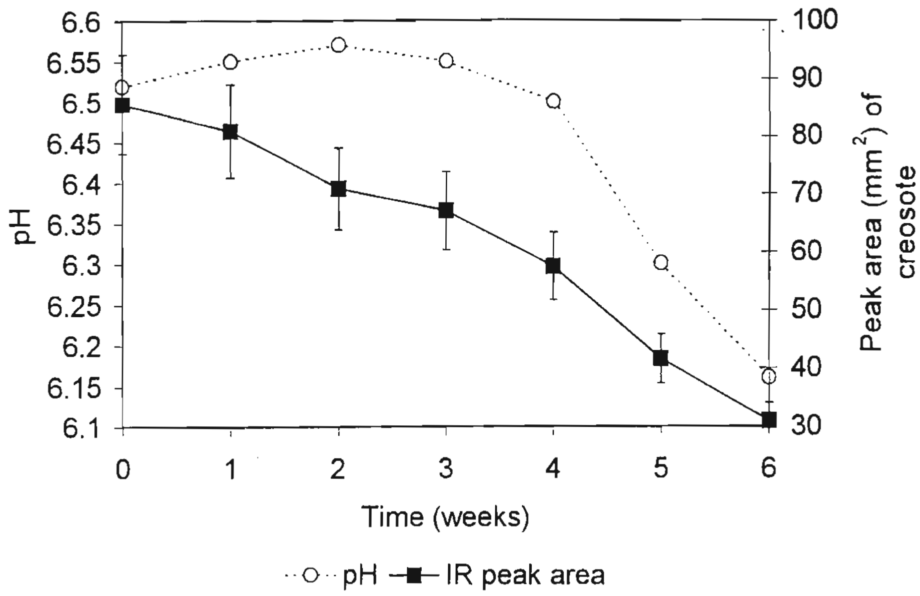



Fig. 6.2 (D) Changes in pH and peak area of creosote in the C:N ratio 10:1. Error bars indicate ± 1 Standard Error.

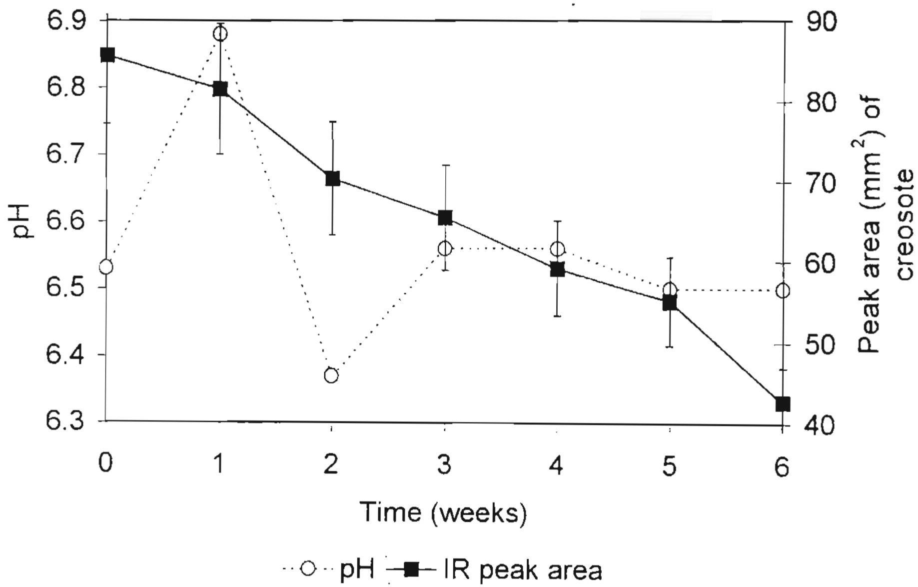



Fig.6.2 (E) Changes in pH and peak area of creosote in the C:N:P ratio 10:1:2. Error bars indicate ± 1 Standard Error.

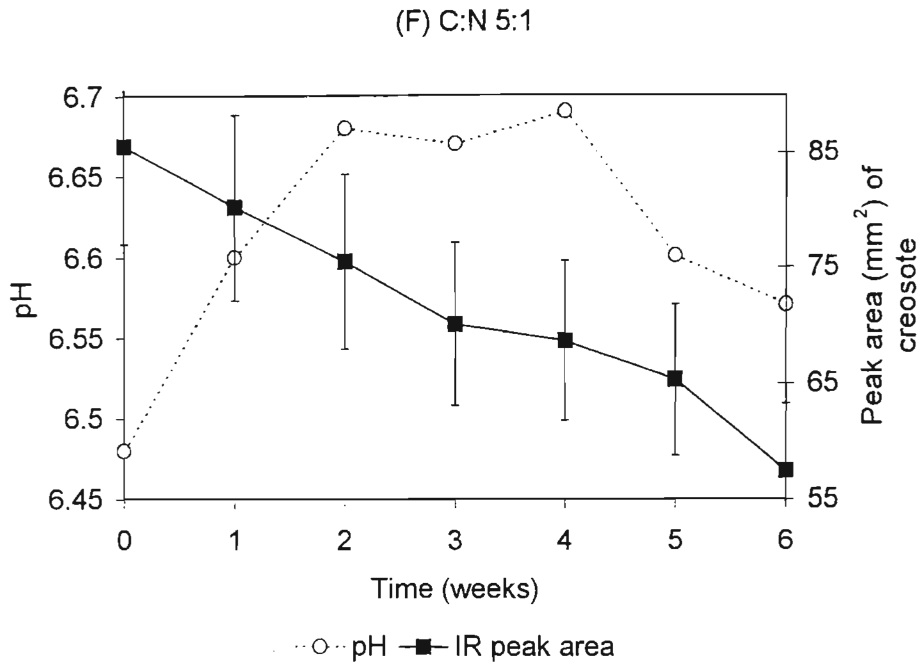



Fig. 6.2 (F) Changes in pH and peak area of creosote in the C:N ratio 5:1. Error bars indicate ± 1 Standard Error.

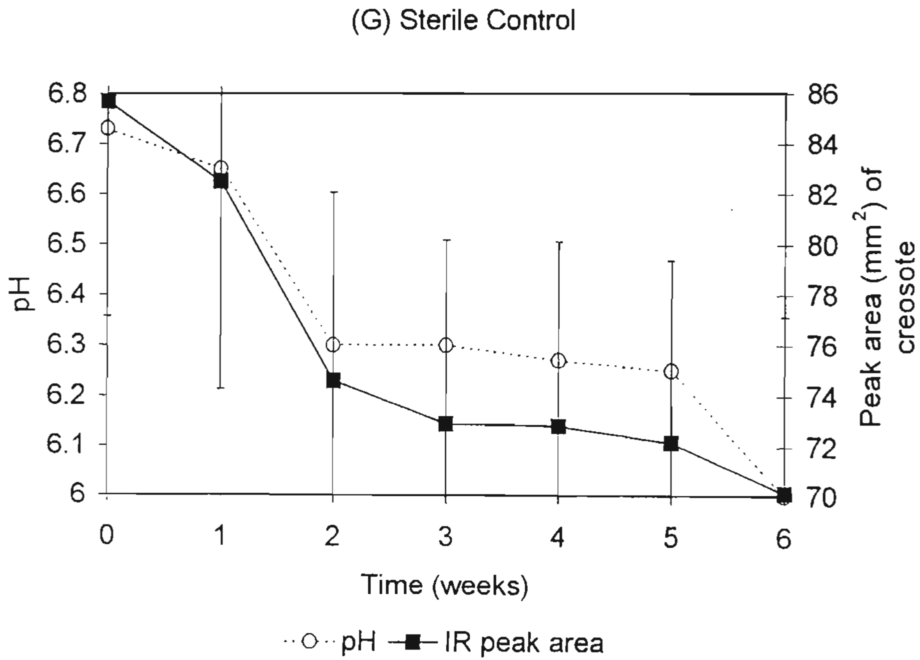



Fig. 6.2 (G) Changes in pH and peak area of creosote in the sterile control. Error bars indicate ± 1 Standard Error.

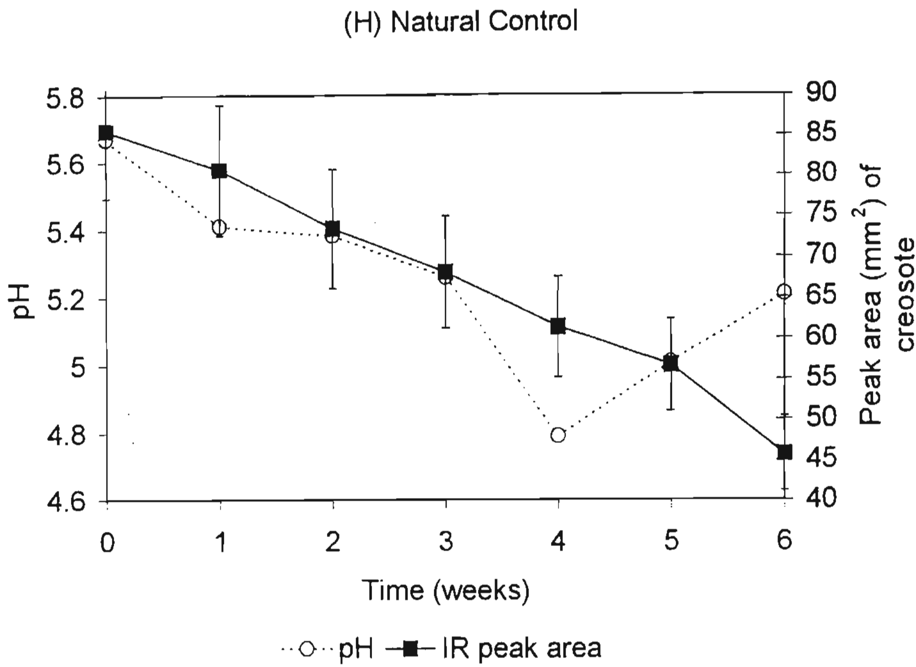



Fig. 6.2 (H) Changes in pH and peak area of creosote in the natural control. Error bars indicate ± 1 Standard Error.

which had 0.3 mg $kg^{-1}$ left at the end of the sixth week. Concentration of o-cresol decreased beyond detectable limits by the end of the third week in all C:N treatments including the natural control. All the C:N treatments except 5:1 reached undetectable levels by the end of the second week (Fig.6.2). Phenolics have been widely reported to be degraded by soil microorganisms at varying concentrations and after relatively short periods of time ( Edgehill, 1983, 1994; Vipulanandan, *et al.*, 1994; Dyreborg, *et al.*, 1995; Häggblom and Valo, 1995).

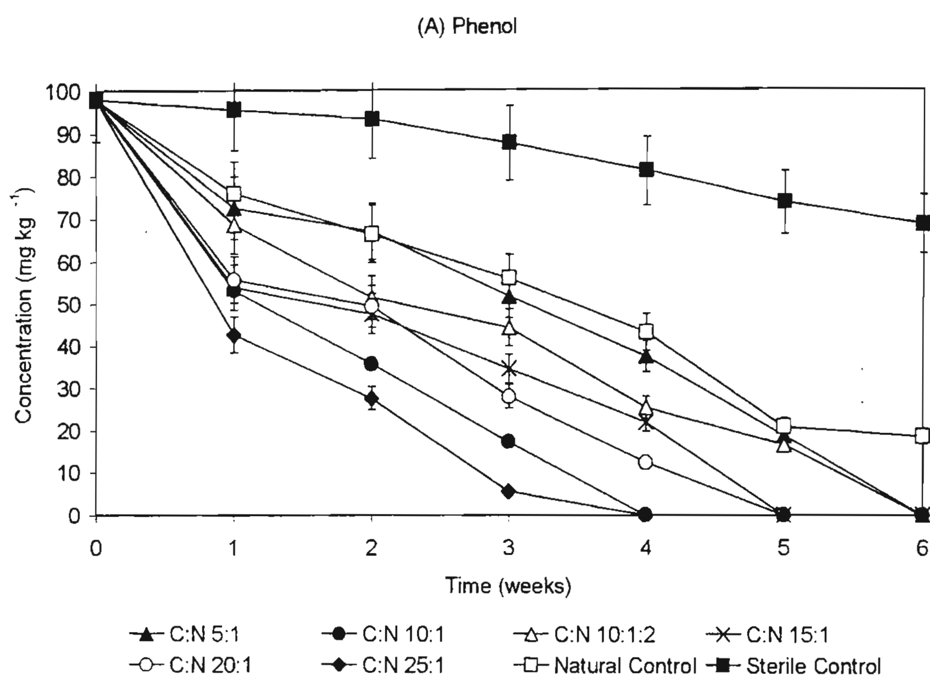



Fig. 6.3 (A) Changes in the concentration of phenol in different C:N ratios in the creosote contaminated soil. Error bars indicate ± 1 Standard Error.

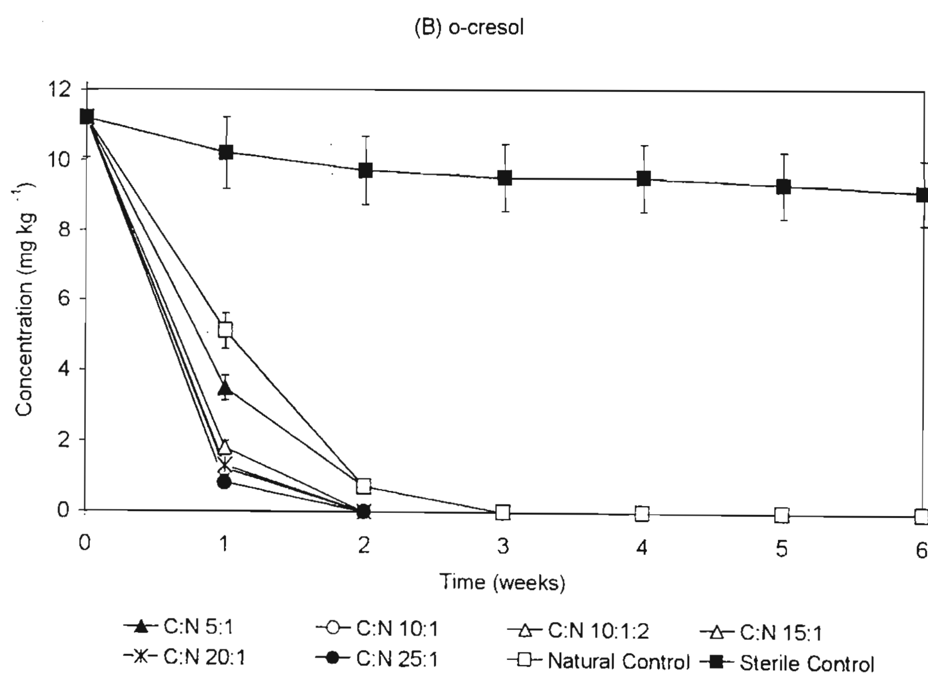



Fig. 6.3 (B) Changes in the concentration of o-cresol in different C:N ratios in the creosote contaminated soil. Error bars indicate ± 1 Standard Error.

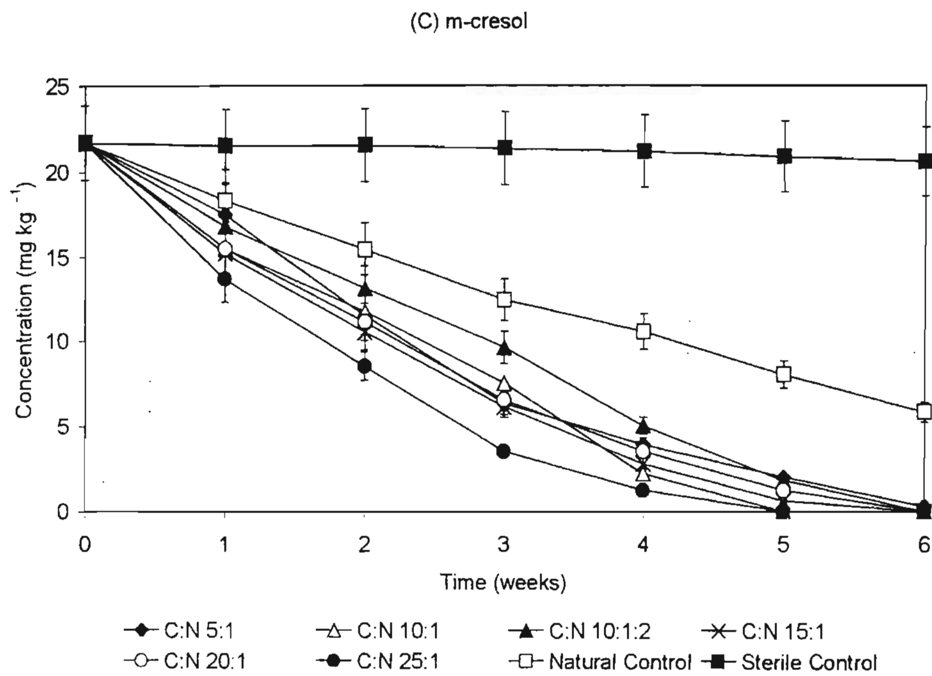



Fig. 6.3 (C) Changes in the concentration of m-cresol in different C:N ratios in the creosote contaminated soil. Error bars indicate ± 1 Standard Error.

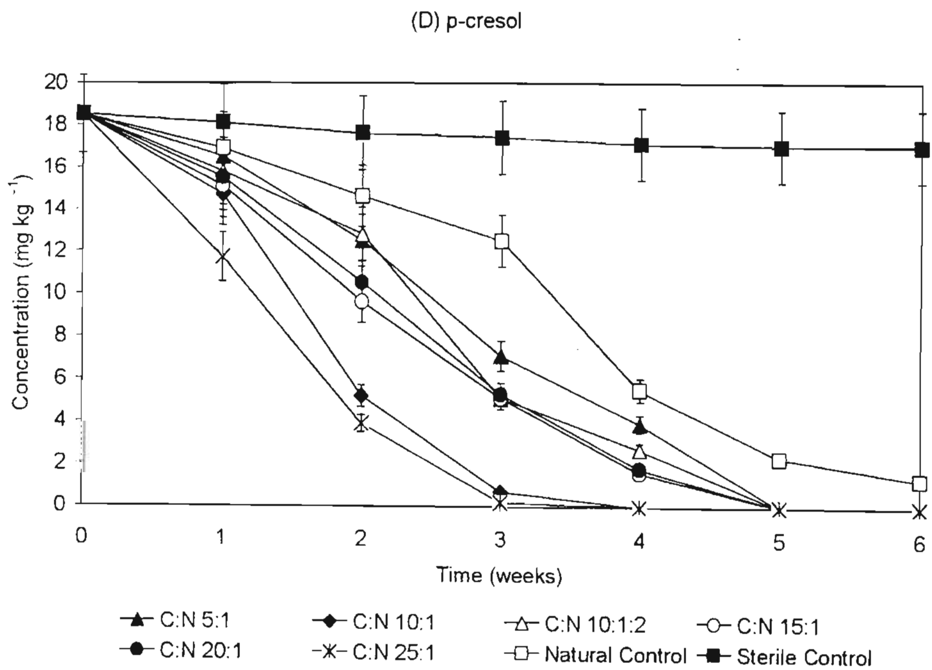



Fig. 6.3 (D) Changes in the concentration of p-cresol in different C:N ratios in the creosote contaminated soil. Error bars ± 1 Standard Error.

The relative ease with which soil microorganisms have been able to degrade phenolic compounds can be attributed to their simple structure when compared to the other aromatic compounds with more benzene rings. Those with simpler substitutions are also known to be more readily attacked by degrading microorganisms than those with complex substitutions (Gibson and Subramanian,1984; Sutherland *et al.*, 1995). The position of substitution and the substituting group have also been suggested to play important roles in the susceptibility of a phenolic compound to microbial degradation (Knackmuss, 1992; Alexander, 1999).

Among the PAHs studied, naphthalene was found to be the most susceptible to microbial degradation. Apart from the controls and C:N 5:1, naphthalene was completely removed by the end of the sixth week. The success with which naphthalene was degraded is attributed to its simple structure, consisting basically of two benzene rings, its low molecular mass and its high solubility in aqueous media (31 mg $L^{-1}$ at 25 $^{O}$ C) (Alexander,1994; Eriksson *et al.*, 2000). The degradation of naphthalene by biological processes has been well-studied and documented in the literature (Ghosha and Luthy, 1996,1998; Manohar and Karegoudar,1998; Annweiler *et al.*, 2000). Lajoie and Strom (1994), for example, reported the reduction of 202ppm of naphthalene from coal tar oil to concentrations less than 1ppm in two weeks. Results from this experiment demonstrate that high concentrations of naphthalene can be removed from contaminated soils over a short period of time by optimizing soil physical and chemical conditions. The removal of more than 70% of the compound from the contaminated soil in three different treatments by the end of the first week in comparison with the results from the controls, as shown in Fig. 6.3 (E), demonstrates that soil nutrient

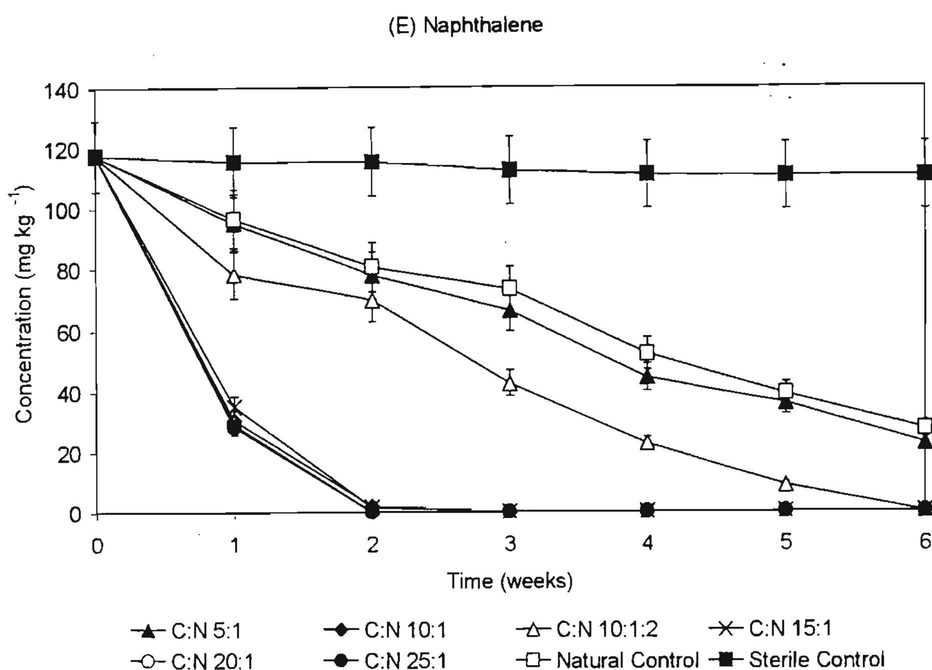



Fig. 6.3 (E) Changes in the concentration of naphthalene in different C:N ratios in the creosote contaminated soi. Error bars indicate ± 1 Standard Error.

manipulation can speed up the degradation of naphthalene in contaminated soils. Although the intermediates of naphthalene degradation were not studied, it is assumed that intermediates produced were readily mineralized or biotransformed, since there was no observed inhibition in microbial growth during the period of study. Intermediate products of the degradation of naphthalene are known to interfere with the further degradation of naphthalene if allowed to accumulate in the reaction medium (Auger *et al.*, 1995; Goshal and Luthy, 1998).

Figures 6.2 (F) and (G) show that anthracene is much less susceptible to degradation than phenanthrene. Even though about 81% of anthracene was removed by the end of the sixth week of incubation in C:N 25:1, most of the other treatments still contained appreciable concentrations of the compound (Figure 6.2 (F)). This slow degradation is attributable to its low solubility (0.05mg $L^{-1}$)

in aqueous systems which renders it only slowly available for microbial attack. Phenanthrene on the other hand, was rapidly degraded ( Figure 6.2 ( G )) in most of the treatments in spite of its high initial concentration (215 mg kg$^{-1}$) in the contaminated soil. This is due to its higher solubility (1.1 mg L$^{-1}$) in aqueous media, making it more available for microbial attack than anthracene. Although, both compounds consist of the same number of rings and have the same molar mass, their structures are different. The difference in spatial arrangement of the ring structure possibly accounts for the difference in solubility. This difference in the rate of degradation of the two compounds has been reported previously in the literature ( Mueller *et al.*, 1991; Lajoie and Strom, 1994).

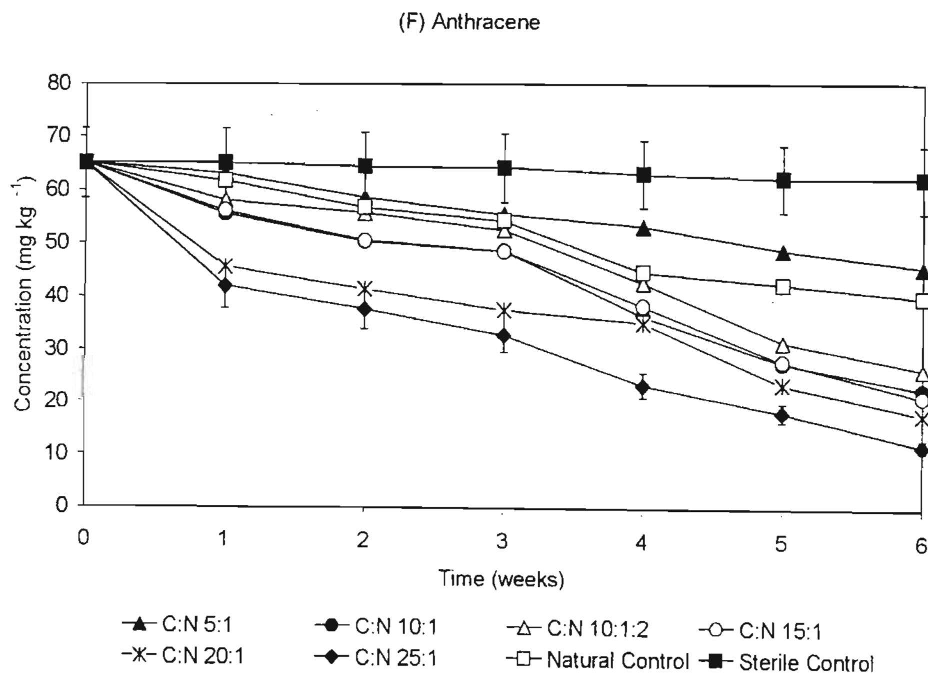



Fig. 6.3 (F). Changes in the concentration of anthracene in different C:N ratios in the creosote contaminated soil. Error bars indicate ± 1 Standard Error.

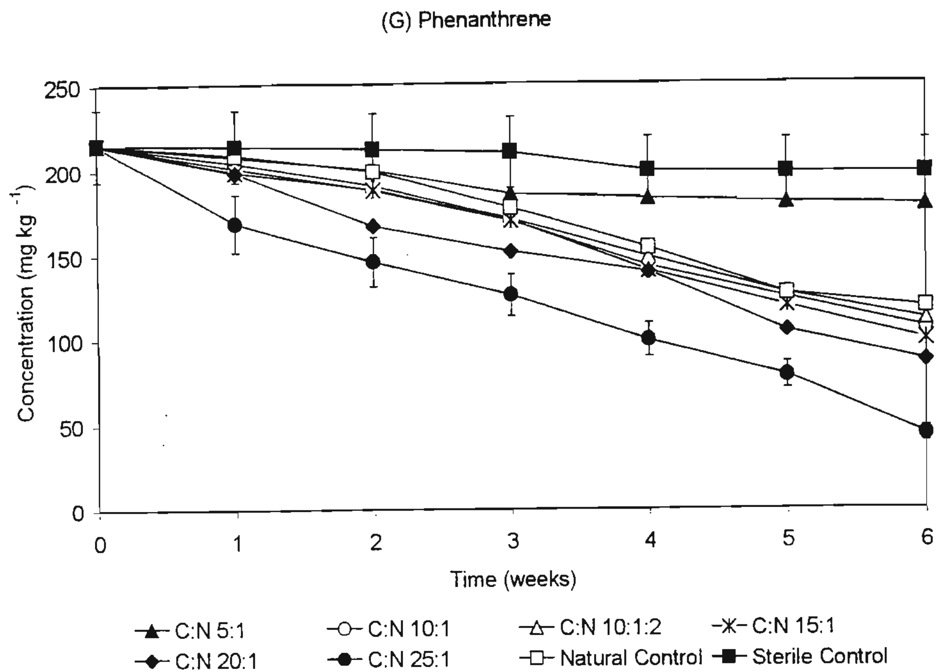



Fig. 6.3 (G). Changes in the concentration of phenanthrene in different C:N ratios in the creosote contaminated soil. Error bars indicate ± 1 Standard Error.

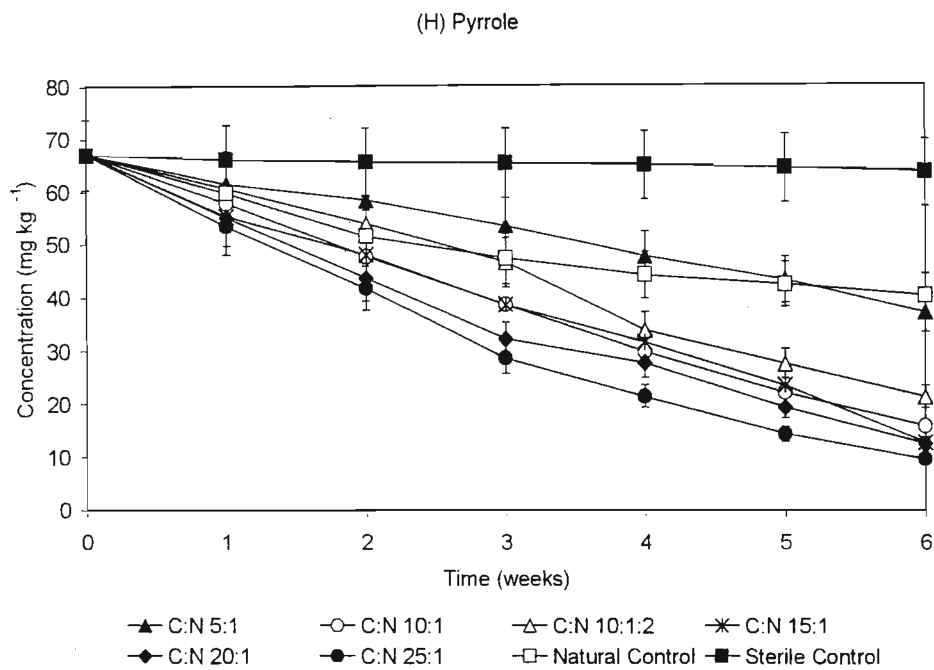



Fig. 6.3 (H). Changes in the concentration of pyrrole in different C:N ratios in the creosote contaminated soil. Error bars indicate ± 1 Standard Error.

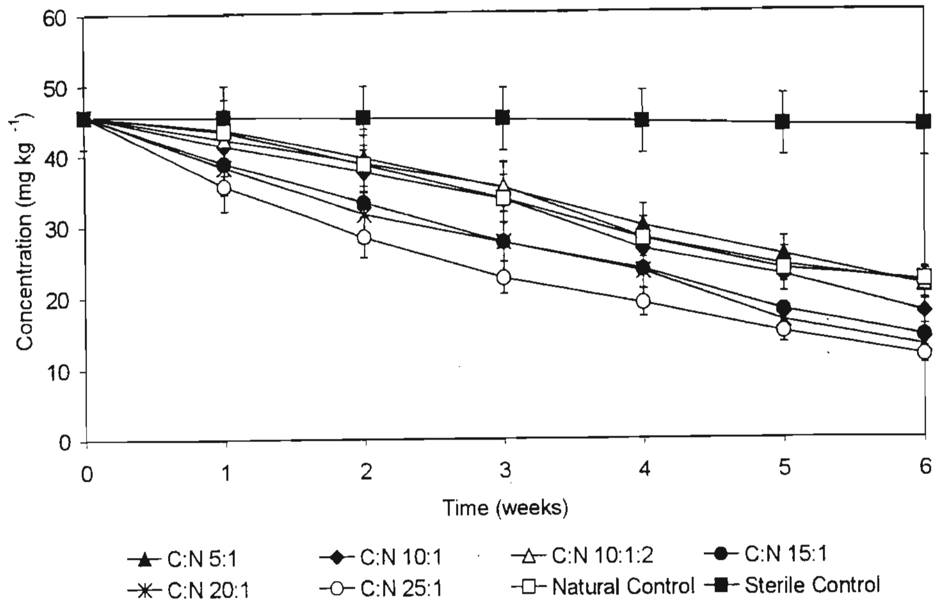



Fig. 6.3 (I). Changes in the concentration of fluorene in different C:N ratios in the creosote contaminated soil. Error bars indicate ± 1 Standard Error.

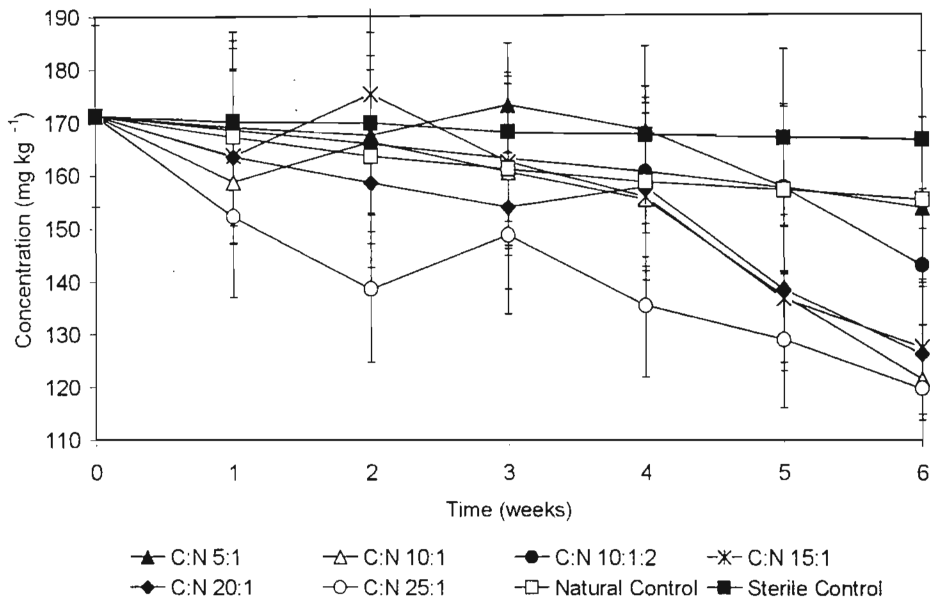



Fig.6.3 (J) Changes in the concentration of pyrene in different C:N ratios in the creosote contaminated soil. Error bars indicate ± 1 Standard Error.

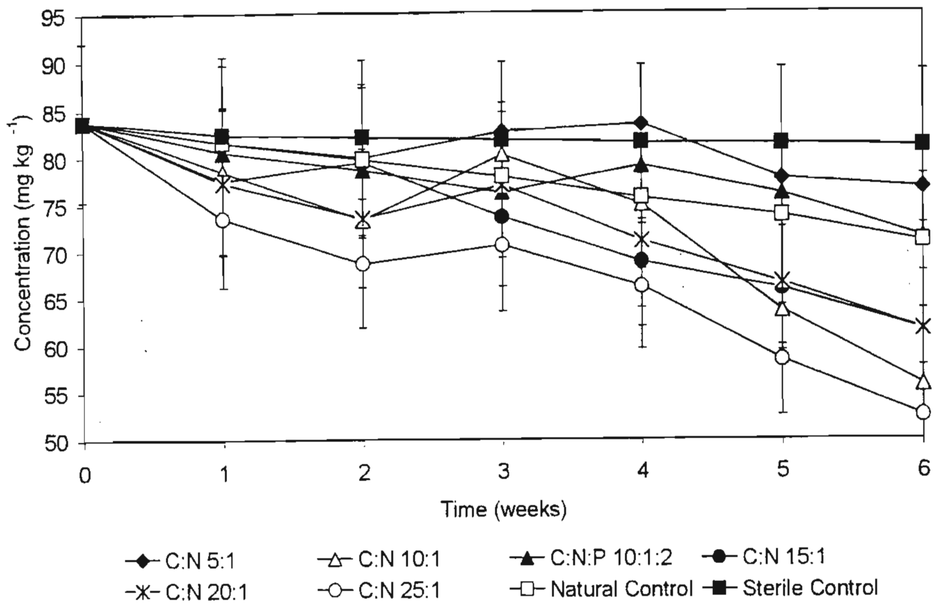



Fig. 6.3 (K) Changes in the concentration of chrysene in different C:N ratios in the creosote contaminated soil. Error bars indicate ± 1 Standard Error.

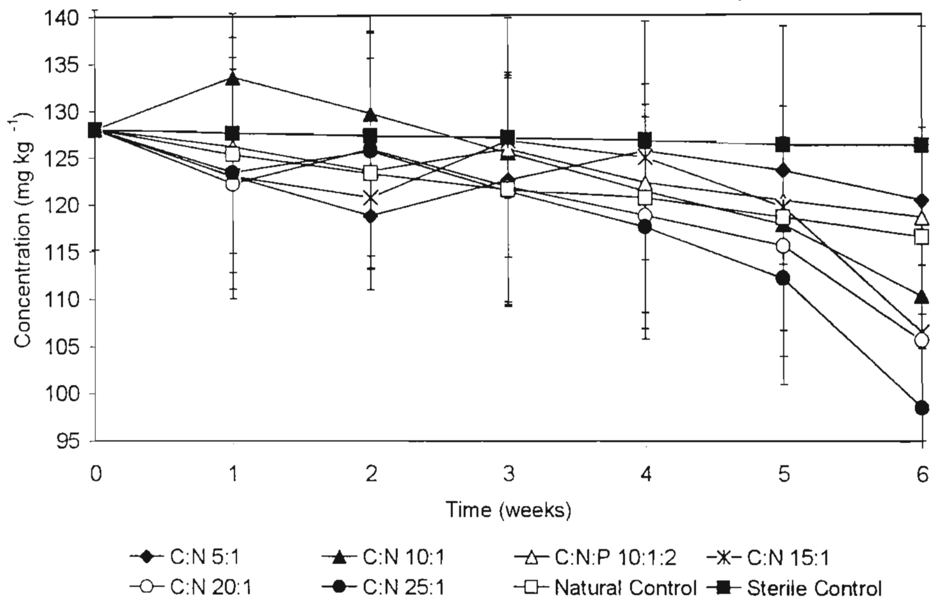



Fig. 6.3 (L) Changes in the concentration of fluoranthene in different C:N ratios in the creosote contaminated soil. Error bars indicate ± 1 Standard Error.

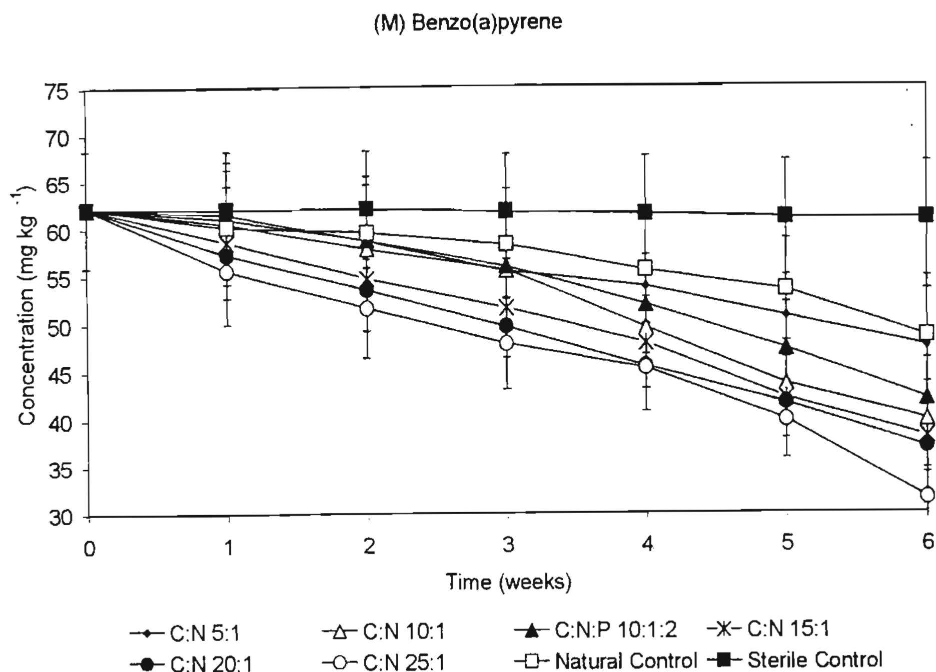



Fig.6.3 (M) Changes in the concentration of benzo(a)pyrene in different C:N ratios in the creosote contaminated soil. Error bars indicate ± 1 Standard Error.

The removal of the higher molecular mass compounds is shown in Figure 6.2 (H) to (M). Although the higher molecular mass compounds were observed to be more recalcitrant, nutrient amendment increased the rate of degradation of the compounds. The C:N ratio of 25:1 was found to be the most effective amendment ratio tested. The effectiveness of nutrient amendment decreased with an increase in molar mass, number of rings and the complexity of the structure. That is: phenols< naphthalene< phenanthrene< anthracene<pyrolle<fluorene<pyrene<chrysene<fluoranthene >benzo(a)pyrene. The highest supplementation C:N 5:1 was also found to be the least effective in enhancing degradation. This ratio was found to be inhibitory in most cases. Very high nitrogen content have been reported to impair microbial utilization of carbon in soil (Lees, 1996). Nutrient supplementation is known to be most effective when applied in quantities close to the required concentration (Baker and Herson, 1994).

Among the higher molecular mass compounds, concentrations were observed to increase rather than decrease at certain times during the period of study. Such increases were, however, followed by decreases in the subsequent week. This phenomenon was found in all treatments but was more frequently observed at in the C:N ratio 5:1. It did not occur for pyrrole, fluorene and benzo(a)pyrene. This behaviour is attributed to faster degradation of associated compounds, improved desorption, or other compounds being transformed at certain stages into the compound, showing the increase. This phenomenon has been previously reported during the degradation of some compounds like fluoranthene (Mueller *et al.*, 1991). The nutrient amendments used were found to enhance degradation of all the compounds, particularly at the lower supplementation levels, with a C:N ratio of 25:1 showing best results for all the compounds studied. The effectiveness of the treatment can greatly be attributed to the soil type, whose composition and physical structure allowed minimal application of nutrients for the results obtained.

Results from soils set at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and moisture contents set at 45, 50, 55, 60, 65, 70, 75, 80% of field capacity, respectively, showed that microbial activity was highest between 60 and 70% of field capacity (Figure 6.1). Previous studies have generally shown that soil microbial activity is optimum at between 50% and 70% field capacity (Atlas and Bartha, 1972; Lajoie and Strom, 1994). The optimum moisture content varies from one soil to another, with soil physical properties playing a major role in determining the water holding capacity and the interaction with the soil microbial population. Dibble and Bartha (1979) reported high microbial activity and hydrocarbon degradation at 30% soil moisture content. However, Deuel *et al.* (1978) reported that optimal activity can also be observed at 100% field capacity. Although soil-slurry reactors have been reported to be very effective for treating creosote contaminated soils, such a treatment is not appropriate for the

present research because the objective is to develop a cost-effective treatment for large-scale bioremediation.

The microorganisms involved in the utilization of creosote hydrocarbons in the soil demonstrated a wide range of pH tolerance, with the counts of microorganisms continuing to increase up to the sixth week between the pH 5.5 and 8.0; maximum activity was recorded between pH 6.5 and 7.0. Biodegradation of hydrocarbons has been widely reported to proceed effectively at pH 7 ( Atlas and Bartha, 1972; Dibble and Bartha, 1979; Alexander, 1999; Tate, 1995). At extremes of acidity and alkalinity, activity generally declines (Alexander, 1994). At more moderate pH values, biodegradation tends to be fastest. If a compound in a particular environment can be metabolized by a diverse group

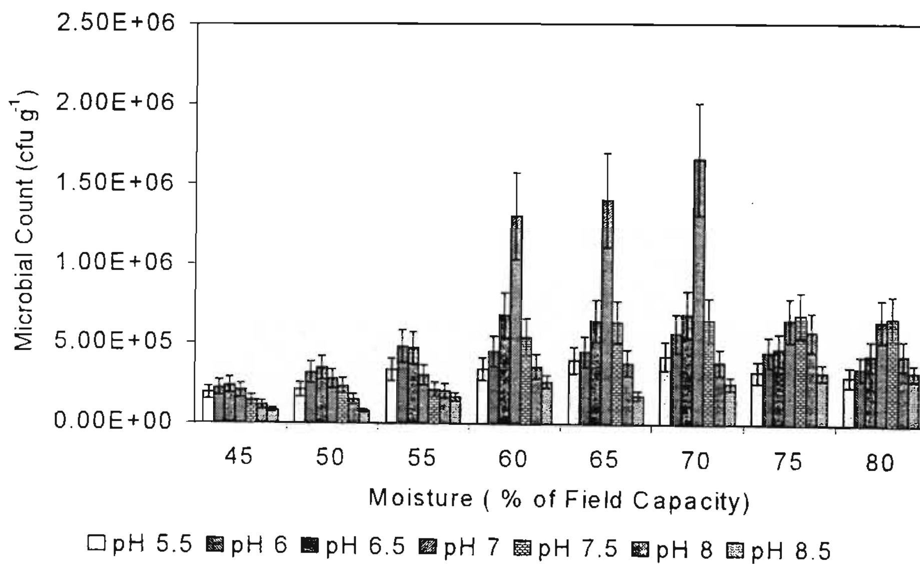


Fig. 6.4 Effect of moisture and pH on microbial activity in the creosote contaminated soil. Error bars indicate ± 1 Standard Error.

of organisms, the range of pH at which degradation occurs frequently is broader than if only one species can bring about the transformation (Alexander, 1999). The optimal pH for microbial activity was found to vary with change in moisture content of the soil. For example, at 60 to 70% moisture content, a pH of between 6.5 and 7.0 was found to be optimal. At lower water contents (45 - 55%), optimal activity was recorded at pH 5.5 to 6.5. Microbial activity in this range was, however, found to be much lower than was observed at 60 to 70% moisture content and pH 6.5 to 7.0. At higher moisture contents (70 to 80 %) the pH range for optimal activity shifted to 6.0 to 7.5. This moisture range exhibited a wider pH tolerance, but microbial counts were found to be much lower than was recorded in the 60 to 70% moisture range. These responses are thought to be due to changes in the composition microbial species prevalent in the soil with changes in pH and moisture content. Temperature was kept constant at $30^{O} \pm 2^{O}C$ throughout the incubation period. The effect of temperature on all the treatments was thus uniform. All jars were tilled in the same pattern and, as a result, oxygen distribution was assumed to be uniform in all the treatments.

The overall decrease in mass in all samples can be attributed to loss in hydrocarbon content, since all samples were moistened with the same amount of water at the same time to keep the soil at the same moisture content. All samples were seen to have a moisture content of about 61% during analysis. Loss of water could not therefore have effectively accounted for the difference in mass of the jars. Fig. 6.5 shows the mean mass loss in all the jars over a period of six weeks. The mass loss was observed to be progressive and reached the peak (6.71g) in C:N 25:1 at the end of the incubation period. The pattern of mass loss was observed to correlate well with microbial population and the decrease in creosote concentration.

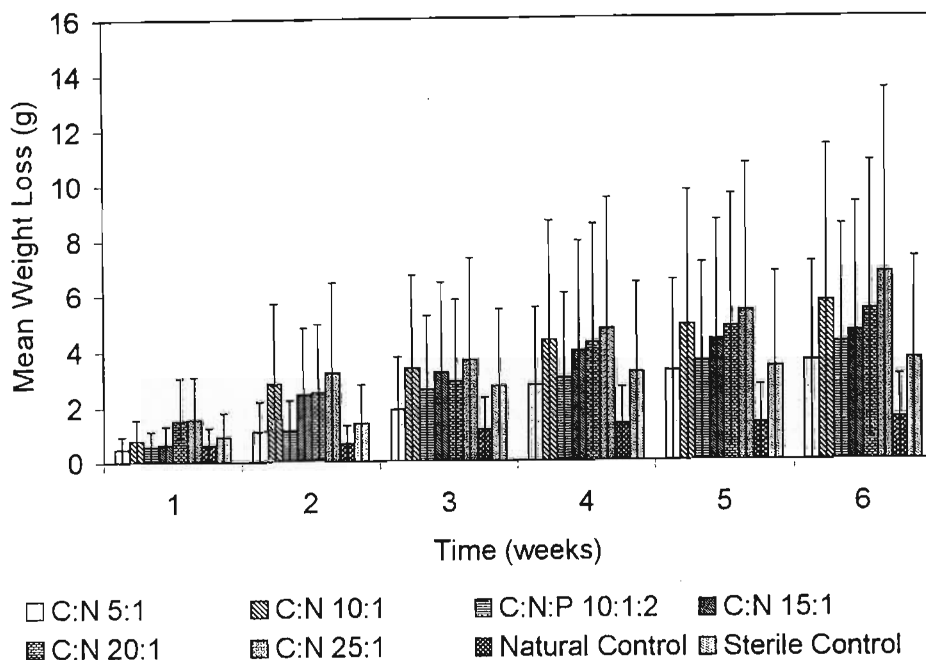



Fig.6.5 Mean mass loss of soil samples over the period of treatment. Error bars indicate ± 1 Standard Error.

## 6.4 CONCLUSIONS

The results from the analyses of the carbon content of the soil confirmed that the soil was heavily contaminated with creosote, with a mean carbon content of 130 000mg C kg $^{-1}$ soil. This amount of carbon was found to make the nitrogen component of the soil limiting for microbial growth. Supplementation with nitrogen was necessary for effective growth of the microorganisms. The results show that nutrient supplementation at lower concentrations of N (as was represented in the C:N ratio of 25:1) was most effective in enhancing biodegradation of the creosote hydrocarbons. The effectiveness of supplementation on microbial growth decreased with increases in the ratio of N and P to C. The highest concentration of N (C:N 5:1) was found to be inhibitory to microbial activity. Decreases in creosote concentration during the study were found to correlate with increases in

microbial activity, with the highest decrease of 68.7% recorded at C:N of 25:1 and the least (32.7%) at C:N of 5:1. The decrease in creosote concentration was also found to directly correlate with a decrease in soil pH. It was established that creosote degradation can proceed in the soil without nutrient supplementation, particularly where moisture and oxygen are adequate. Changes in soil water content were found to affect the pH optimum for creosote biodegradation. Optimal pH and moisture range for microbial degradation of creosote in soil were found to be 6.5 - 7.0 and 60 and 70%, respectively. Although an overall decrease in the mass of the soil samples correlated with the decrease in concentration of creosote, this could not be conclusively established since mass loss can be attributed to a number of other parameters in the system. Decreases in the concentration of the selected creosote compounds were found to follow the pattern of decrease in the total creosote hydrocarbon. However, the phenolics and the lower molecular mass PAHs were more susceptible to microbial degradation than the higher molecular mass PAHs. It is concluded that nutrient supplementation can effectively enhance the degradation of creosote in contaminated soil and that management of aeration, moisture content and pH are also important considerations. These results, and results from other laboratory trials, will be considered during the landfarming project.

# CHAPTER 7

# PILOT-SCALE LANDFARMING FOR THE BIOREMEDIATION OF CREOSOTE-CONTAMINATED LAND

## 7.1 INTRODUCTION

Landfarming, also known as land treatment or land application, is an above-ground remediation technology for soils which reduces concentrations of petroleum constituents through bioremediation (Hansen, *et al.*, 2000; Harris, 2000; Talley *et al.*, 2000). This technology usually involves spreading excavated soil in a thin layer on the ground surface and stimulating aerobic microbial activity within this soil through aeration and/or the addition of minerals, nutrients and moisture (EPA,1995). The enhanced microbial activity results in degradation of the adsorbed petroleum product through microbial respiration (EPA,1995). Soil excavation may not be necessary in stimulating microbial activity if the contaminated soil is shallow (i.e. less than 1 m deep). Excavation is usually applied to contaminated soils deeper than 1.5 m. Thus landfarming can be an *ex situ* solid-phase bioremediation technique where contaminated soil is treated above ground, using conventional soil management practices such as tilling, irrigation and fertilization, to enhance the microbial degradation of contaminants (Brunsbach and Reineke, 1993; Talley *et al.*, 2000; Kuyukina *et al.*, 2001). By contrast, it can also be an *in situ* technology where the contaminated soil is treated in place without excavation or movement of the soil from the site (Baker and Herson, 1994; Harmsen and Ferdinandy, 1999; Winningham *et al.*, 1999; Kuyukina *et al.*, 2001). *In situ* remediation techniques are known to be cost effective and easy to apply (Baker and Herson, 1994).

Landfarming has been proved to be effective in reducing concentrations of nearly all the constituents of petroleum products typically found in underground storage sites (EPA, 1995). Lighter fractions of petroleum commonly volatilize during aeration processes such as tilling and ploughing and are, to a lesser extent, degraded by microbial respiration. Higher molecular mass or heavier fractions are usually degraded by biological processes but take long periods (Wang *et al.*, 1990; Testa *et al.*, 1991; Baker and Herson, 1994; EPA, 1995; Winningham *et al.*, 1999; Harris, 2000).

Although originally a petroleum spill cleaning procedure, landfarming has been applied to the remediation of soils contaminated with a wide range of other chemical pollutants, including organic compounds from a variety of sources such as coal tar and other coal gasification products (Fan and Tafuri, 1994; Lajoie *et al.*, 1994; Harris, 2001). However, the success of the technique in degrading petroleum products in soils may not necessarily be repeated with other pollutants. Mixed results have been obtained from attempts to remediate creosote by landfarming, as creosote has a large number of compounds similar to those found in petroleum (Mueller *et al.*, 1991; Winningham *et al.*, 1999; Harris, 2000; Kuyukina *et al.*, 2001).

The objective of the present study is to develop a remediation strategy which will be cost-effective and easy to apply for small-scale operators using creosote to treat utility poles in South Africa. It is therefore looking at employing conventional landfarming principles, in conjunction with biostimulation and bioaugmentation, for the purpose of remediating creosote- contaminated soils. The determination of the effects of: aeration through mechanical tilling; moisture at 70% field capacity; hydrogen peroxide ($H_2O_2$) as a supplemental source of electron acceptor; indigenous soil

microbial enrichment as a biosupplement; organic fertilizers in the form of sewage sludge, cattle manure, poultry manure as biosupplements; inorganic fertilization through mono-ammonium phosphate as nutrient supplement, are the objectives of this study.

The study is also aimed at comparing the efficacies of biostimulation and bioaugmentation in the landfarming process, with a view to determining which will be the most effective measure to reduce creosote concentrations in the specific soil studied.

## 7.2 MATERIALS AND METHODS

### 7.2.1 Soil samples

About 500 kg of soil heavily contaminated with creosote ( 258 257.4 mg $kg^{-1}$), was collected from site by digging 12 holes of approximately 30cm x 30cm x 30cm deep. The 12 holes were dug in markedout quadrants across an area of about 20m x 20m to ensure that the samples collected represented the contamination situation in the site. The samples were bulked and thoroughly mixed in an electric concrete mixer to homogenize the soil before it was stored at $4^{0}$ C until required. A one kilogram sample of the soil was taken for creosote analysis.

### 7.2.2 Experimental design

Eighteen polyvinyl chloride (PVC) vessels of about 30 litre capacity each, with a diameter of 56 cm

and a depth of 22cm, were filled with 25kg of creosote-contaminated soil (the same as in section 7.2.1). All treatments were duplicated. To each of the soil reactors was added 175g of agricultural lime ( $CaCO_3$ ) to raise the soil pH to approximately 7. Table 7.1 shows the experimental design and the different treatments applied. To the soil in Treatment 1 was added 57 g of sodium azide to establish a metabolically inhibited control (sterile control).

Table 7.1. Experimental design and treatment for each soil reactor

| Duplicate Treatments (A&B) | Water | Sodium azide | MAP | Tilling (daily) | $H_2O_2$ | Indigenous microbial culture | Sewage sludge | Cow manure | poultry manure |
|---|---|---|---|---|---|---|---|---|---|
| 1 | no | yes | no | no | no | no | no | no | no |
| 2 | no | no | no | no | no | no | no | no | no |
| 3 | yes | no | no | yes | no | no | no | no | no |
| 4 | yes | no | yes | yes | no | no | no | no | no |
| 5 | yes | no | yes | yes | yes | no | no | no | no |
| 6 | yes | no | yes | yes | no | yes | no | no | no |
| 7 | yes | no | yes | yes | no | no | yes | no | no |
| 8 | yes | no | yes | yes | no | no | no | yes | no |
| 9 | yes | no | yes | yes | no | no | no | no | yes |

The sodium azide was applied twice, at the beginning of the experiment and in the seventh week of the experiment. The application was done by dissolving the sodium azide in distilled water and diluting to 2 L before mixing thoroughly with the soil. The method was adapted from Snyman (1996).

The pH values of the soil in the reactors were determined every week and when necessary, agricultural lime (7g $kg^{-1}$) was added to raise the pH to about 7. The pre-treatment level was about 5. Ambient air temperature was measured daily at noon. The temperature of the soil in the reactors was measured daily at noon to determine whether or not there were changes in the temperature of the soil in the reactors during the study period. Between 250 and 500 ml of water were added to the replicate reactors in Treatments 3-9 every two days to keep the soil moisture at about 70 % field capacity, as determined by mass. Aeration of soil in Treatments 3-9 was effected by turning the soil daily with a hand trowel.

Mono-ammonium phosphate (MAP) fertilizer ( 1g $kg^{-1}$ soil ) was added to Treatments 4-9 as a means of introducing ammonia and phosphate to the soil. The choice of MAP was made on the basis of the results obtained from the treatment of a soil heavily contaminated (>250 000mg $kg^{-1}$) with crude oil and in which nitrogen was found to be deficient (Snyman, 1996). The availability of MAP fertilizer in the market and the constraint in trying to formulate a special composition of N and P fertilizer also played a role in the decision to use MAP. The objective of the research, which is the development of a cost-effective treatment technology that will be readily available to and applicable by the local, small-scale wood treatment operator, was borne in mind when making the decision to use MAP. The application of MAP was repeated once every month for the duration of the experiment. As an alternative source of electron acceptor, hydrogen peroxide was introduced into the soil in Treatment 5, at a concentration of 500mg $L^{-1}$ (600 µl of a 39% solution) once a week (Thomas and Ward, 1989).

Treatment 6 received a weekly dose of 50 ml of an indigenous microorganism-biosupplement, which was prepared by adding 2g of a creosote-contaminated soil sample to an enrichment broth containing (g $L^{-1}$) $NaNO_3$, 2; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $Na_2HPO_4$, 1; $FeSO_4 \cdot 7H_2O$, 0.01; sucrose, 3; 30ml creosote [adapted from Harrigan and McCance (1966)]. The mixture was homogenized in a blender for about 20 seconds. A second creosote-contaminated soil sample (2g) was inoculated into 1 litre of soil extract broth (Parkinson *et al.*,1971) which contained 30ml creosote as additional carbon source. The broth was sonicated to release soil-adsorbed creosote and disperse the creosote properly before inoculation. The microbial suspension was incubated and aerated by means of a sintered glass diffuser at 25$^{\circ}$C for 4 days. This facilitated enrichment of both fungi and bacteria . The enrichment reactors were two 500ml glass filter funnels with a taps at the bottom to allow for decanting the microbial suspension without allowing the unemulsified creosote to contaminate the suspension. The population of bacteria was estimated by plate counts on nutrient agar.

Treatment 7 received a 10% (w/w) addition of sewage sludge which was dosed twice, the first at the beginning of the experiment and the second in the seventh week, during the treatment period. The sewage sludge was collected from the waste water treatment plant in Hammarsdale, KwaZulu-Natal Province, South Africa. The treatment plant handles both domestic and industrial waste water. Treatments 8 and 9 received 10 % (w/w) cow manure and poultry manure respectively. Both treatments were applied at the beginning and in the seventh week during the duration of the experiment.

Samples were taken at weekly intervals from all the soil reactors for analysis. The soil samples were

analysed for changes in concentration of creosote and for changes in concentration of selected creosote components. Counts of total heterotrophic microorganisms, creosote-degrading bacteria and fungi were made on nutrient agar and mineral salts agar supplemented with 30ml $L^{-1}$ creosote. Antibiotics and fungicides were incorporated into the media for isolating fungi and bacteria, as described by Atlas and Bartha (1979).

**7.2.3 Soil nutrient analyses**

Determination of total nitrogen was done by a soil digestion method (Forster, 1995). About 0.2 g of ground (2mm) creosote-contaminated soil was placed in a digestion tube and weighed. To this was added 4.4 ml of digestion mixture. The digestion mixture was prepared by adding 0.42 g selenium powder and 14 g lithium sulphate to 350ml of 30 % hydrogen peroxide and mixing thoroughly. Concentrated sulphuric acid (420 ml) was slowly added while cooling on ice. Six blanks (i.e. digestion mixture without samples) for the correction of results were prepared. The mixture was digested at 360$^{O}$C for 2 hours. After cooling, the mixture was transferred to a 100 ml volumetric flask with about 50 ml distilled water and allowed to cool again. The volume was made up to 100 ml with distilled water, mixed thoroughly and allowed to settle until the supernatant was clear. (Forster, 1995). Ammonium in the extract was determined by distillation and titration (Keeney and Nelson, 1982).

### 7.2.4 Isolation and characterization of indigenous soil microorganisms

Soil samples of 1g each were diluted serially ($10^{-1}$ to $10^{-9}$), using aqueous physiological saline solution (0.85% m/v) and plated out on soil extract agar and nutrient agar to estimate total colony forming units(cfu). Soil extract agar (Parkinson *et al.*, 1971) was prepared by autoclaving 500 g soil in 1 litre of distilled water at 121°C for 20 minutes. The suspension was suction-filtered through a Büchner funnel lined with No.5 Whatman's filter paper. One gram of agar was added to the filtrate and the pH was adjusted to 6.8 with NaOH. Glucose (1.0g), yeast extract (5.0g) and $K_2HPO_4$ (0.2g) were added to the filtrate before autoclaving again (Parkinson *et al.*, 1971). The dilutions were also plated on potato dextrose agar (PDA) which contained 500 mg of chloramphenicol per litre PDA and agar to estimate the total numbers of yeasts and fungi present in the soil (Davis and Westlake, 1978). Plates were incubated at 25°C for 24 hours for bacteria and at 25°C for 4 days for fungi. Pure cultures were obtained from colonies on the plates by means of dilution streaking. Gram stains and light microscope examinations were carried out on all bacterial cultures. Cultural and morphological characteristics of all fungal isolates were used to identify the fungi to genus level ( Rapper and Thom, 1968 ).

### 7.2.5 Plate counts of total heterotrophs and creosote-degrading species

A 1 g sample of soil was taken from each reactor on a weekly basis and analysed for the total number of indigenous microorganisms present, using standard dilution plating techniques on nutrient agar. The soil was also analysed for total number of creosote degrading microorganisms by inoculating

a modified version of the medium, described by the Organisation for Economic Co-operation & Development (OECD), containing 30ml creosote oil as the sole carbon source. The OECD medium was prepared by adding 17g agar, 4ml $FeCl_3$ (0.25g $L^{-1}$), 1ml each of $MgSO_4 \cdot 7H_2O$ (22.5g $L^{-1}$), $CaCl_2$ (27.5g $L^{-1}$) and $(NH_4)_2SO_4$ 40g $L^{-1}$ to 2ml of the following mixture: $KH_2PO_4$ (8.5g $L^{-1}$), $K_2HPO_4$ (21.75g $L^{-1}$), $NaH_2PO_4 \cdot 7H_2O$ (33.4g $L^{-1}$) and $NH_4Cl$ (1.7g $L^{-1}$) and diluting to one litre with distilled water. The mixture was autoclaved at 121°C for 20 minutes and cooled. Creosote oil was filtered through a hydrophilic membrane (0.4μm) pore filter and agar was added to it before the plates were poured.

### 7.2.6 Determination of concentrations of creosote and selected creosote components in the soil

Total creosote concentration in the soil was analysed using the USEPA 4181.1 (1982) method as follows: creosote-contaminated soil (2g) and 2g anhydrous $Na_2SO_4$ were placed in a 30ml amber glass vial. Carbon tetrachloride (10ml) was added and the vial was sealed with a teflon lined screw cap. The sealed vial was vortexed for 15 seconds and then placed in a sonicating bath (Whaledent Biosonic) for 15 minutes before remixing on the vortex mixer for about 15 seconds . It was then placed in the sonicating bath for another 15 minutes. The solvent was transferred to a clean, dry vial containing 1g activated Florosil™ (Sigma) and 0.6ml water [i.e. 6% water (w/w)]. The sealed vial was shaken for one minute and allowed to stand overnight at ambient temperature. This silica "clean-up" procedure was used to remove interfering humic materials (EPA,1985). The extract was finally filtered through a Whatman GF/C glass fibre filter. The filtrate was made up to 10 ml in a volumetric flask and the absorbance determined with a Nicolet Avater 320 Infra-red Spectrophotometer at wave

numbers between 2760 and 3070 $cm^{-1}$ and an integration value for the absorbance peak area was automatically generated.

Determination of changes in the concentration of selected creosote hydrocarbons was done by Soxhlet extraction and GC/FID. The GC was a Varian-3800 with argon as the carrier gas and fitted with a 30 m capillary column with 0.25mm internal diameter and 0.25μm film thickness, and a flame ionization detector (FID). Two temperature programmes were run in order to obtain good separation and quantification of the more volatile compounds. The first temperature programme was: $60^{O}C$, 4min., followed by ramping at $10^{O}C$/min. up to $235^{O}C$, maintained for 40min.; injector temperature $220^{O}C$. The second temperature programme was used for the analysis of the more volatile compounds, viz. $20^{O}C$; 1 min $40^{O}C$, 1min., $10^{O}C$/min., ramping up to $200^{O}C$, maintained for 20 min.; injector temperature $220^{O}C$ (Eriksson *et al.*,2000).

## 7.3 RESULTS AND DISCUSSION

### 7.3.1 Characteristics of the contaminated soil

The concentration of hydrocarbon in the composite soil sample was 258 257.4mg $kg^{-1}$ [25.83% (w/w)]. Individual samples from the study area had creosote concentrations varying from 180 000 mg $kg^{-1}$ (18%) to 380 000mg $kg^{-1}$ (38%) (Section 2.3.2.1).

The extractable phosphorus content of the soil was 4.75mg $kg^{-1}$ soil. This meant that the phosphorus

content of the soil had to be increased, because the typical phosphorus concentration of a soil ranges from 12 to 18 mg $kg^{-1}$ soil ( Snyman, 1996 ). Total nitrogen was found to be 0.081%. Inorganic nitrogen normally represents about 2% of the total nitrogen in soils ( Keeney and Nelson, 1982 ).

Table 7.2. Characteristics of the creosote contaminated soil.

| Parameters | Means |
|---|---|
| Total creosote | 258,257.4 mg $kg^{-1}$ |
| Total organic carbon | 5.3% |
| pH | 5.45 |
| Total nitrogen | 0.08% |
| Extractable phosphorus | 4.75 mg $kg^{-1}$ |
| Clay | 18.75% |
| Fine silt | 18.75% |
| Sand | 62.5% |
| Total exchangeable cations | 4.61 cmol/L |
| Exchange acidity | 0.19 cmol/L |
| Acid sat. | 12.5% |
| Loss on drying at 105°C ($H_2O$ & Volatile organics) | 37.3% |
| Non-volatile fractions & water of crystallization at 800°C | 17.5% |
| Total heterotrophic microorganisms | 2.18 x$10^6$ cfu $g^{-1}$ |

This meant that additional nitrogen was necessary in the soil. On drying at 105$^{o}$C, 37.3% of the total mass of the sample was lost (Table 7.2). This represents mainly water and volatile organic matter. A further 17.5% was lost on ignition at 800$^{o}$C representing the non-volatile organic matter.

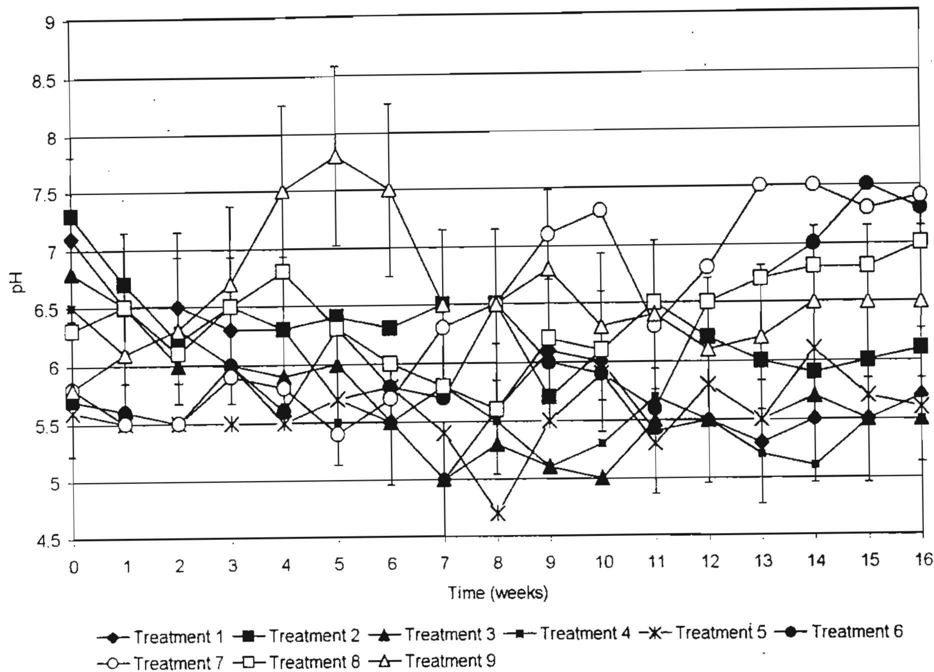



Fig.7.1 Changes in pH of soil in treatment reactors during pilot-scale landfarming. Error bars indicate ± 1 Standard Error.

The soil pH in most of the treatments fluctuated between 7 and 5.5. However, higher pH values occurred in Treatments 6, 7 & 9, where the highest recorded values were 7.5 in Treatments 6 and 7, and 7.8 in Treatment 9. The lowest pH encountered outside this range was 4.7 in Treatment 5, 5.0 in Treatment 3 and 5.1 in Treatment 4 (Fig.7.1). These pH values are, however, not outside the conventional values reported to sustain microbial activity in the soil (Alexander, 1999). This can be substantiated by the fact that microbial activity in the reactors continued to flourish during the entire period of the experiment. The addition of lime, however, kept the pH controlled at about pH 7 during

the durationof the experiment. As was observed in the results of pH measurements in Chapter 6, there was a general decrease in pH in this experiment. A similar explanation as that given in Chapter 6 may apply in this case. Thus the decrease in pH in Treatment 1 (sterile control) can only indicate that natural attenuation, or very slow degradation, occurred naturally through some other action such as photoxidation.

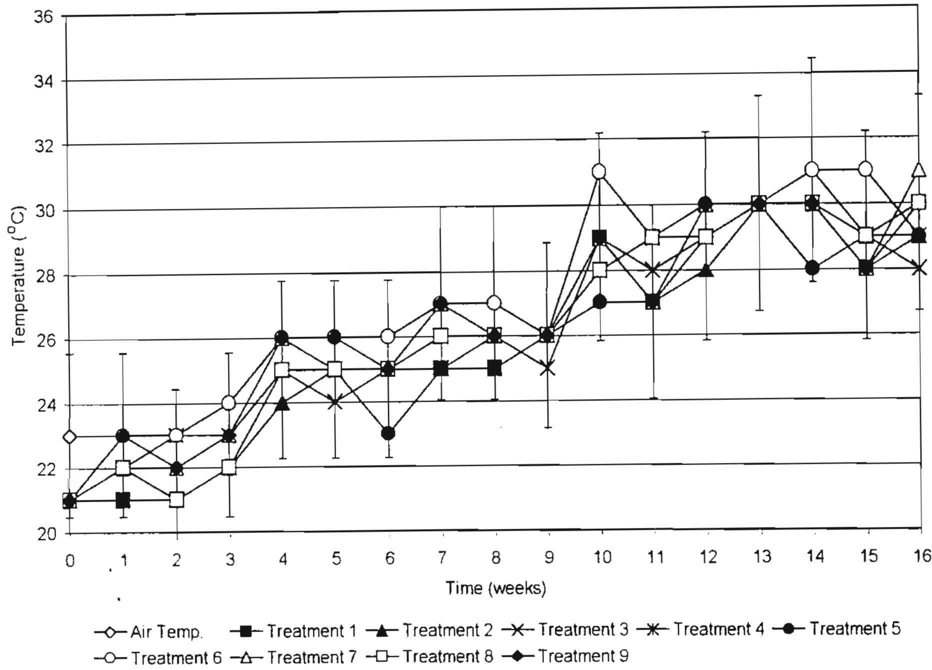



Fig.7.2 Changes in temperature of air and soil in treatment reactors during pilot-scale landfarming. Error bars indicate ± 1 Standard Error.

Temperature changes in the air and in the soils in the reactors during the experiment are shown in Fig. 7.2. Soil temperatures were observed to be slightly below the mean day air temperature, in some cases. However, in a few instances soil temperature, was slightly above the air temperature but usually by not more than 1 or $2^{0}$ C. These cases, where soil temperatures were higher than the ambient temperature, did not show any particular pattern. However, this response was observed mainly in treatments that received biosupplementation. The overall temperature pattern in the soils

followed the mean day temperature of the air, with the lowest temperature recorded in the late winter months and the highest in the summer months (Fig. 7.2). The experiment was started in July (late winter in the southern hemisphere).

### 7.3.2 Degradation of contaminant creosote oil

The decrease in creosote concentration in contaminated soil is shown in Fig.7.3 - 7.6. A comparative study was carried out of the six treatments (Treatments 4 - 9) that received the different forms of supplementation and the sterile control that was treated with sodium azide. The sodium azide acted as an electron transport inhibitor by interfering with electron flow in cytochrome oxidase, as was suggested by Stryer (1988). This condition created a biologically sterile medium in the reactor, as it caused a shortage in ATP and consequently cell death.

The concentration of creosote in reactor1(sterile control) was seen to decrease from an initial 258 257.4mg $kg^{-1}$ to 215150mg $kg^{-1}$, representing a 16.7% loss in creosote content of the soil (Fig.7.3). This was considered to be relatively high for a sterile medium. However, it indicates that a some quantity of volatile organic components of the creosote remained in the contaminated soil in spite of its age, as this reduction can only be attributed to volatilization. Another possible explanation is that seepages from the discharge unit which still held a large quantity of creosote, may have continued to contaminate the soil further with the lower molecular mass, more volatile fractions of creosote. It is also possible that the volatile fractions may have been "locked up" in interstitial spaces and closed

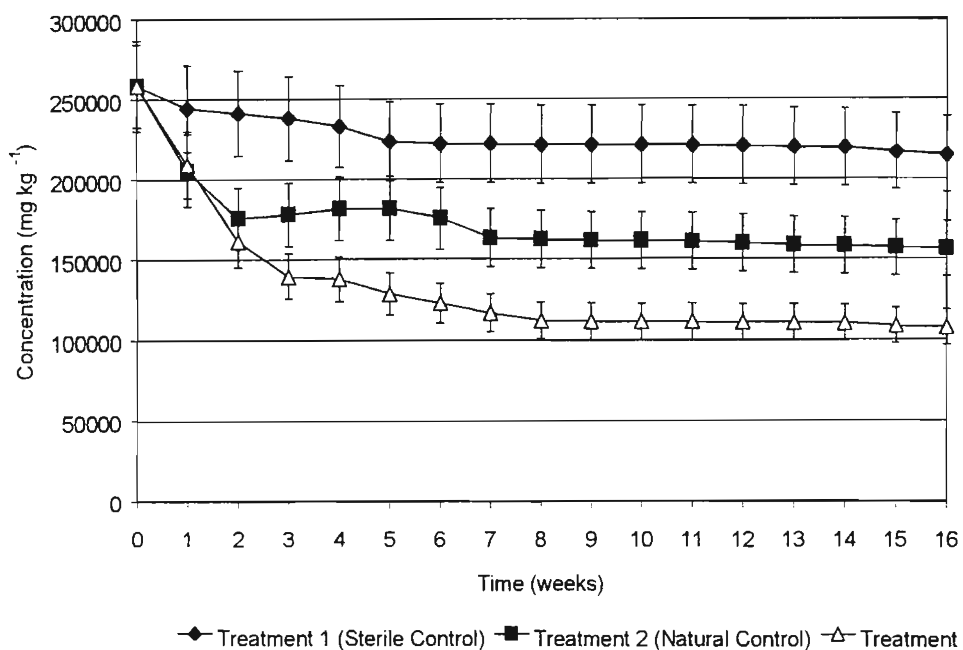



Fig. 7.3 Changes in creosote concentration in soil in Treatment 1, 2 & 3. Error bars indicate ± 1 Standard Error.

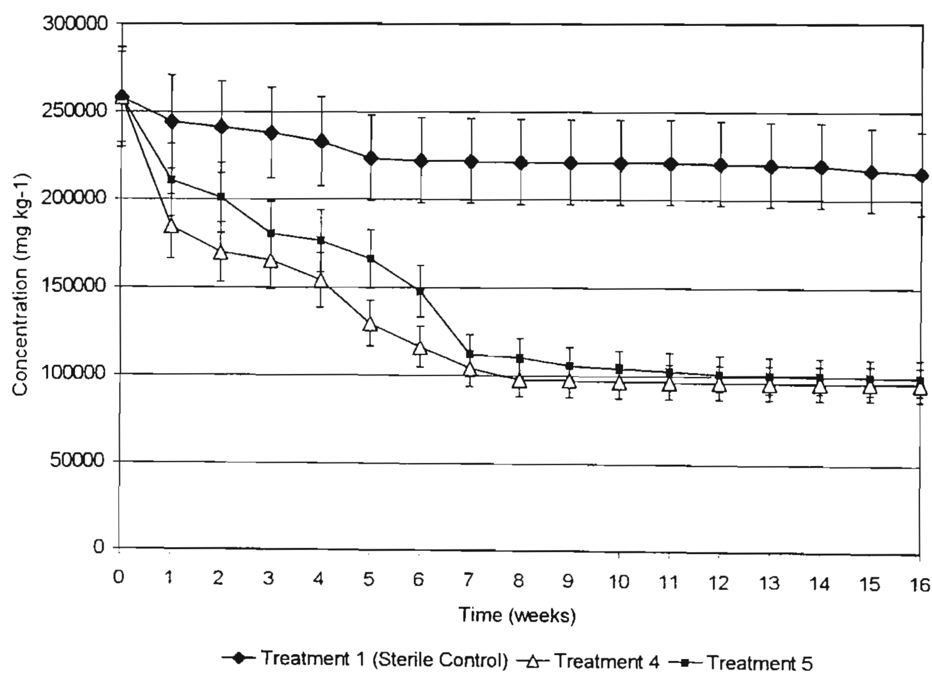



Fig. 7.4 Changes in creosote concentration in soil in Treatments 1, 4 & 5. Error bars indicate ± 1 Standard Error.

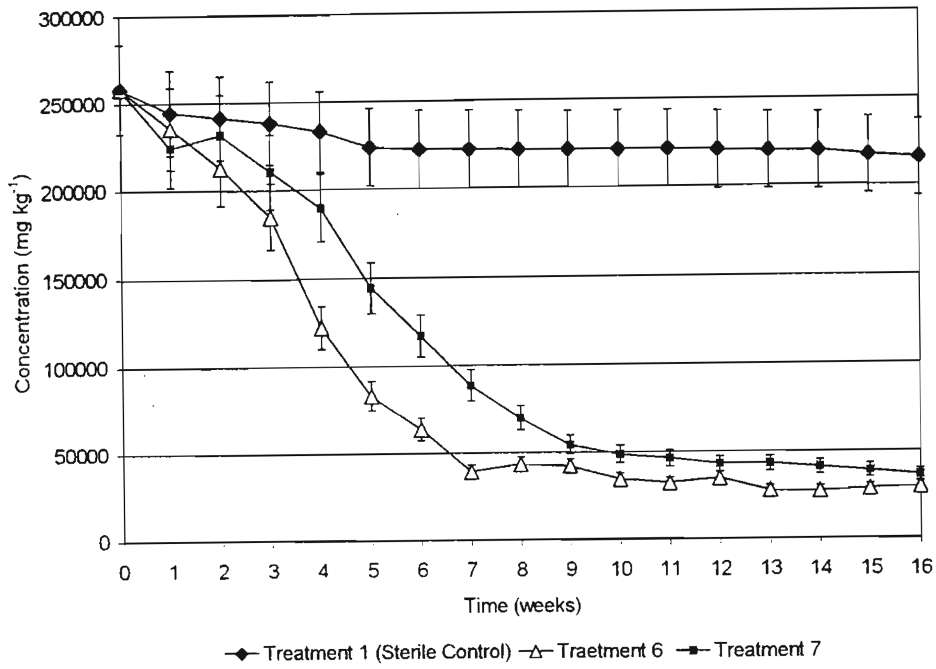



Fig.7.5 Changes in creosote concentration in soil in Treatments 1, 6 & 7. Error bars indicate ± 1 Standard Error.

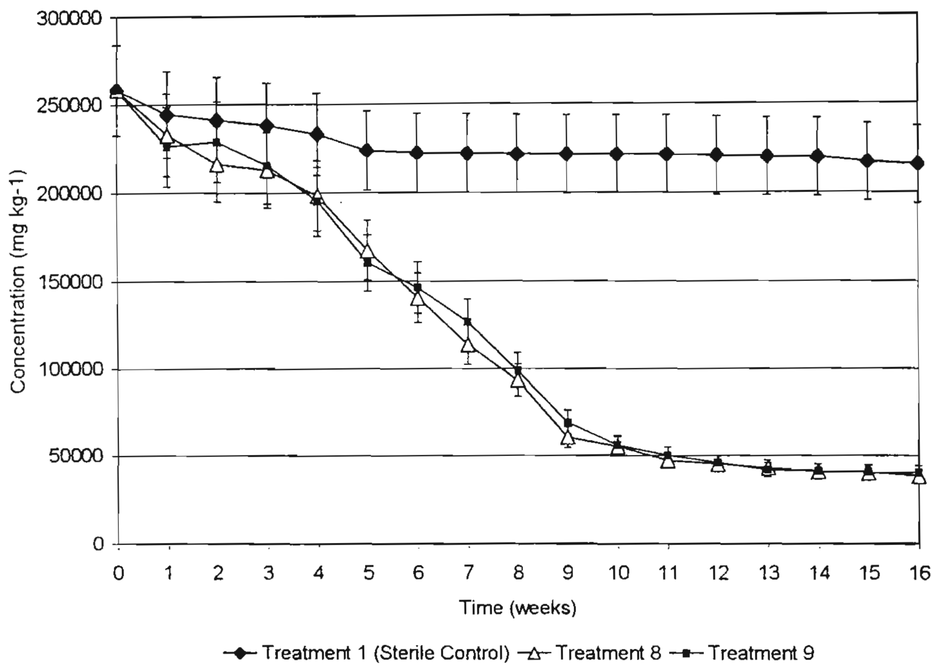



Fig.7.6 Changes in creosote concentration in soil in Treatments 1, 8 & 9. Error bars indicate ± 1 Standard Error.

pore spaces in the soil matrix and only became released when the soil was turned over and homogenized. The process of aeration and homogenization may have resulted in the oxidation and/or breakdown of higher molecular mass components into volatile fractions or undetected intermediates or end-products. Volatilization was not expected to be high in the controls as it was assumed that the bulk of creosote compounds left in the soil would be the higher molecular mass fractions that are not volatile and known to degrade slowly.

In Treatment 2, which represents natural control, 39.5% of the initial creosote concentration was lost during the treatment period (Fig. 7.3). This increased decline in creosote concentration, when compared to the sterile control, can be attributed to biodegradation, as can be seen from the results of the plate counts of the microorganisms in this treatment. Both the numbers of total heterotrophic microorganisms and hydrocarbon degraders were seen to increase substantially in the first 0-3 weeks (Fig.7.7 and 7.8). These increases soon levelled off, maintaining a relatively unchanging population size, probably as a result of stabilization of conditions such as aeration and moisture, since this treatment was neither aerated nor watered. The natural soil conditions prevailing in Treatment 2 were observed to allow an increase in the microbial population at the expense of the contaminating hydrocarbon. Pretreatment such as homogenization must have enhanced aeration in the soil and/or the accessibility of hydrocarbons to the microbial population and thus promoted creosote degradation (Fig.7.3).

Treatment 3, which was regularly watered and aerated throughout the treatment period, but did not receive any nutrient supplement, showed increased microbial numbers (Fig.7.7 &7.8), as well as improved creosote reduction (58.7%) (Fig.7.3), compared to Treatments 1 and 2 (Fig.7.3). These

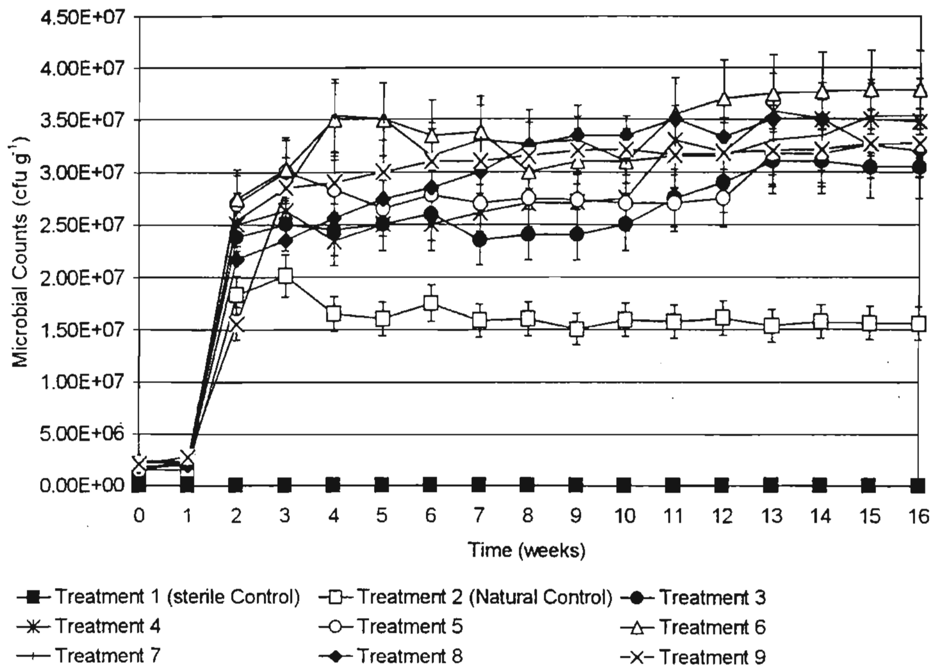



Fig.7.7 Counts of total heterotrophic microorganisms in soil in treatment reactors during pilot-scale landfarming. Error bars indicate ± 1 Standard Error.

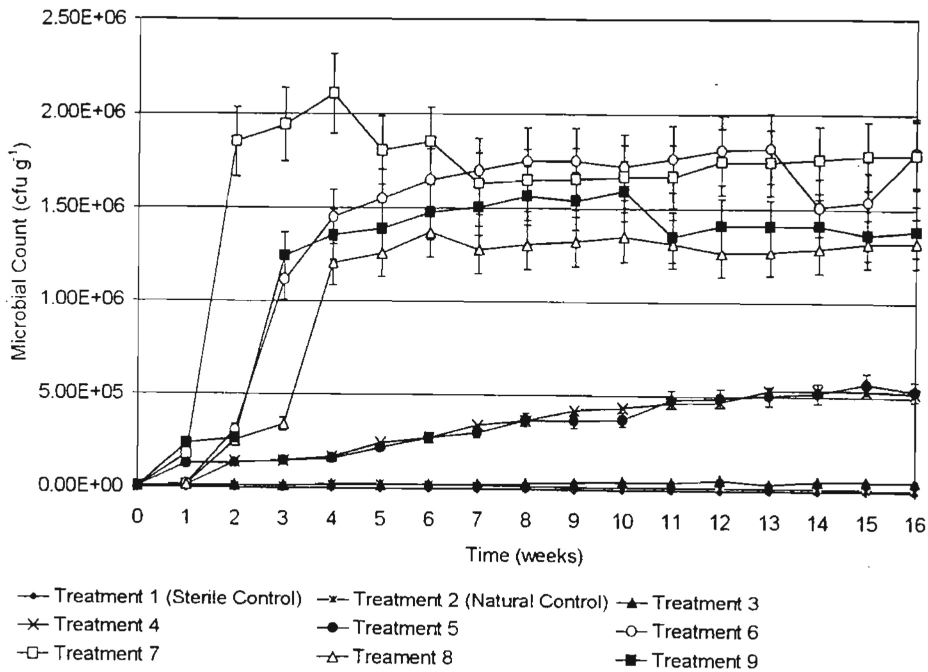



Fig.7.8 Counts of creosote degrading microorganisms in soil in treatment reactors during pilot-scale landfarming. Error bars indicate ± 1 Standard Error.

results clearly show that aeration and water are important requirements for microbial activity in hydrocarbon utilization in contaminated soils. These results also establish that mechanical tilling is a very cheap and effective means of promoting creosote degradation. Tillage will help supply oxygen, which is an electron acceptor, for the oxidation of the contaminating hydrocarbon compounds. In addition, creosote compounds may have been physically inaccessible to the microbial biomass by being held within the structure of soil aggregates. This is common when relatively immiscible liquid phases such as dense non-aqueous phase liquids (DNAPL) become trapped in interstitial pores (Kimball, 1995). Hydrocarbons adsorbed to soil particles that later become very tightly compacted become less available to microbial biomass on the inaccessible surfaces. Aeration and moisture availability in such a soil matrix become limited and thus inhibit further microbial activity (Tate, 1995; Alexander, 1999; Harmsen and Ferdinandy, 1999; Baeseman and Novak, 2001). Tillage would have broken up aggregates, thus exposing the hydrocarbons to microbial attack. The results from these treatments also establish that the high concentration of creosote found in the soil after a long period of disuse of the facility was due to poor aeration and/or physical inaccessibility which has hindered the biological breakdown of the contaminating creosote compounds.

Treatments 4, 5, 6, 7, 8 & 9 all received fertilizer treatment in the form of mono-ammonium phosphate (MAP) to supplement the nitrogen and phosphorus contents of the soil. They were all aerated daily and supplied with between 250 and 500 ml of water every two days, as determined by the water content of the soil throughout the experimental period. Each of these sets of treatments, apart from Treatment 4, received a further nutrient supplementation. Treatment 4 showed a marked improvement (62.9%) over Treatment 3 (58.7%) in creosote degradation. Treatment 3 was aerated

and watered but was not supplemented with any nutrients. Microbial populations were considerably higher than in Treatment 3 (Fig. 7.7 & 7.8). The nitrogen and phosphorus content of the soil was earlier found to be insufficient in relation to the amount of carbon that had been introduced into the soil by the contaminant creosote. This deficiency was thought to be one of the factors responsible for the poor natural attenuation that took place in the soil over the long period of contamination. Thus the enhancement of microbial activity and the consequent improved creosote degradation in Treatment 4 (Fig.7.4) was attributed to the supplementation of the soil in the reactors with nitrogen and phosphorus by means of MAP. MAP has been used in a number of instances to boost the nutrient content of hydrocarbon-contaminated soils during remedial processes (Snyman, 1996).

Treatment 5 was supplied with MAP and hydrogen peroxide ($H_2O_2$) was added once a week to provide an alternative source of electron acceptor. Hydrogen peroxide is known to decompose to oxygen and water and is soluble in water. Thus large amounts of oxygen are made available for biodegradation by its addition. However, $H_2O_2$ is highly reactive and subject to decomposition by the action of light, metal ions and enzymes. It is known to be more reactive than oxygen (Fiorenza and Ward, 1997). The soil in the reactor was supplied with about 500 ml of water and aerated daily throughout the experimental period. Results from this treatment showed that hydrogen peroxide as an alternative electron acceptor did not appreciably enhance biodegradation of the creosote. The 61.6% reduction in creosote concentration achieved in Treatment 5 (Fig.7.4) is only a slight improvement over the 58.4% reduction observed in Treatment 3, which was only watered and aerated throughout the treatment period. This low performance by hydrogen peroxide suggests that oxygen supply was not limiting to creosote biodegradation. Thus, as expected, daily tillage was sufficient to

provide an adequate supply of oxygen for creosote degradation. In addition, some of the lower molecular mass components may have been lost due to continued tilling of the soil to facilitate aeration. Toxicity and consequent poor adaptation of the resident microorganisms to the hydrogen peroxide could also have occurred, since it is known to cause toxicity to microorganisms during decomposition as a result of the free hydroxyl (OH*) radical being formed (Halliwell and Gutteridge, 1985; Harris, 2000). This action has been reported to damage DNA (Ananthaswamy and Eisenstark, 1977; Demple and Linn, 1982; Demple *et al.*, 1986). If the initial DNA damage is reversible, then the exogenous $H_2O_2$ can be removed by increased rates of catalase production and the DNA damage can be repaired (Demple and Linn, 1982). The inability of $H_2O_2$ to enhance creosote degradation could also be due to the presence of a sufficient supply of gaseous oxygen from the atmosphere, which was readily available to the microorganisms through the tilling process. The soil physical conditions may have contributed to the limited success of $H_2O_2$ in the trial. However, the slight improvement over Treatment 3 is possibly due to the additional oxygen from the hydrogen peroxide.

Treatments 6 & 7 received MAP, daily aeration and watering once in two days. In addition to these treatments, they were supplemented with indigenous microbial biosupplement and sewage sludge, respectively. Both treatments contained the highest counts for total heterotrophic and creosote degrading microorganisms. (Fig. 7.7 & 7.8 ). Microbial counts increased rapidly between the second and third week of experimentation and continued to increase more slowly until the sixteenth week. A rapid increase in counts of creosote degrading microorganisms was observed to continue in Treatment 7 until the fourth week, reaching a peak of $2.1 \times 10^6$, before declining slightly over the next three-week period and then increasing gradually for the remaining period of the experiment.

This rapid increase indicates a fairly rapid adaptation of microorganisms from the sewage sludge to the contaminating creosote. Although there was a slight fall in microbial population, which coincided with a period during which most of the cocci became scarce, the counts continued to increase gradually afterwards. The increase in numbers of creosote-degrading microorganisms in Treatment 6 continued slowly after the third week. Results in Fig. 7.7 & 7.8 show that the organisms from the sewage sludge inoculum proliferated more rapidly than those from the enriched indigenous soil biosupplement. This may be due to the conditions established in the enrichment reactor to improve the growth of the organisms which were not present in the pilot scale soil reactor system.

These two treatments (6 & 7) showed substantial degradation of creosote and concentrations decreased from 258 257.4mg $kg^{-1}$ to 29 137.5mg $kg^{-1}$ and 35 956mg $kg^{-1}$ , respectively. This represents a total reduction of 88.7% and 86.1%, respectively (Fig. 7.5). Enrichment cultures from contaminated soils have previously been used in accelerating the degradation of contaminant hydrocarbon in soils (Snyman, 1996). This process has also been used in different soil reactors for the same purpose, in many cases acting purely as microbial inocula for biodegradation processes. Even though microbial activity in the reactor supplemented with sewage sludge was slightly higher than that in the treatment with indigenous biosupplement, the latter showed a higher percentage reduction in creosote content of the soil. This can be attributed to the specificity of the indigenous soil organisms to creosote hydrocarbons compared to those of sewage origin, which must still adapt to the new hydrocarbon source.

Treatments 8 and 9, which received cattle and poultry manure, respectively, showed substantial

microbial activity and creosote removal from the soil. Both treatments were found to reduce the creosote concentration from 258 257.4mg $kg^{-1}$ to 38 163mg $kg^{-1}$ and 40 067mg $kg^{-1}$, respectively, representing a total reduction of 85.0% and 84.5%, respectively (Fig.7.6). These decreases are comparable with those observed in Treatments 6 and 7, which showed reductions of 88.7% and 86.1%, respectively. The decrease in creosote concentrations in Treatments 8 and 9 was rapid up to the ninth week, reaching 60 373mg $kg^{-1}$and 68 982mg $kg^{-1}$, respectively. The degradation rate then slowed until the sixteenth week, with only very slight decreases being recorded over the remaining seven weeks of the experiment. This slow degradation rate over the second half of the experiment can be attributed to the very low concentration of hydrocarbon remaining in the soil, which led to carbon limitation for the degrading microorganisms. Microbial utilization of carbon as a growth substrate in the soil was not expected to deplete only the carbon supply of the soil but also other utilizable soil nutrients such as nitrogen. These conditions could lower the microbial population of the soil, leading to reduced degradation of the creosote. Low concentrations have been reported to hinder microbial utilization of hydrocarbons in the environment (Alexander, 1999). Another possible explanation is that the metabolites of the degrading microorganisms may have accumulated in the soil and become inhibitory to the organisms (Veldkamp and Jannasch,1970). This is, however, less likely because the microbial population continued to increase, although very slowly until the end of the experiment.

The results from the reactors supplemented with organic fertilizers (sewage sludge, cattle manure and poultry manure) demonstrate creosote degradation where the active microorganisms already have a substantial amount of utilization carbon supplied in the manure. These substrates also have high

amounts of nitrogen and phosphorus and other required mineral nutrients that will tend to stimulate microbial activity. This nutritionally rich environment will naturally cushion the organisms from the initial shock experienced on transfer to a medium containing a new recalcitrant carbon source. Adaptation to the new substrate would, therefore, be achieved in a reasonably short time. The variety of organisms isolated from these reactors in the first week of operation and the high level of creosote degradation observed in these reactors suggest that the organisms only require a short period of adaptation to the new carbon substrate. This may have been due to co-metabolism of the new carbon substrate along with the carbon substrates available in the manure. This could have been achieved in a number of ways. The production of requisite enzymes to facilitate the break-down of the creosote substrate may have occurred while the microorganisms were still metabolising the original substrate. Microorganisms produce many enzymes, regardless of whether their specific substrates are present. These are called *constitutive enzymes* (Alexander, 1999). It is possible that such enzymes required for the breakdown of the polluting creosote hydrocarbons have been produced as *constitutive enzymes* while metabolizing the manure substrate. This situation could reduce the acclimation period significantly. Another possibility is the production of *inducible enzymes* as a result of the introduction of the creosote hydrocarbons. These enzymes may have been present in very limited amounts, typically because their production has been suppressed by the products of catabolism of another substrate (Alexander, 1999). Such enzymes can be readily induced by the abundant presence of their specific substrate since enzyme induction is usually carried out in very short periods of time; it is commonly completed in minutes ( Richmond, 1968). The other possibility is the creation of new environmental conditions brought about by the mixing of the creosote contaminated soil and the manure. This will have affected the nutrient and substrate composition available to the microbial

community and consequently created an entirely new environment that may have encouraged new species to invade and colonize the substrate. Since there was no taxonomic characterization of the isolates, it was not possible to confirm whether news species colonized the substrate or not.

From a comparison of Treatment 1 with the others, it can be seen that the volatile fractions of the pollutant, which were removed by volatilization, constitute a reasonable proportion of the total contaminating hydrocarbons. However, the non-volatile fractions had to be degraded by soil microorganisms, as is evident from the results from Treatments 2 to 9, which showed continued microbial activity and further creosote degradation. This latter effect was, however, not observed in Treatment 1 (sterile control), in which only 43 107mg $kg^{-1}$ of the original creosote present was removed.

Analyses of selected creosote components monitored in the degradation studies, showed that phenol and the other phenolic compounds, o-cresol, p-cresol and m-cresol, were reduced to undetectable levels (<1.0mg $kg^{-1}$) in all the reactors that were either treated with MAP or watered and aerated regularly, or which received some form of biosupplement (Fig.7.10, 7.11, 7.12 & 7.13) .The reduction in creosote components observed in Treatment 3, which was regularly watered and aerated but did not receive any biosupplementation, was found to be much more than in Treatment 1 (sterile control) (Fig.7.9) and about twice that observed in Treatment 2 (natural control) (Fig.7.10), indicating that regular aeration and watering enhanced degradation of the compounds. As already suggested, this may indicate that compaction of the soil resulting from the activities of factory workers over a long period of time had reduced the total porosity of the soil, thus creating relatively anoxic conditions. which would have contributed to inhibition of microbial proliferation and the concomitant inhibition

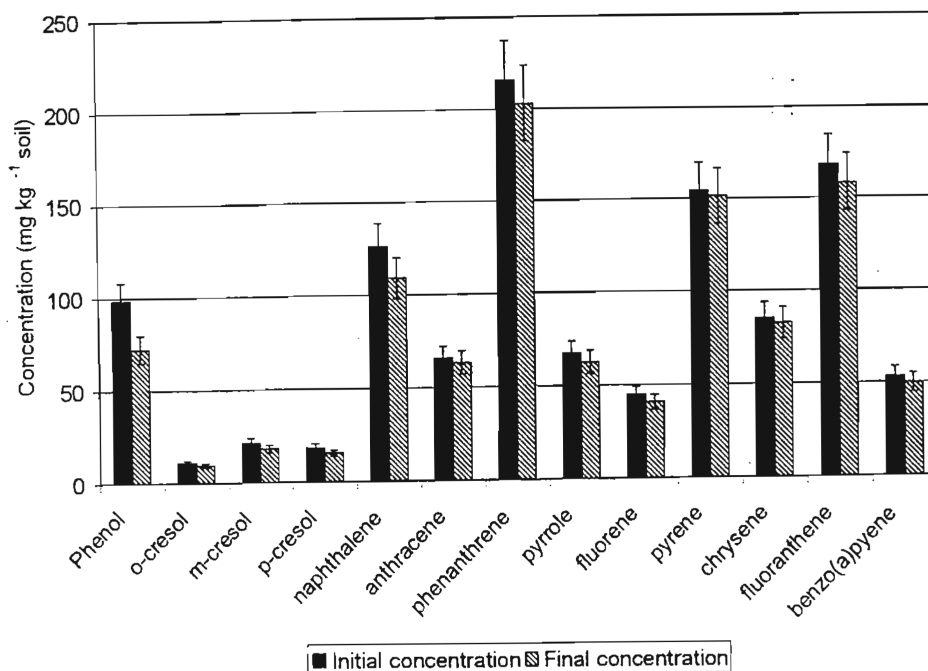



Fig.7.9 Changes in concentrations of selected creosote components in Treatment 1 (sterile control) during pilot-scale landfarming. Error bars indicate ± 1 Standard Error.

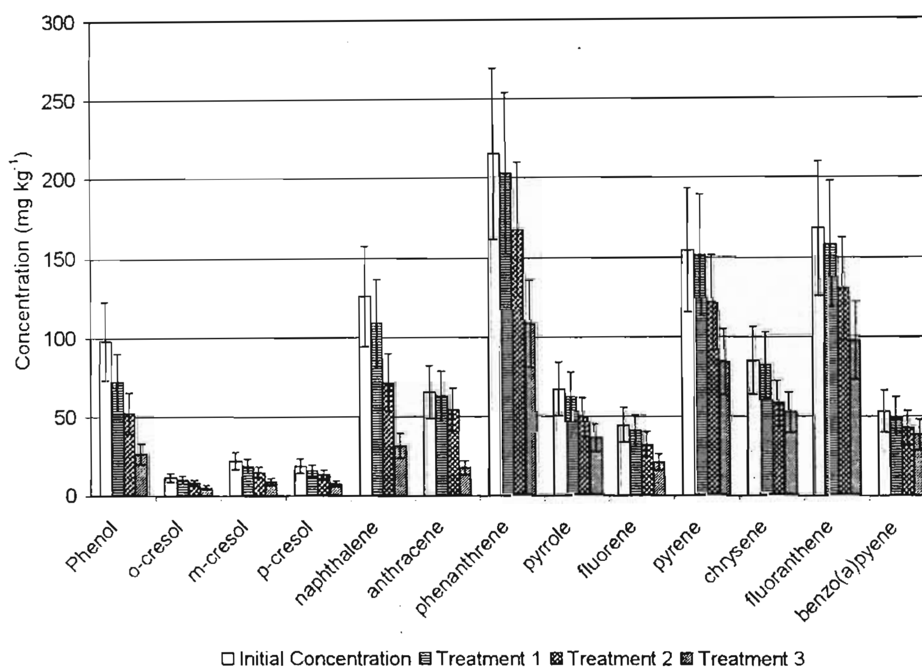



Fig.7.10 Changes in concentrations of selected creosote components in Treatments 1, 2 & 3. Error bars indicate ± 1 Standard Error.

of creosote degradation. Indeed, creosote degradation has been reported to be very slow or nonexistent under anaerobic conditions ( Arvin *et al.*, 1988; Dyreborg and Arvin, 1995). Physical

inaccessibility of hydrocarbons within soil aggregates may also have been a factor limiting their degradation in undisturbed soil (Harmsen and Ferdinandy, 1999).

Among the polycyclic aromatic hydrocarbons (PAHs) studied, naphthalene was removed to below detectable limits in Treatments 4-9 (Fig. 7.11 & 7.12). As previously stated, this suggests that the lower molecular mass compounds, i.e. those with 1-2 ring structure, including the phenolic compounds, which are also relatively soluble in aqueous media, are more susceptible to microbial degradation in the environment (Baker and Herson, 1994; Bossert and Bartha, 1986; Wodzinski and Johnson, 1968). Complete degradation of these compounds has been reported by Mueller *et al.* (1991), Kennes and Lema (1994), Annweiler *et al.* (1995) Hughes *et al.* (1997) and Atagana *et al.* (2000). Naphthalene, which is more soluble than the other PAHs studied, has been shown to desorb more than the other PAHs from surfaces of soil particles, thus making it more available for microbial action than the other compounds studied. Mueller *et al.* (1991) have reported a high recovery rate (92 %) of naphthalene in contaminated soil.

Fluorene, phenanthrene, anthracene and pyrrole were also degraded substantially by the soil organisms (Fig. 7.11 & 7.12). These compounds are all three-ringed structures but they have different solubility in aqueous solutions (Sanseverino *et al.*, 1994). Fluorene, phenanthrene and pyrrole, which are fairly soluble, were degraded rapidly by the soil organisms, with residual concentrations being below 1.0 mg $kg^{-1}$ in all the biosupplemented treatments. Anthracene, which is much less soluble, was found to be less degraded.

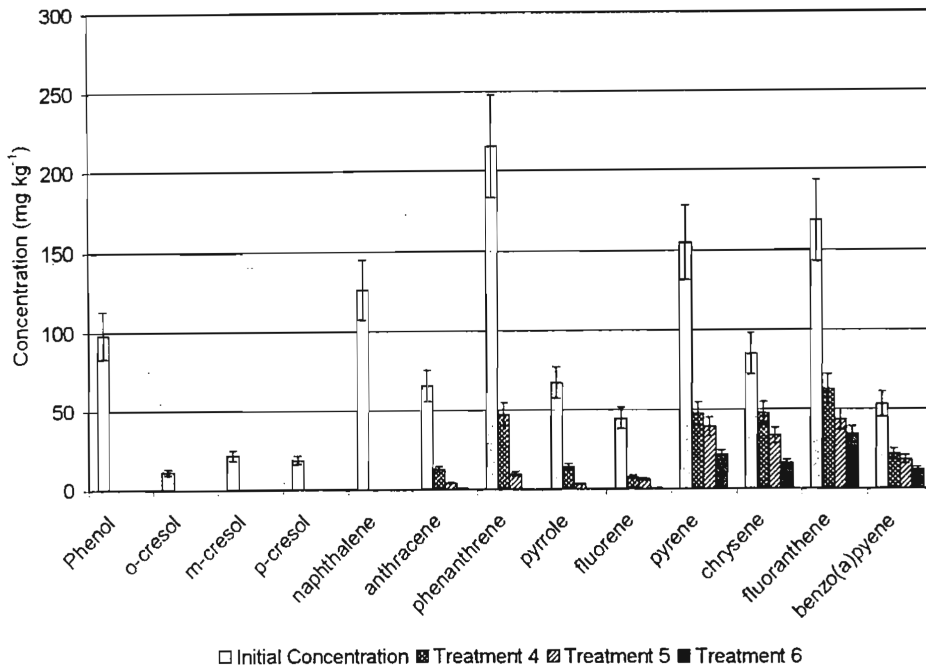



Fig.7.11 Changes in concentrations of selected creosote components in Treatments 4, 5 & 6. Error bars indicate ± 1 Standard Error.

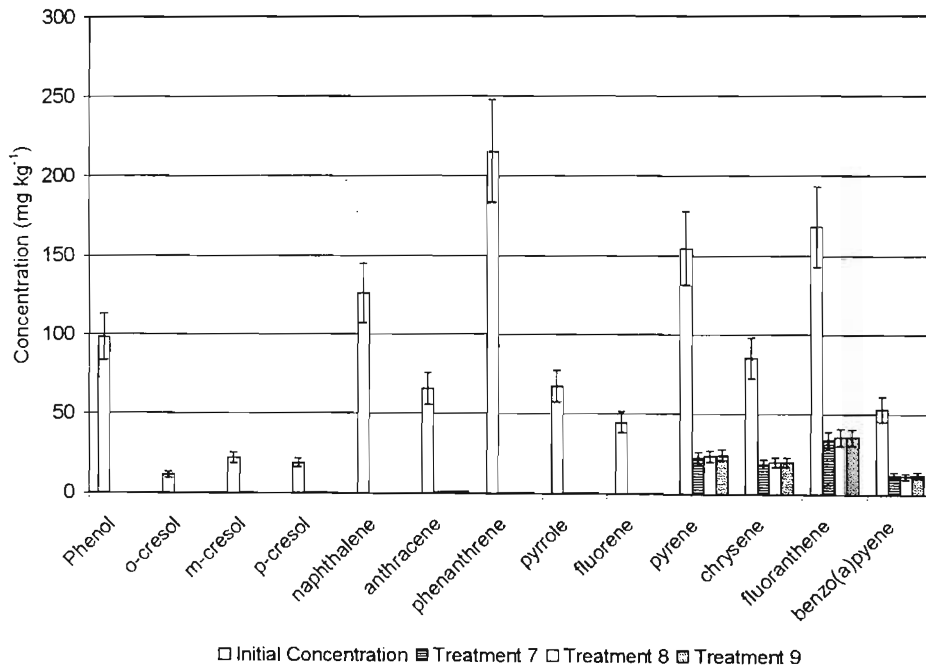



Fig.7.12 Changes in concentrations of selected creosote components in Treatments 7, 8 & 9. Error bars indicate ± 1 Standard Error.

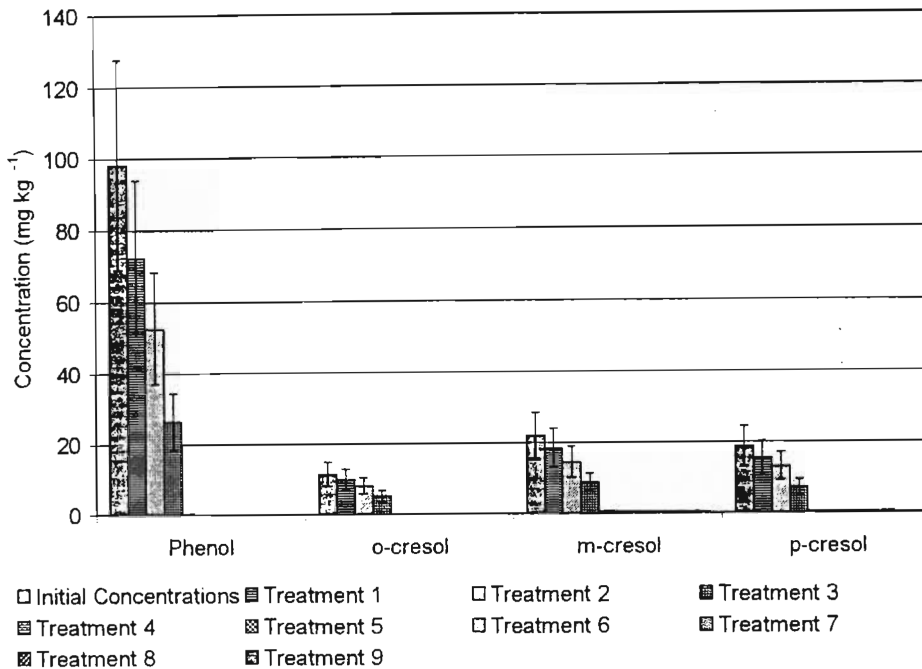



Fig.7.13 Changes in concentrations of phenolic compounds in soil in treatment reactors during pilot-scale landfarming. Error bars indicate ± 1 Etandard Error.

Although the concentrations of anthracene in the soil were less than those of the other three-ringed compounds studied, the residual concentrations were higher than other three-ringed compounds in the treated reactors. Pyrolle has been reported to be only slowly degraded by soil microorganisms (Arvin *et al.*, 1988; Flyvbjerg *et al.*, 1993). In Treatment 1, the sterile control, removal of these compounds was unexpectedly high. However, Mueller *et al.* (1991) have reported the volatilization of these compounds from surface soil. As has earlier been reported, the non-uniformity in degradation of these compounds by soil organisms can be attributed to differences in their solubility which is a function of the spatial distribution of their ring structures (Baker and Herson, 1994; Sutherland *et al.*, 1995; Connolly *et al.*, 1999).

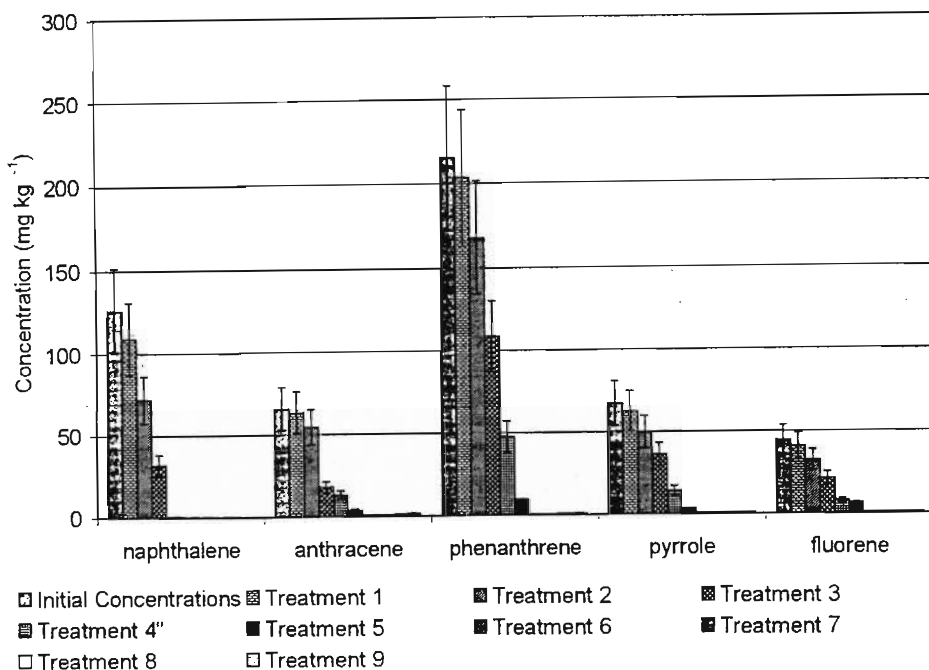



Fig.7.14 Changes in concentrations of selected lower molecular mass components of creosote in soil in treatment reactors during pilot-scale landfarming. Error bars indicate ± 1 Standard Error.

The removal of the higher molecular mass compounds, pyrene, chrysene, fluoranthene and benzo(a)pyrene, was relatively slow. Trzesika-Mlynarz and Ward (1995) reported a mixed microbial culture that degraded benzo(a)pyrene, pyrene, fluoranthene, anthracene, phenanthrene, acenaphthene and fluorene. Although significant amounts of these compounds were removed from the experimental soils, reasonable amounts persisted to the end of the sixteenth week (Fig.7.15). The highest percentage removal was observed for pyrene (between 68.1 and 83.9% in Treatments 4-9). Apart from Treatment 6, which received an indigenous soil biosupplement, chrysene was found to be the least degraded among the higher molecular mass compounds studied in all the treatments (Fig.7.15). The aqueous solubility of the compound (0.002mg $kg^{-1}$) is also implicated in this behaviour (Sanseverino *et al.*, 1994; Eriksson *et al.*, 2000). The spatial arrangement of the ring structure, which

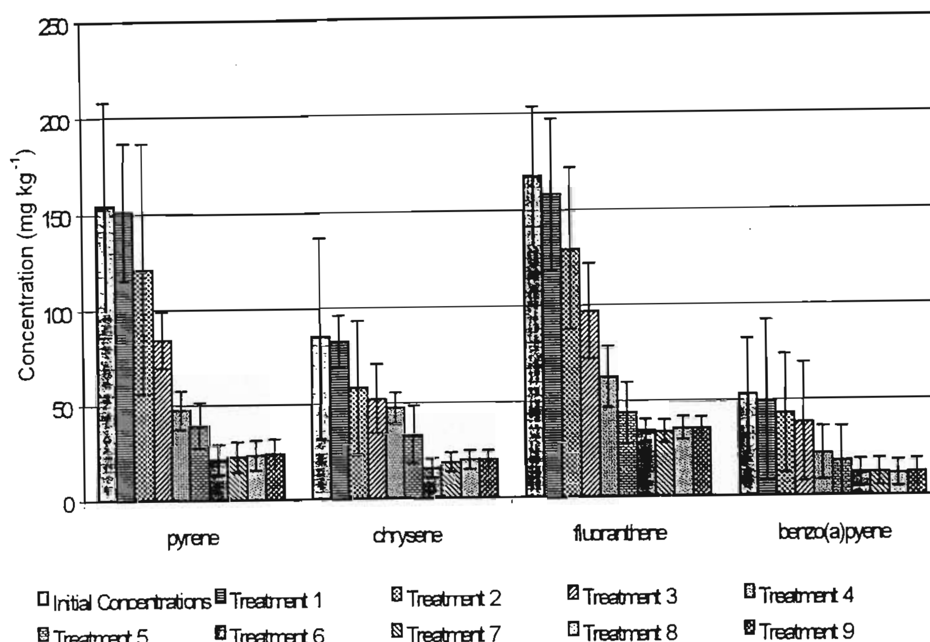



Fig.7.15 Changes in concentrations of selected higher molecular mass components of creosote in soil in treatment reactors during pilot-scale landfarming. Error bars ± 1 Standard Error.

determines the type of intermediate that will be produced, could also be responsible for the slow rate of degradation. Although fluoranthene has a higher water solubility and lower molecular mass than benzo(a)pyrene (Sanseverino *et al.*, 1994; Connolly *et al.*, 1999), both were degraded to very similar levels in all the supplemented treatments (Fig.7.15). This observation suggests that other factors apart from solubility and molecular mass, which greatly influence bioavailability, may be important in the microbial utilization of PAHs in soil. Indeed, the biodegradability of PAHs in soil depends on their physical and chemical properties, concentration, rates of diffusion in soil or water, soil type, pH, temperature, availability of water and oxygen and other seasonal factors (Weissenfels *et al.*, 1992; Cerniglia, 1993; Sutherland *et al.*, 1995; Meyer *et al.*, 2001). These compounds have been reported by many workers to be metabolised in the environment by different microbial species, producing

their alcohol derivatives as intermediates or carbon dioxide as the final product (Bumpus *et al.*, 1985; Sanglard *et al.*, 1986; Warshawsky1990; Walter *et al.*, 1991; Cerniglia *et. al.*, 1992; Sutherland *et al.*, 1995; Reid *et al.*, 1999; Hansen *et al.*, 2000; Alshawabkeh, 2001; Boronin, 2001).

In the last weeks of treatment, concentrations of anthracene, phenanthrene, and fluorene remained constant. As noted previously, this can be attributed to the very low concentration levels reached for these compounds, making them unavailable to microbial action (Alexander, 1999). However, other compounds which were degraded to non-detectable concentrations are believed to have reached such levels because of their high solubility and also because of possible co-metabolization in the presence of other compounds (Alexander, 1999; Sellers, 1999). The concentrations of higher molecular mass ( 4 & 5 ring) compounds continued to decrease up until the end of the experiment. However, by the end of the experiment the decrease in concentrations of chrysene and benzo(a)pyrene became very slow. Initially, fluoranthene concentrations decreased at about the same rate as pyrene but degradation of the latter accelerated during the last three weeks of the experiment. These changes in removal rate can be attributed to differences in the rate of desorption, toxicity to specific organisms and, possibly, absence of the main carbon source for organisms co-metabolizing the substrate (Baker and Herson, 1994; Alexander, 1999).

### 7.3.3 Isolation and characterization of indigenous soil microorganisms degrading creosote oil

The organisms isolated from the treatments in the first week were predominantly spherical bacteria (Plate 1A). They included both Gram-positive and Gram-negative forms. The Gram-positive

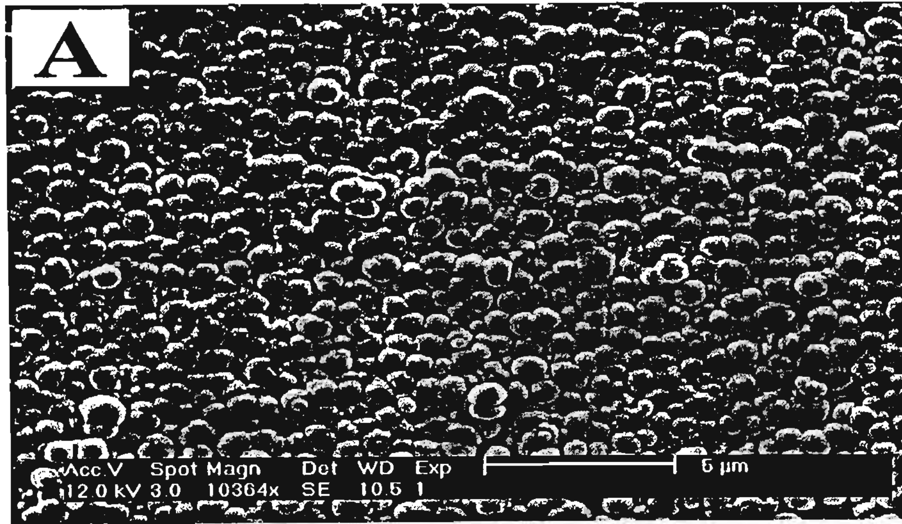
organisms were mainly encountered in Treatments 7, 8 & 9, which were supplemented with sewage sludge, cattle manure and poultry manure, respectively. A few short rods (Plates 7.1B) and moulds were also isolated. By the end of the third week, the populations had become diversified to include more rods, actinomycetes, yeasts and moulds (Plates 7.1C & D and 7.3 A, B & C). Isolates from the treatments at the end of the sixteenth week continued to show a reasonable amount of diversity, with the filamentous forms becoming more prominent than in earlier isolates (Plates 7.2A & B). It was observed, however, that the few organisms found in the sterile control throughout the experimental period were mainly Gram negative cocci (Plate 7.2C) and very rarely rods(Plate 7.2 D).

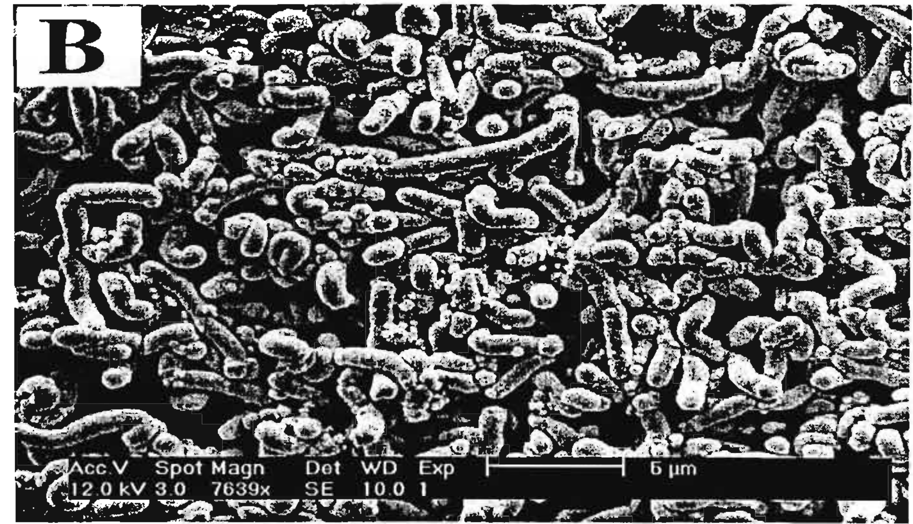
The characteristics of the organisms isolated from the soil in the reactors showed that there was a wide variety of organisms involved in the utilization of the polluting carbon. Since the emphasis was on biostimulation of soil microbial consortia that could degrade the polluting hydrocarbon, the identification of individual species of bacteria was not carried out.

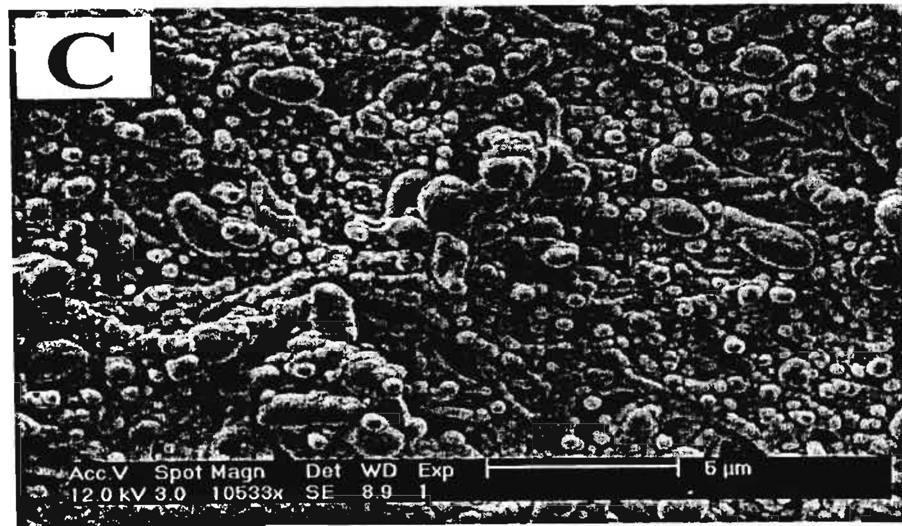
The bacterial isolates represented a wide variety of forms, possibly including *Pseudomonas* spp., *Bacillus* spp., *Mycobacterium* spp., *Micrococcus* spp., *Rhodococcus* spp., *Alcaligenes* spp., *Acinetobacter* spp., *Flavobactrium* spp., *Actinomyces* spp. *Arthrobacter* spp. and *Norcadia* spp., which are commonly encountered in hydrocarbon contaminated soils. The dominant form observed in the treatment supplied with$H_2O_2$ were short rods, which persisted throughout the treatment period. These isolates probably belong to the genus *Pseudomonas*, as they have been reported by different workers to be present in soils contaminated with creosote or other hydrocarbons and also to be involved in the utilization of hydrocarbon in soil (Britton, 1984; Nohynek *et al.*, 1995; 1996; Briglia *et al.*, 1996; Annweiler *et al.*, 2000). *Pseudomonas cepacia* has been reported to degrade most of the

Plate 7.1A & B Bacterial isolates from creosote contaminated soil during pilot-scale landfarming

Plate 7.1 A & B Bacterial isolates from creosote-contaminated soil during pilot-scale landfarming from the first week of treatment, C & D Isolates from the third week of treatment.



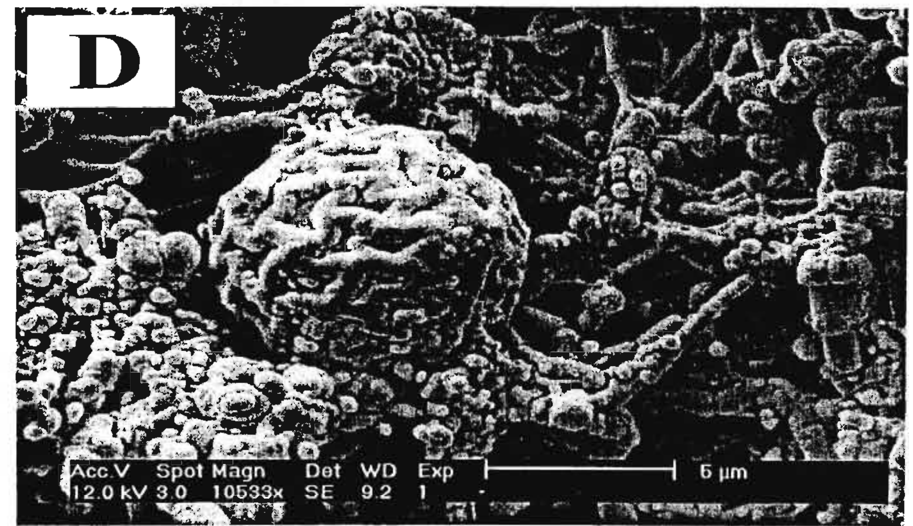
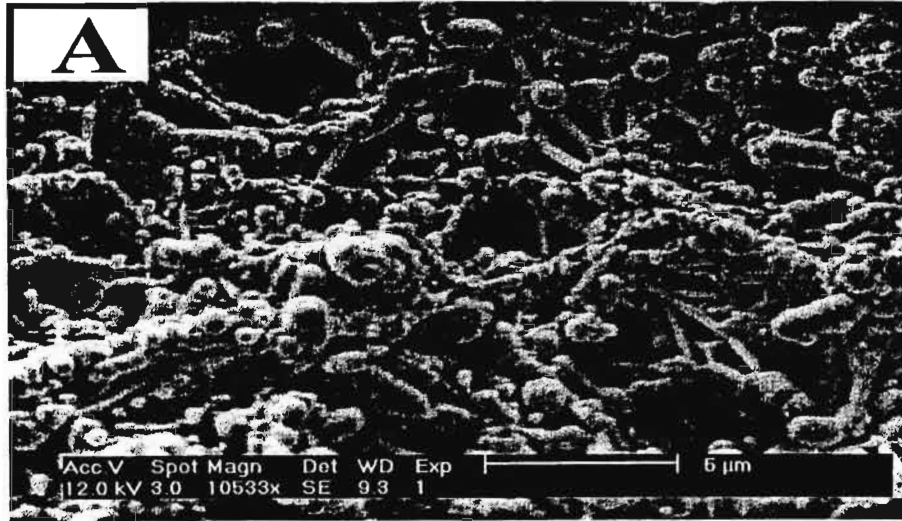
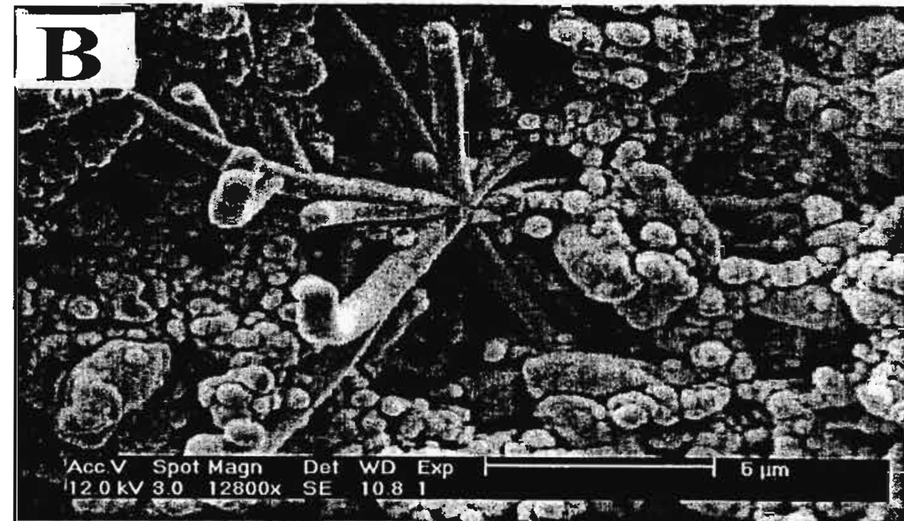
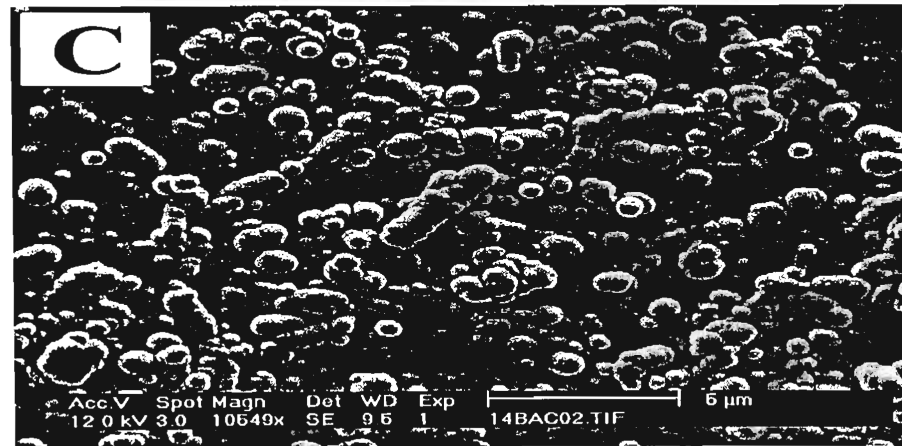



Plate 7.2 A & B Isolates from soil in the sixteenth week of pilot-scale landfarming, C Isolates from the natural control, D Isolates from the sterile control.



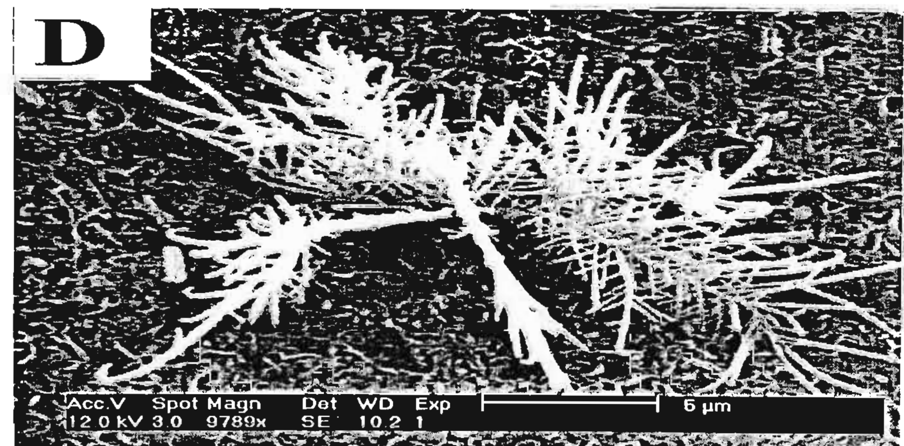
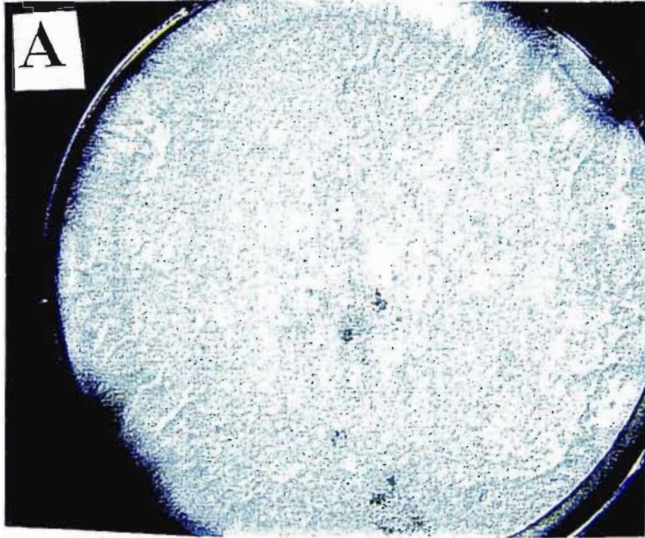
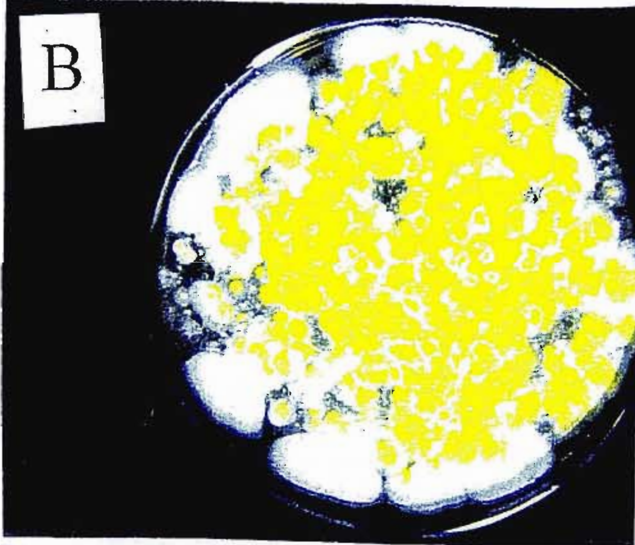
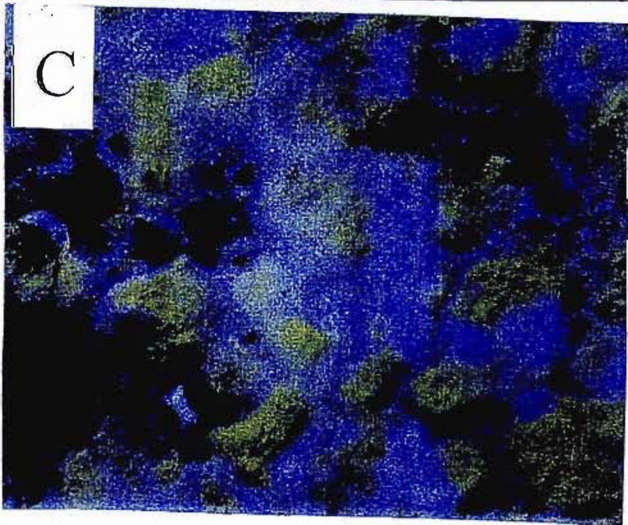



Plate 7.3 Fungal isolates from creosote-contaminated soil during pilot-scale landfarming.



components of creosote, including the PAHs, except chrysene and pyrene, which were not effectively degraded ( Ellis, 1994). Other species of *Pseudomonas*, including *P. fluorescens*, have been reported to degrade naphthalene (Sanseverino *et al.*, 1994), *Pseudomonas* sp. strain HL7b has been shown to degrade the 4 ring compound, fluoranthene (Foght and Westlake, 1988; Trzesicka-Mlynarz and Ward, 1995). A mixed culture containing *P. putida*, *P. aeruginosa*, *Flavobacterium* sp. and a fourth unidentified species, all of which were Gram negative, was reported to degrade a range of PAHs including benzo(a)pyrene, anthracene, phenanthrene, acenaphthene and fluorene (Trzesicka-Mlynarz and Ward, 1995).

The fungal species isolated included: *Mucor* sp., *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp., *Fusarium* sp., *Candida* sp., *Saccharomyces* sp., *Monocillium* sp., *Alternaria* sp. and some unidentified species. Fungal species are known to tolerate extreme pH conditions better than bacterial species. Initially, the experimental soil had a pH of 4.0 which would have favoured fungal proliferation more than bacterial growth (Davis and Westlake, 1978; Alexander, 1999). The amendment of the soil with lime to raise the pH to about 7 did not appear to inhibit fungi in the soil (Fig. 7.4). *Penicillium* sp. have been reported by many workers to degrade 4-5 ring PAHs in soil (Pothuluri *et al.*, 1994; Launen *et al.*, 1995; Kiehlmann *et al.*, 1996; Wunder *et al.*, 1997; Boonchan *et al.*, 2000). *Saccharomyces cerevisiae* and *Candida lipolytica* have been reported to metabolize benzo(a)pyrene (Cerniglia and Crow, 1981; King *et al.*, 1984; Clemente *et al.*, 1999). Although soil fungi have been shown to effectively degrade both aliphatic, and up to 3 ring aromatic compounds, those capable of metabolizing 4 and more ring PAHs have been relatively less characterized (Launen *et al.*, 1995). Some yeast species have been isolated that have the capability of oxidizing

benzo(a)anthracene (MacGillivray and Shiaris, 1993). Launen *et al.* (1995) observed that, although a variety of fungal species were isolated from the soil, they oxidized the PAH substrates at different rates. They also observed that the activity of the different strains could change over a period of time. Even though the organisms continued to grow in liquid culture, they could not metabolise the carbon substrate they were found to metabolise previously at the earlier screening.

Statistical analysis showed that Treatments 1 and 2, which were the sterile and natural controls, respectively, supported significantly lower microbial activity levels and creosote/creosote component removal rates from the soil than all the supplemented treatments. Treatment 6, which was supplied with indigenous microbial biosupplement, supported significantly higher levels of microbial growth and removal of creosote and its components from the contaminated soil than did the other treatments. The Student-Newman-Keuls' range test (0.05 significance level) indicated no significant differences in performance among the other treatments. However, LSD (0.05) values showed that Treatments 5 and 9 were not significantly different from each other but did differ from Treatments 3, 4, 7 and 8 which are, in turn, not significantly different from each other. Analysis of Variance (ANOVA) showed that the degradation of creosote components in the control experiments was significantly lower than in the other treatments. Treatments 5-9 were generally the more effective in creosote component degradation. These results show that the addition of 10% organic manure in the form of sewage sludge, cow manure or poultry manure, the addition of an indigenous microbial inoculum and the application of hydrogen peroxide greatly enhanced the degradation of creosote in the contaminated experimental soil.

## 7.4 CONCLUSIONS

From the results obtained in these experiments, it can be concluded that:

- oxygen and moisture are of paramount importance in the overall microbial degradation of creosote hydrocarbons in soil.
- mechanical tilling can greatly enhance the porosity of the creosote-contaminated soil, thereby improving aeration, moisture distribution and consequent degradation of creosote in the soil. The addition of hydrogen peroxide did not significantly improve the oxygen level of the soil.
- tilling and pretreatment such as homogenization of contaminated soils can cause the volatilization of the lower molecular mass components of creosote previously "locked up" in the soil matrix.
- inorganic nutrient supplements in the form of mono-ammonium phosphate can ameliorate nitrogen and phosphorus deficiencies, thereby increasing microbial activity and enhancing degradation of creosote.
- the use of organic manure or indigenous microbial biosupplements, in conjunction with inorganic fertilizers, can greatly increase microbial activity and result in the further degradation of creosote.
- there was no difference between the performances of the different organic manures used.
- there was no difference in the performance of the different organic manures and the indigenous microbial biosupplements.
- a wide variety of microorganisms, including bacteria, actinomycetes, fungi and yeasts, are responsible for the overall degradation of creosote in contaminated soils.

- creosote-contaminated soils can be effectively treated by landfarming to remove the contaminating creosote compounds from the soil.
- the lower molecular mass compounds are more amenable to microbial degradation than the higher molecular mass compounds present in creosote.

## 7.5 Recommendations

It is evident from the results that creosote was not completely removed from the soil at the end of the treatment period. It is also evident that the remaining fractions of creosote in the soil are predominantly the higher molecular mass components. It is therefore recommended that a treatment method that is capable of degrading the higher molecular mass components of creosote be investigated.

# CHAPTER 8

# BIOREMEDIATION OF CREOSOTE-CONTAMINATED SOIL BY FUNGI

## 8.1 INTRODUCTION

Fungi are known for their diversity and remarkable ability to degrade complex and persistent natural materials such as lignin, chitin and microcrystalline cellulose. In contrast to bacteria, fungi are able to extend the location of their biomass through hyphal growth. These features distinguish fungi as organisms having great potential for use as components of a technology capable of treating soil contaminated with organic pollutants (Glaser and Lamar,1995). Fungi are probably as important as bacteria for the decontamination of terrestrial environments. The biomass of fungi in normal soil is estimated to be five to ten times higher than that of bacteria, but the fungal activity is about one magnitude lower, as indicated by specific respiration rate (Fritsche,1992; Kotterman *et al.,* 1999). The activities of aerobic bacteria in metabolizing xenobiotics are well studied, whereas corresponding knowledge about fungi is limited (Fritsche,1992; Alexander,1999; Launen *et al.*, 1999).

Bioremediation technologies have commonly relied on bacterial activity to decontaminate the environment. While knowledge of bacterial bioremediation has continued to improve, very little attention has been given to fungal remediation. Although many reports claim the success of fungi in metabolizing different hydrocarbons, only a few reports are available where direct application of

fungi in bioremediation has been carried out. The potential for these organisms to degrade high molecular mass polycyclic aromatic hydrocarbons (PAHs)( Cerniglia and Gibson, 1979,1980;Wood and Wiseman, 1979; Field *et al.*, 1992; Pothuluri *et al.*, 1992; MacGillivray and Shiaris, 1993; Cerniglia *et al* 1994; Andersson and Henrysson 1996,) and other recalcitrant organic compounds in the laboratory, through the use of their extracellular and other enzyme systems, presents an attractive area for investigation. Thus the application of fungi in remediating high molecular mass polycyclic aromatic hydrocarbons which are resistant to bacterial degradation in soil constitutes the focus of this chapter. Cerniglia (1997) presents a list of PAHs that have been reported to successfully support the growth of a large number of fungi and yeasts and in the last five years this list has grown (Borazjani and Diehl, 1999; Clemente *et al.*, 1999; Harmsen *et al.*, 1999). This report gives a further indication that these organisms, if managed under appropriate conditions, will help address the issue of the recalcitrance of some of the PAHs in soil.

Both bacteria and fungi are ubiquitous in soil and members of both groups contribute to the biodegradation of hydrocarbons. In many soils fungi appear to be more important than bacteria in community respiration (Alexander,1999), but studies of degradation of hydrocarbons in the environment are commonly based on bacterial activity. This may, in part, be due to the difficulties presented by fungi increasing their biomass through hyphal lengthening and branching rather than binary fission and the organisms undergoing morphological changes during their life cycle (Alexander,1999). Such heterogeneity is related to changes in physiological activities in the course of fungal growth (Fritsche,1992). Fungi possess a number of characteristics that are potentially useful for application in bioremediation. They are able to grow under environmentally stressed

conditions such as low nutrient availability, low water activity and at low pH values, where bacterial growth might be limited (Davis and Westlake, 1978). Their hyphal growth is chemotactically directed to areas of the soil covered by the carbon substrate, thus optimizing surface contact with the contaminant and increasing the bioavailability of the compound. The ability of mycelial fungi to penetrate insoluble substances, such as oil tarballs, initiates a succession of other microorganisms and enhances degradation of the oil. Such a relationship was observed by Kirk (1969), who found that bacteria became attached to the mucilaginous hyphae of *Corollospora maritima* during the degradation of oil. This suggests that soil contaminants are cometalized by soil microorganisms. This means that the substance may not necessarily support the growth of organisms, but could be transformed in the presence of a second, more suitable substrate that is used by the organism as a source of carbon and energy (Fritsche, 1992). Under the co-metabolic conditions fungal degradation is a transformation-detoxification process rather than catabolism (Fritsche, 1992).

Saprophytes of the Zygomycotina and Deuteromycotina, and the white rot fungi of the Basidiomycota, have been found to be very important in the degradation of contaminants in soil (Fritsche, 1992). The transformation of the most significant pollutants in soil by members of these groups has been shown to be carried out by two main enzyme systems, including the intracellular cytochrome $P_{450}$ monooxygenases and heme-containing lignin peroxidase, excreted by white rot fungi (Fritsche, 1992; Sutherland *et al.*, 1995; Eggen *et al.*, 1999; Rodriguez-Vazquez *et al.*, 1999).

Eukaryotes, in general, do not degrade polycyclic aromatic hydrocarbons (PAHs)(Gibson and Subramanian, 1984). Instead, they metabolize these compounds, using well-described

monooxygenase enzyme systems, to hydroxylate metabolites that can be excreted directly or as conjugates with more polar molecules (Gibson and Subramanian, 1984). Hydroxylation and excretion are standard eukaryotic strategies for xenobiotic detoxification and most fungi fit this pattern (Hammel, 1995). Aryl oxidative enzymes of fungi have been said to be similar to those found in the mammalian liver, showing broad substrate specificities (Gibson and subramanian, 1984; Hummel, 1995; Cerniglia, 1997). In both cases, aryl compounds are oxidized to arene oxides by the cytochrome P450 enzyme system (Auret *et al.*, 1971). The oxides can isomerize to phenols or undergo enzymatic hydration by the enzyme epoxide hydratase to yield dihydrodiols in which the hydroxyl groups have a trans relative stereochemistry (Gibson and Subramanian, 1984).

Several fungi, including the genera *Penicillium* and *Cunninghamella*, exhibit greater hydrocarbon biodegradation than bacteria (Riser-Roberts, 1992, Boonchant *et al.*, 2000). Many other species of fungi and yeasts have the potential ability to oxidize hydrocarbons in soil. They include *Verticillium*, *Beauveria*, *Cladosporium*, *Candida*, *Mortieriella*, *Saccharomyces*, with varied potentials to oxidize different hydrocarbons (Hofrichter *et al.*, 1993; Cerniglia, 1997; Boonchan *et al.*, 2000). In spite of the number of mycelial fungi and yeasts with hydrocarbon-oxidizing potential, relatively little attention has been given to their action on soil pollutants. Research on fungal bioremediation of hydrocarbon pollutants has been centred on the white and brown rot fungi (Gold *et al.*, 1989; Hofrichter *et al.*, 1993; Bogan and Lamar, 1999), which in nature are not inhabitants of the soil environment. This concentration of effort on the wood rot fungi is due to their ability to produce hydrogen peroxide and enzymes that have been found to degrade lignin to its final product, carbon dioxide (Gold *et al.*, 1989).

The low specificities of the enzymes produced by the white rot fungi, such as *Phanerochaete chrysosporium*, are believed to enable them to degrade a range of recalcitrant, anthropogenic compounds (Field *et al.*, 1992; Bogan and Lamar, 1995). The degradative capability of the white rot fungi was first researched in detail because of its application in the bleaching of kraft pulp mill effluents (Lundquist *et al.*, 1977; Eaton, 1985). This research demonstrated the ability of *P. chrysosporium* to degrade chlorinated organic compounds in effluents from the kraft pulp bleaching process. Pulp mill and bleach plant effluents are highly coloured, due to the presence of polymeric lignin degradation products and contain chlorinated aromatics which are highly toxic and mutagenic (Eaton, 1985). Dye-degrading bacteria, unlike the white rot fungi, usually exhibit strict specificity towards a single dye and are of little use for the treatment of a mixture of dyes which normally occurs in such industrial effluents. Conversely, *P. chrysosporium* and several other white rot fungi are able to decolourise many types of recalcitrant dyes (McGugan,1997). The potential of the white rot fungi for bioremediation of environmental pollutants was first reported by Bumpus *et al.*(1985). The white rot fungus *P. chrysosporium* has been reported to degrade a number of xenobiotics, including DDT, PCP, PCB, TCCD and PAHs (Higson, 1991; Pozdnyakova *et al.*, 2001). The capabilities of white rot fungi to degrade recalcitrant molecules has led to attempts to use these microorganisms for soil decontamination processes (Martens and Zadrazil, 1992). Field *et al.* (1992) demonstrated up to 99% degradation of anthracene by a number of white rot fungi including *Phanerochaete chrysosporium*, *Trametes versicolor* and *Bjerkandera adust*. PAHs of creosote origin were shown to be degraded by cultures of *P. chrysosporium* in studies by Bogan and Lamar (1995, 1999). They considered the primary enzymatic constituents of the organism as lignin peroxidase and manganese peroxidase, as was earlier suggested by Tien and Kirk (1988) and Paszezynski *et al.*

(1985). They found that the recovery rates of the compounds tested corelated with their ionization potentials and that transformations observed were due mainly to enzyme-dependent processes. Novotný *et al.* (1999) found that of three white rot fungi, *Pleurotus ostreatus, P. chrysosporium* and *Trametes versicolor*, *P. ostreatus* was the most effective in removing PAHs from soil and that anthraquinone accumulated during the process. The phenolic compounds, which constitute about 10% of creosote, have been reported to be degraded by a number of yeasts and filamentous fungi. *Trichosporon cutaneum, Candida* sp. *and Rhodotorula* sp. are able to utilize phenol as sole source of carbon and energy (Fritsche, 1992). *Fusarium flocciferum* was reported by Anselmo (1984) to grow on phenol. Strains of *Penicillium* (Scow, 1990; Hofrichter *et al.*, 1993) and white rot fungi (Valli and Gold, 1991) have also been shown to metabolise phenols. The latter authors, for example, demonstrated the degradation of 2,4-dichlorophenol by a white rot fungus, in which both chlorine atoms were first removed before ring cleavage occurred. *Phanerochaete chrysosporium* was shown by Kennes and Lema (1994) to degrade *p*-cresol and phenol, simultaneously.

It is thus obvious that individual strains of different fungi and yeasts are capable of metabolizing creosote components in the environment. However, natural soil conditions allow for the growth of a wide variety of organisms. Depending on the prevailing physical and chemical conditions, a select group of organisms will thrive, to the exclusion of the less adapted ones ( Tate, 1995; Prescot *et al.*, 1999). Considering the fact that fungi have shown reasonable potential to detoxify hydrocarbon pollutants in the environment, and the particular potential to oxidise high molecular mass PAHs, the present study was aimed at screening fungi isolated from creosote-contaminated soil for: (i) their ability to grow on creosote-contaminated soil, and (ii) the capacity of the selected strains to oxidise

creosote in soil. It was also aimed at determining the extent to which the selected fungal strains can oxidise the higher molecular mass PAHs in soil and the optimum environmental conditions that will enhance such degradation.

## 8.2 MATERIALS AND METHODS

### 8.2.1 Enrichment and Isolation of Soil Fungi

#### 8.2.1.1 Soil Samples

Four categories of soil were collected from the experimental site to a depth of 30 cm with a spade. The samples were defined as: (a) heavily contaminated soil with concentrations of creosote >250 000mg $kg^{-1}$ , (b) moderately contaminated soil with concentration of creosote between 5 000 and 250 000mg $kg^{-1}$, (c) lightly contaminated soil with concentrations of creosote <5 000mg $kg^{-1}$, and (d) uncontaminated (control) soil collected from the same site but with no visible trace of creosote contamination. The samples were stored in heat-sealed polyethylene bags at $4^{O}C$ until required.

#### 8.2.1.2 Batch culture enrichment

Samples of soil (10g) from each contamination level were placed in sterile 250ml Erlenmeyer flasks containing 100ml of sterile nutrient broth. The medium was aerated by connecting an aquarium pump through a system of tubes to air stones which were submerged in the medium in the different

flasks. All flasks were then incubated for 72 hours at 32°C. Soil extract broth was prepared by mixing fresh garden soil (1kg) with 1 *l* of tap water and autoclaving at 121°C (15lb psi) for 30 minutes. To buffer the system, calcium carbonate ($CaCO_3$) (3g) was added to the mixture and stirred. The soil suspension was allowed to settle before the supernatant was filtered through a double thickness of Whatman's No. 1 filter paper. The filtration process was repeated until the filtrate became clear. The soil extract was autoclaved again and the pH was adjusted to about 4.6 with HCl. Penicillin (0.01g); chloramphenicol (0.5g); and streptomycin (0.025g) were dissolved in 2.5 ml of distilled water, filter sterilized through a 0.4μm millipore filter (Raymond *et al.*, 1976) and incorporated in the soil extract after cooling to about 40°C. Four flasks, each containing 200 ml of the soil extract diluted to 350 ml with deionized water, were each inoculated with 10g of one of the soil types and incubated under the same conditions, as described above for the nutrient broth cultures.

### 8.2.1.3 Isolation and identification

*(a) Nutrient medium*

The solid medium used for the isolation of fungi was the modified Czapeks medium, as described by Raymond *et al.* (1976). The medium contained per litre deionized water: 1g $K_2HPO_4$, 3g $NaNO_3$, 0.5g $MgSO_4.7H_2O$, 0.5g KCl, 0.01g $FeSO_4.7H_2O$, 20g agar. The medium pH was adjusted to 4.6 with 0.1M HCl before autoclaving for 15 minutes at 121°C (15lb psi).The medium was allowed to cool to about 40 °C before antibiotic addition, as described in section 8.2.1.2.

*(b) Hydrocarbons*

All hydrocarbons used were of analytical grade except creosote oil, which was purchased directly from a hardware store and was of the same grade as that used at the wood treatment plant. The hydrocarbons used for analysis were: phenol, o-cresol, m-cresol, p-cresol, naphthalene, anthracene, phenanthrene, pyrrole, fluorene, pyrene, chrysene, fluoranthene and benzo(a)pyrene.

*(c) Solid media*

Five grams of each hydrocarbon were separately added to 15ml aliquots of diethylether and then 5g fine colloidal silica was added to the mixture. Each mixture was slurried in a mortar and placed in a fume cabinet until the solvent had evaporated, to leave the hydrocarbon adsorbed to the silica. The hydrocarbon-adsorbed silica was then added to the nutrient medium described in section 8.2.1.3 and autoclaved for 15 minutes at 121$^{0}$C (15lb psi), after adjusting the pH to 4.6. This method is a modification of that described by Lees (1996). The nutrient media, each containing a different hydrocarbon, were then poured into separate plates. A set of STD Czapeks agar plates were individually overlaid with 0.1 ml of the above hydrocarbons, including creosote filtered through a 0.4μm filter. Potato dextrose agar (PDA) was used for the identification of the isolates.

For direct isolation of fungi, a 1 : 25 soil : sterile distilled water slurry was sonicated for 5 minutes at 25$^{0}$C to release the fungal spores bound to soil particles and 0.1 ml of the suspension was inoculated onto the STD Czepeks agar plates and incubated at 25$^{0}$C for 48 hours.. The nutrient

medium plates described above were inoculated with 0.1 ml of each enrichment culture, as in section 8.2.1.2, and incubated for 48 hours at 25°C. Subcultures were made from these plates.

### 8.2.2 Evaluation of Degradative Capabilities of Fungal Species

#### 8.2.2.1 Selection of strains with hydrocarbon-degrading capabilities

*(a) Oil-agar plates*

Creosote oil (2 ml) was Soxhlet-extracted (Lees, 1996) and added to 100ml molten Czapeks agar in a 150 ml Erlenmayer flask. The mixture was sonicated for 5 minutes to facilitate the formation of an emulsion. The medium was applied as a 3 mm overlay to STD Czapeks agar plates before the droplets in it could coalesce. This method was modified from Lees (1996).

*(b) Strain selection*

Active mycelium of about 1 $cm^2$ was aseptically cut from five day old cultures and used to inoculate plates from 8.2.2.1 (a) above. The plates were incubated at 25°C. Growth was monitored daily and the rate of growth was determined by measuring the diameters of the expanding colonies over a period of 10 days.

### 8.2.3 Demonstration of Biodegradation of Creosote in Soil by Fungi Isolated From Soil

*8.2.3.1 Soil reactors*

Thirty-six 3 litre polyvinyl chloride (PVC) vessels, with a diameter of about 33 cm and depth of 13cm, were each filled with 2.5kg of the heavily contaminated (>250 000mg $kg^{-1}$ soil, fresh mass) soil from the experimental site. Polystyrene bases, with a regular pattern of perforation to allow passage of leachate, were fitted into the vessels and supported from below to prevent sagging. This was done to prevent the soil from becoming waterlogged during the experiment. Black polyethylene sheeting was used to cover the vessels to prevent photo-oxidation of the creosote components. Distilled water was used to maintain the soil at 70 % of its water holding capacity. The pH was measured at the start of the experiment using a Crison Micro pH 2000.

*8.2.3.2 Fungal Bulking*

About 300 g of barley grain, soaked in water for 48 hours and drained, were placed in each of 30 polyethylene bags. The bags were plugged with cotton wool stoppers before being sterilized at 121$^{o}$C for 15 minutes (15 Ib psi) in an autoclave. The cotton wool plugs were to allow for gaseous exchange. Each bag was aseptically inoculated with one of the selected fungal isolates by slicing the PDA colonized by the organisms into small 5 mm pieces. Subsequent bags were inoculated with 50g of the colonized grains. The bags were incubated in the dark for three weeks at 25$^{o}$C to allow complete colonization of the grains. The colonized grains were then inoculated into the

contaminated soil at the rate of 6g of barley to 100g of soil (fresh mass). At this ratio the organisms did not become well established in the contaminated soil. After a number of preliminary trials, it was found that the organisms became well established at a ratio of 10 g barley to 100 g soil and so this latter ratio was used in the main experiment.

*8.2.3.3 Treatments*

Treatments 8, 10, 11, 12, 13, 14, 15, 16 and 18 received supplementary nutrient amendment to give a C:N:P ratio of 25:5:1, as recommended for aerobic metabolism (Kennedy *et al.*,1975), by adding $NH_4NO_3$ (7.5g) and $K_2HPO_4$ (1.5g) (dissolved in 150 ml of distilled water) and mixing it into the soil after each sampling event. Moisture in all treatments was maintained at 70% of field capacity for the duration of the experiment. Aeration was achieved by turning the entire content of the reactor with a small garden trowel once a week. The trowel was flamed over a spirit lamp to reduce the possibility of contamination. The eighteen duplicated treatments were as shown in Table 8.1. All treatment were incubated at ambient temperature for 70 days.

*8.2.3.4 Sampling*

Once every two weeks, five random samples were collected as cores (100mm x 15mm) from each reactor, using a 300mm x 15mm glass tube. Each set of samples was mixed together thoroughly, placed in a plastic bag and heat sealed before being stored at approximately -17 $^{0}$ C. Soil samples were also taken before and after nutrient addition, for pH measurements.

Table 8.1 Treatments in soil reactors

| Treatments in duplicates | Barley addition | Fungal inocula from isolates from contaminated soil | Nutrient addition ($NH_4NO_3$ + $K_2HPO_4$) |
|---|---|---|---|
| 1 | yes | *Cladosporium sp.* | no |
| 2 | yes | *Fusarium sp.* | no |
| 3 | yes | *Penicillium sp.* | no |
| 4 | yes | *Aspergillus sp.* | no |
| 5 | yes | *Pleurotus sp.* | no |
| 6 | no | no | no (control 1) |
| 7 | yes | *Cladosporium sp.*, *Fusarium sp.*, *Penicillium sp.*, *Aspergillus sp.*, *Pleurotus sp.* | no |
| 8 | no | no | yes (control 2) |
| 9 | sterile barley | no | no (control 3) |
| 10 | yes | *Cladosporium sp.* | yes |
| 11 | yes | *Fusarium sp.* | yes |
| 12 | yes | *Penicillium sp.* | yes |
| 13 | yes | *Aspergillus sp.* | yes |
| 14 | yes | *Pleurotus sp.* | yes |
| 15 | sterile barley | no | yes (control 4) |
| 16 | yes | *Pleurotus* sp.(from Cedara) | yes |
| 17 | yes | *Pleurotus* sp.(from Cedara) | no |
| 18 | yes | *Cladosporium sp.* *Fusarium sp.* *Penicillium sp.* *Aspergillus sp.* *Pleurotus sp.* | yes |

* KwaZulu-Natal Department of Agriculture, Cedara.

* All isolates were identified at KwaZulu-Natal Department of Agriculture, Cedara.

*8.2.3.5 Analysis of the samples from soil reactors*

*(i) Soil extraction*

Soxhlet extraction was performed, as outlined in EPA Method 3540. A duplicate soil sample (5 g), described in section 8.2.3.4, was placed in an extraction thimble (Whatman cellulose). A glass wool plug was placed above the sample to prevent dispersion of the soil. Dichloromethane (75 ml) was placed in a round-bottom flask containing two boiling chips. The flask was attached to a Soxhlet extractor fitted with a condenser and the sample was extracted for 8 hours. After cooling, the extract was passed through magnesium sulphate ($MgSO_4$) to remove residual water. The extract was filtered and the total volume reduced to about 5 ml in a rotary evaporator (Heidolph B.NR.51111). Residual dichloromethane was evaporated under a low flow of nitrogen by passing gaseous nitrogen through extract. The method was modified, in that $MgSO_4$ was not added to the sample before extraction. All experiments were duplicated.

*(ii) Measurement of residual creosote concentration by infra-red spectrophotometry (IRS)*

Residual creosote concentration was determined by the USEPA4181.1 (1982) method, as described in Chapter 7, section 7.2.6.

*(iii) Measurement of changes in concentration of creosote components by gas chromatography (GC)*

The description of the GC procedure is given in Chapter 7 section 7.2.6.

## 8.3 Results and Discussion

### *8.3.1 Enrichment cultures*

Both the nutrient broth and the soil extract medium supported fungal growth, with colonies appearing between 4 and 21 days after inoculation and subsequent incubation in the dark at 30°C. The soil extract was a more restrictive medium, supporting growth of only a few fungi, presumably those that could metabolize the hydrocarbons present. Growth was observed after 14 to 21 days in the soil extract medium and the time of colony appearance and the amount of growth varied, depending on the hydrocarbon. Generally, growth was observed within 14 to 15 days in the cultures spiked with the phenolics, naphthalene, anthracene, phenanthrene, pyrolle and fluorene, with growth on anthracene and naphthalene becoming prolific by day 15. Although growth was not detected in the cultures spiked with some of the higher molecular mass compounds tested until the eighteenth day, the organisms were found to proliferate rapidly in pyrene and benzo(a)pyrene. Growth in fluoranthene was poor. No growth occurred in chrysene until day 21. The cultures spiked with whole creosote oil started showing the presence of organisms on the fifteenth day. Although difficult to initiate, growth became prolific by the end of day eighteen. Soil extract based media are commonly used in studies involving the isolation of soil organisms capable of utilizing xenobiotic substances found in heavily contaminated soils. Although fungal growth was found in all the cultures, it was evident that the higher molecular mass polycyclic aromatic hydrocarbons (PAHs) supported much less growth than the lower molecular mass compounds and the phenolics.

Growth in nutrient broth was more prolific than in soil extract medium and a number of opportunistic microorganisms were detected. One isolate was suspected to be an actinomycete, but its identification was not conclusive. No bacterial growth was observed. The extensive fungal growth observed in the nutrient broth was probably due to the high nutrient status of the medium. The presence of required nutrients in this medium is another possible reason why growth was very prolific in the medium. Lees (1996) reported profuse growth of fungi and bacteria in nutrient broth spiked with the aliphatic hydrocarbons, hexadecane, pristine and paraffin, in spite of the antibiotics chloramphenicol, streptomycin and penicillin incorporated in the medium.

### 8.3.2. Isolation and Identification

From both enrichment media, seventeen strains of fungi were isolated. The isolates were identified as belonging in the following genera: *Penicillium, Aspergillus, Fusarium, Trichoderma, Pleurotus, Cladosporium, Phanerochaete*, *Candida* and *Monicillium*. Table 8.2 show the distribution of the fungi in the different cultures. Most of the isolates grew well on the phenolic compounds. However, *Pleurotus* grew only poorly on all the phenolics. *Phanerochaete* grew profusely on phenol and poorly on m-cresol and p-cresol, but showed no growth in o-cresol. *Aspergillus, Cladosporium, Penicillium* and *Fusarium* grew most prolifically on the phenolic substrates (Table 8.2). These organisms have been previously reported to grow on a number of different hydrocarbons originating from different sources (Cerniglia, 1997). There is reasonable evidence to show that *Penicllium* can metabolise phenol and its compounds (Hofrichter *et al.*, 1993). For example, *Penicillium* isolated from hydrocarbon-contaminated soil was reported by Hofrichter *et al.* (1993) to grow on phenol and

substituted phenols. Anselmo *et al.* (1984, 1985, 1989) described the growth of *Fusarium flocciferum* with phenol as sole carbon source. The mineralization of phenol by *Penicillium* was demonstrated by Scow *et al.* (1990).

The presence of the white rot fungi, *Pleurotus* and *Phanerochaete*, in the soil was not unexpected, since large quantities of untreated wood were stacked at the wood treatment facility. However, their ready isolation from the soil indicates that their propagules can survive the toxicity of creosote in the soil. These organisms' ability to degrade lignin has attracted the attention of degradation technologists, who use them in hydrocarbon degradation. Both organisms have been shown to grow in phenolic compounds (Kennes and Lama, 1994). The disparity in the growth of *Phanerochaete* on phenol and the substituted phenols mentioned above may be due to the substitution of methyl groups on the compounds. This affects some of their physical properties such as solubility.

Growth of the organisms in the two- and three-ringed PAHs is shown in Table 8.2. *Cladosporium* and *Aspergillus* grew most successfully on these compounds, with *Cladosporium* showing the best growth on naphthalene and fluorene. The growth of the other organisms on these compounds ranged from moderate to no growth. *Phanerochaete* grew only on naphthalene. Two and three-ringed PAHs have been reported by several workers to support the growth of these organisms (Cerniglia and Crow, 1981; Cerniglia *et al.*, 1982; Dhawale *et al.*, 1992; Bezalel *et al.*, 1996; Bogan and Lama, 1996; Casilas *et al.*, 1996; Cerniglia, 1997).

Table 8.2 Growth of nine fungal isolates after 48 hours on solid media described in 8.2.1.3(c) overlaid with different hydrocarbons.

| Organism | Phenol | o-cresol | m-cresol | p-cresol | Naphth. | Anthra. | Phenanth | Pyrrol |
|---|---|---|---|---|---|---|---|---|
| *Aspergillus* | +++ | ++ | ++ | ++ | ++ | + | ++ | ++ |
| *Cladosporium* | ++ | +++ | ++ | ++ | +++ | ++ | ++ | ++ |
| *Fusarium* | + | ++ | ++ | ++ | ++ | - | - | + |
| *Candida* | ++ | ++ | + | - | + | + | ++ | - |
| *Monicillium* | ++ | + | + | ++ | + | + | + | + |
| *Trichoderma* | ++ | ++ | - | + | ++ | + | - | - |
| *Penicillium* | ++ | ++ | + | +++ | + | + | + | + |
| *Pleurotus* | + | + | + | - | ++ | + | + | + |
| *Phanerochaete* | +++ | - | + | + | + | - | - | - |

| Organisms | Fluorene | Pyrene | Chrysene | Fluoranth | Benzo(a)p | Creosote |
|---|---|---|---|---|---|---|
| *Aspergillus* | ++ | ++ | + | + | + | ++ |
| *Cladosporium* | +++ | ++ | ++ | ++ | ++ | +++ |
| *Fusarium* | + | + | - | + | - | + |
| *Candida* | - | - | + | - | - | + |
| *Monicilium* | - | ++ | - | + | - | + |
| *Trichoderma* | ++ | + | + | - | ++ | + |
| *Penicillium* | ++ | ++ | + | + | + | ++ |
| *Pleurotus* | ++ | ++ | + | + | + | ++ |
| *Phanerochaete* | - | + | - | - | ++ | +++ |

* Profuse growth +++ Moderate growth ++ Sparse growth + No growth -

Growth of these fungi on the higher molecular mass PAHs ( i.e. those with four and five benzene rings) was less prolific than on the lower hydrocarbons studied. *Penicllium* and *Candida* were found to grow only on pyrene and chrysene, respectively. Of all the fungi investigated, *Cladosporium*

showed the greatest metabolic diversity, growing well to moderately on all the compounds tested. *Phanerochaete* and *Pleurotus* also performed reasonably well, with sparse to moderate growth occurring on most of the compounds. Generally, fewer organisms grew on the PAHs than on the phenolic compounds. All the organisms, however, could utilize naphthalene. Naphthalene has been reported by many workers to support the growth of, and be mineralized by, representative of several different genera of fungi (Hofmann,1986 Cerniglia, 1997; Kotterman *et al.*, 1999). The lignin-degrading enzymes of the white rot fungi have been particularly employed in the degradation of high molecular mass PAHs. Field *et al.* (1992) suggested that since the enzyme system of the white rot fungi is non-specific in action, it should be able to act non-specifically on a variety of hydrocarbon substrates. Pyrene, chrysene, benzo(a)pyrene and fluoranthene have been reported to support the growth of different fungi (Pothuluri *et al.*, 1990; Launen *et al.*, 1994; Sack and Fritsche, 1997).

All the organisms investigated grew on whole creosote, but to different degrees. However, *Cladosporium* and both white rot fungi, *Phanerochaete and Pleurotus*, grew well on this substrate. Although many workers have reported the growth of many fungi on creosote and its constituent compounds, the effect of concentration on its vulnerability to microbial degradation remains unknown. More specifically, the concentration at which the substance becomes toxic to wood-degrading microorganisms is uncertain.

### 8.3.3 Evaluation of the Degradative Capabilities of Fungal Species

A few of the isolates were observed to grow profusely and to sporulate over the ten-day period,

thereby giving rise to many daughter colonies. This pattern of growth indicates that the organisms were having difficulty utilizing the available carbon substrate and, as a result, were extending their growth to reach out for an alternative source of carbon (Lees,1996). It can be argued that the organisms were able to partially utilize the substrate and hence they were able to initiate such growth. However, the production of spores and subsequent appearance of daughter colonies could be seen as a response to the unavailability of the carbon substrate for utilization, as many fungal species are known to produce spores under conditions of nutrient deprivation (Alexopoulos,1996). That the colonies continued to thrive for up to ten days is an obvious indication that they were progressively adapting to the substrate in the absence of an alternative carbon source.

Most of the organisms, on the other hand, produced very dense, compact, circular colonies. These colonies were interpreted as indicating more ready adaptation to the xenobiotic carbon substrate. Based on these observations, it was difficult to eliminate any of the isolates from the biodegradation trial on creosote-contaminated soil. However, it was decided that only those organisms that produced discrete dense colonies would be used in the subsequent trial investigating the degradation of creosote in soil.

### 8.3.4 Demonstration of Biodegradation of Creosote in Soil by Fungi Isolated From Soil

*(i) Growth of fungi in soil treatment reactors*

The growth of the fungal inoculum in the soil reactors varied according to the treatment applied.

Growth was first observed after 14 days in all treatments inoculated with non-basidiomycete fungi. After 20 days of incubation, mycelium had spread profusely, covering most of the soil surface. No growth was observed during the first 20 days of incubation in the reactors inoculated with basidiomycetes. At this stage no contaminant organisms were detected in these reactors in which the inoculated fungi had grown. Although mycelial growth was apparent in all the reactors inoculated with non-basidiomycete fungi, it was patchy, with large areas of uncolonized soil visible in those treatments which were not nutrient supplemented (Treatments 1-5), compared with the dense growth observed in Treatments 10-14, which were nutrient supplemented. The use of nutrient supplements to increase the growth of fungi in soil was also demonstrated by Lamar *et al.* (1987). In Treatment 7, which was inoculated with all five fungal isolates, *Cladosporium* sp., *Fusarium* sp., *Penicillium* sp. and *Aspergillus* sp. became established first, while *Pleurotus* sp. only became noticeable after 21 days.

With the exception of Treatments 6 and 8, prolific fungal growth had developed in all the treatments by day 38, when the fifth tilling was carried out. At this stage *Pleurotus* sp. was the dominant fungus in Treatments 7 and 18. *Pleurotus* has been reported to easily colonize soil media upon inoculation (Novotný *et al.*, 1999) and dominate the microbial community, even when its common substrates such as woody materials are absent. It was found to have grown well in Treatments 5,14,15 and 16, which were inoculated with only *Pleurotus* sp. During tilling it was observed that fungal mycelium did not penetrate the entire soil mass at the same rate. There was more mycelial proliferation in the upper soil layer than in the middle and bottom layers. However, with tilling, hyphae were distributed to the lower levels of the soil system.

Soil in Treatments 9 and 15 ( controls 3 & 4), to which barley was added, exhibited prolific fungal growth in spite of not being inoculated with any of the isolates. This is in contrast to observation in soils in Treatments 6 and 8 (controls 1&2), to which barley was not added. In these, growth was very sparse and became visible only after 30 days. Samples from both the latter treatments revealed the presence of some bacteria, mycelial fungi and yeast species, following plating on nutrient agar and PDA. The presence of barley in controls 3 and 4 presented an alternative carbon source for the fungi to utilize and so proliferate. These organisms could therefore grow and co-metabolize the creosote in the soil. A wide variety of fungi was observed in these two reactors because they arose from spores originally present in the contaminated soil and not from cultivated and well-established inocula, as was the case in all other treatments. All treatments to which barley was added showed more abundant fungal growth than those that did not receive barley. This confirms the findings of other researchers, who reported improved growth of fungi following addition of substances such as straw, pine bark, wheat, alfalfa and barley to sterile soil ( Morgan *et al.*, 1993; Boyle 1995; Novotný *et al.*, 1999). The mixing of fungi grown in some form of bulking material with contaminated soil is a common practice in bioremediation of soil and has produced effective results in the bioremediation of different substances in soil (Loske *et al.*,1989; Šašek *et al.*,1993; Sack *et al.*, 1997). The lack of an alternative carbon source affected the development of a fungal population in controls 1 and 2, providing the opportunity for opportun

supplements, was more rapid than in Treatment 17 which did not, becoming more dense from day seven. However, both treatments showed very profuse growth by day 25. Fungal growth in these two treatments continued until the end of the experimental period.

*(ii) Degradation of creosote hydrocarbon in soil samples*

All the treatments resulted in the removal of between 65% and 94% of creosote from the soils in the reactors (Fig. 8.1). Removal from the controls ranged from 26% to 58% (Fig. 8.1). Reduction in creosote concentrations in the first 14 days was relatively slow in all the treated reactors, the concentrations differing little from those in the controls (Fig. 8.2-8.3). It is presumed that this was the time taken for the fungi to become established in the soil. Since fungal growth rates are generally slower than those of bacteria the slow colonization of the substrate was not unexpected. Another possible reason for the observed lag period is that the fungal isolates used as inoculum were reintroduced into the contaminated soil after their isolation and purification on media lacking creosote or its components and therefore needed time to readapt to the contaminant hydrocarbons. Reduction in residual hydrocarbon concentrations generally became more rapid after about one month's

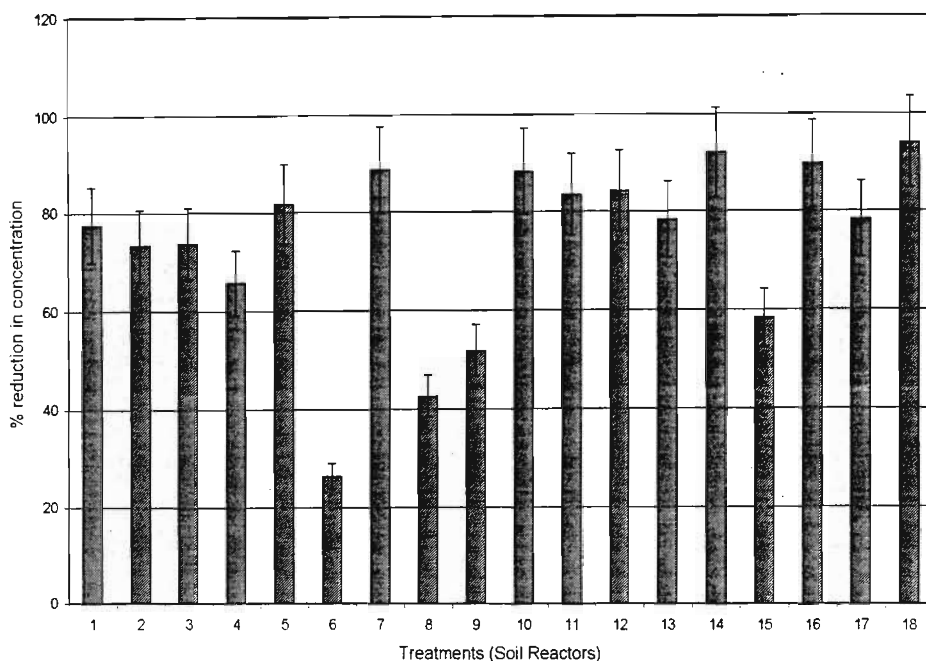



Fig.8.1 Total reduction (%) in concentration of creosote in contaminated soil in treatment reactors after seventy days. Values are means of two ± 1 Standard Error.

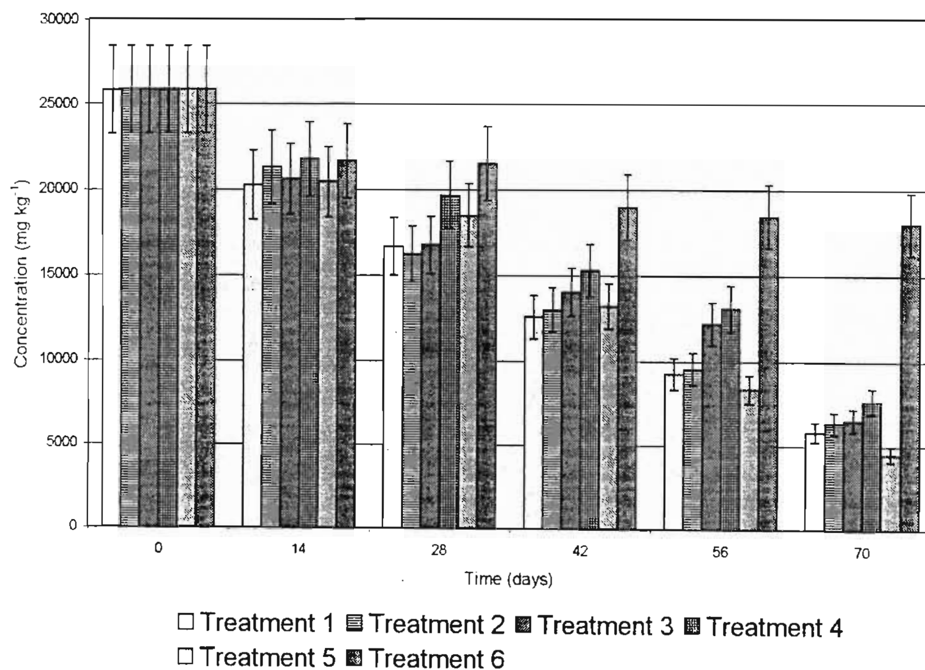



Fig.8.2 Changes in concentration of creosote with time in Treatments 1-6. Values are means of two ± 1 Standard Error.

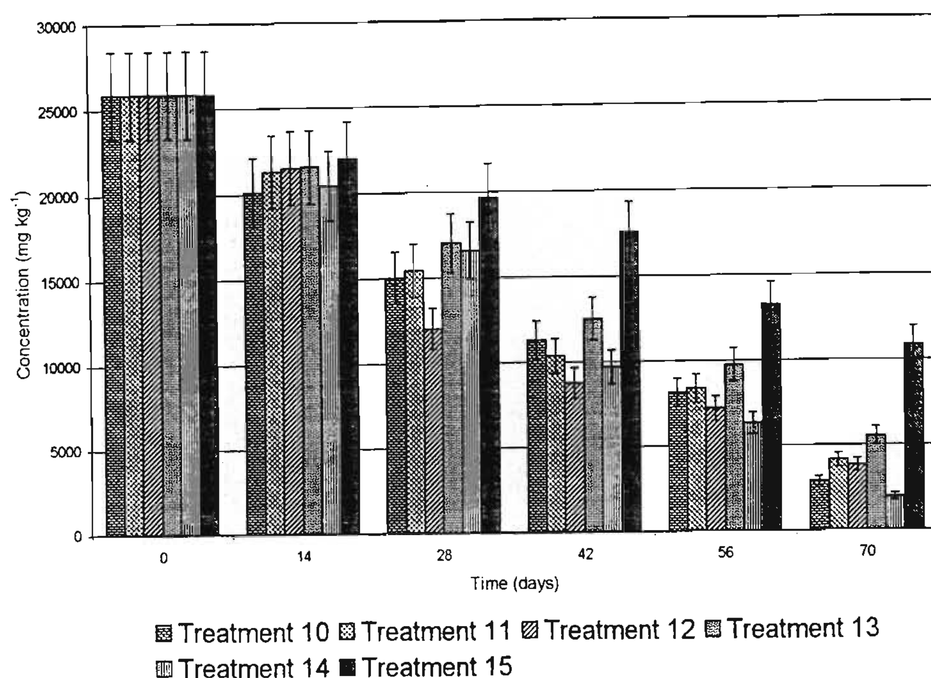



Fig.8.3 Changes in concentration of creosote with time in Treatments 10-15. Values are means of two ± 1 Standard Error.

incubation, when the fungal population had become well established through the soil in all the treated reactors.

The highest reduction (94.1%) in total creosote in all treated soils was observed in Treatment 18 which was inoculated with all five fungal isolates and also received nutrient supplementation and regular aeration during the period of incubation (Fig.8.4). The least reduction among the treated reactors was observed in the soil in Treatment 4, which was inoculated with *Aspergillus* sp. but received no nutrient supplementation ( Fig.8.2). The fact that supplementary nutrients were observed to enhance microbial growth and consequently creosote degradation confirms an earlier report of Lamar *et al.* (1987). Reduction in creosote concentrations in Treatments 1-7, which did not receive

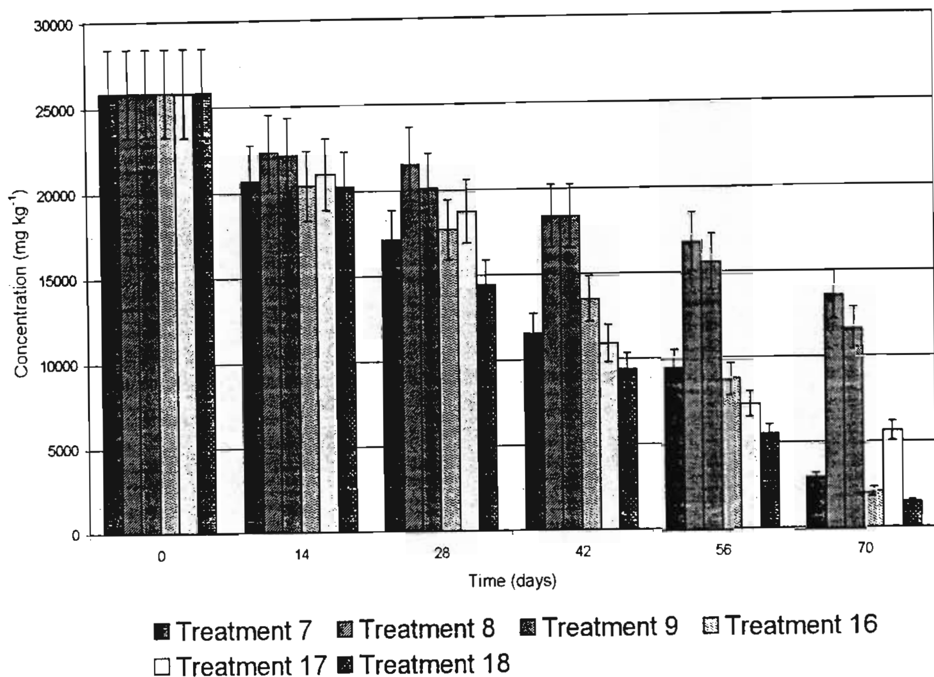



Fig.8.4 Changes in concentration of creosote with time in Treatments 7,8,9,17 & 18. Values are means of two ± 1 Standard Error.

any nutrient supplementation, was found to be generally lower (between 65% and 81%) than in Treatments 8-16 and 18 that received nutrients (between 78% and 94%) (Fig. 8.1). Treatments 7 and 18 were inoculated with the same mixture of five fungal isolates. This also accounts for the similarity in the results obtained from the two treatments (Fig 8.1). However, the addition of supplementary nutrient in Treatment 18 proved to be advantageous, as this greater percentage reduction in creosote concentration than Treatment 7. The introduction of pollutant hydrocarbons into an environment supplies a large amount of carbon, which often results in an excessively high carbon to nitrogen (C:N) and carbon to phosphorus (C:P) ratio. This can be unfavourable for microbial growth (Atlas, 1981). It is therefore necessary to increase nutrient (especially nitrogen and phosphorus) concentrations in the soil by supplementing with nitrates and phosphates to offset the imbalance created by the polluting carbon source(s) to promote microbial growth and biodegradation. The

effects of nutrient supplements on the fungal degradation of hydrocarbons has been reported by other workers (Sack *et al.*,1997 Eggen *et al.*, 1999; Podznyakova *et al.*, 2001). Rodriguez *et al.* (1999) reported a high removal rate of a benzo(a)pyrene in a nitrogen-limited medium and Griffin (1972) reported enzyme activity of white rot fungi at a C:N ratio of 2000:1. Although fungi are known to grow in low nutrient media (Davis and Westlake,1978), nutrient-rich media support better growth. The effect may depend on the type of nutrient supplement and the rate of release of the nutrient into the environment for utilization by the organisms (Davis and Westlake, 1978). McGugan (1997) showed that organic fertilizer, in the form of animal manure, and inorganic fertilizers and a bulking agent had varying effects on the growth of soil fungi and hydrocarbon degradation. He observed more reduction of oil in the presence of manure and bulking agent than with any of the other treatments. In the present study, a combination of organic and inorganic nutrients and bulking agent (viz. barley) was found to support prolific fungal growth and bring about large reduction in creosote concentrations in the soil.

Barley as a bulking agent provided an additional source of carbon for the organisms to grow on while adapting to the hydrocarbon substrate. Most fungi are not known to utilize PAHs and other hydrocarbons as their sole source of carbon and energy and, as a result, the medium must be supplemented with an additional carbon source to allow fungi to metabolize them (Cerniglia, 1997). Since fungi are known to metabolise their substrate by non-specific extracellular enzymes ( Field *et al.*, 1992; Fritsche, 1992; Martens and Zadrazil, 1992), the enzymes produced during growth on one substrate can readily facilitate the metabolism of another compound. Thus the initial oxidation of the creosote hydrocarbons by the fungal isolates was probably mainly by co-metabolization. This

may explain the relatively high rate of reduction of creosote concentration during the first four weeks (Fig. 8.2-8.4). Carbohydrate-rich substances such as barley, wheat and cotton seed are commonly used as sources of carbon for the cultivation of fungi for various purposes ( Lees, 1996; McGugan, 1997; Bogan, 1999; Rodriguez *et al.*, 1999). The advantage of using such nutrient-rich substrates is to facilitate the rapid growth of the fungi and thus the production of the enzymes that will be required for the degradation of the hydrocarbons present. The enhanced growth observed in all the treatments with barley was to a greater or lesser extent manifested in the greater creosote degradation in those treatments. Treatments 6 and 8, which were not supplemented with barley, showed the least reduction in creosote concentration (26.4% and 42.6% respectively). Nutrient (N and P) supplementation did improve degradation in Treatment 8. These results substantiate the results shown in Treatments 1-6 and 10-14, where nutrient supplement increased both the growth of the fungi and degradation rate of the creosote.

Although fungal biomass continued to increase in subsequent weeks, the reduction in hydrocarbon concentration was not as pronounced as during the first four weeks. This may be attributable to a decrease in quantity of carbohydrate base (barley) present which forced the organisms to adapt to the hydrocarbon substrate effectively, during which period enzyme activity decreased. During the last two weeks, it was observed that degradation began to increase again in some of the treatments. This may be an indication that the fungi were becoming better adapted to creosote as a carbon source.

The mixed culture of all the fungi in Treatments 7 and 18 was observed to degrade creosote better than the individual organisms in pure culture. However, after the fourth week, *Pleurotus* sp.

dominated the biomass and, by the end of the experiment, the other organisms had been virtually eliminated from the reactors. Although it can be argued that *Pleurotus* spp. was responsible for the improved performance of these reactors, a comparison with the results from Treatment 14 (*Pleurotus* alone) suggests that the improved activity of the composite mixtures could have been the result of the other organisms contributing, albeit perhaps in a minor way, in a synergistic action with the dominant *Pleurotus* spp. *Pleurotus* has been known to grow actively in hydrocarbon-rich media and has been reported to oxidize hydrocarbon compounds (Bogan and Lamar, 1999; Eggen *et al.*, 1999; Novotoný *et al.*, 1999; Rodriguez *et al.*, 1999). Other treatments containing *Pleurotus* sp. (Treatments 5, 14, 16 & 17) showed some of the lowest residual creosote concentrations. *Pleurotus* spp. Isolated from non contaminated soil were not as effective as those isolated from the contaminated site. This difference in performance could be attributed to the adaptation of the strain from the contaminated site to the polluting hydrocarbon over a long period. Lambert *et al.* (1992) reported that fungi isolated from non-polluted soil did not have any degradative effect when applied to a pollutant. However, the slow adaptation of the fungi to the polluting carbon substrate and its delayed oxidation observed, in the present study, could have been as a result of the presence of an alternative carbon source (barley). These results suggested that factors such as the strain of the organism, and hence the particular type of enzyme produced, may greatly affect the rate of pollutant oxidation by a given species.

*Cladosporium* was found to actively oxidize the creosote both in the presence or absence of nutrient supplementation. Treatments containing this fungus showed a reduction of up to 88.3% in creosote concentrations. *Cladosporium* has been widely reported to grow in hydrocarbon media including

crude oil and creosote ( Parbery, 1971; Cerniglia, 1997). *Fusarium* sp., *Penicillium* sp., *Aspergillus* sp. have also all been implicated in the degradation of hydrocarbons (Anselmo,1989; Hofrichter *et al*., 1993; Cerniglia 1997; Boonchan *et al*., 2000). However, the extent of degradation varied from one substrate to another and from one environment to another, as discussed earlier.

The amounts of the selected creosote compounds recovered at the end of the study showed that most of the fungi isolated degraded the higher molecular mass compounds better than the bacteria (Chapter 7) (Fig.8.5-8.7). The fungi reduced the 4- and 5- ringed compounds by as much as 94% after 70 days in some of the reactors, about half the time taken by the bacteria to achieve the same level of removal or less removal (Fig.8.7). Some of these compounds, which are usually resistant to microbial attack, can be oxidized by fungi because of their use of non-specific enzymes. All the phenolic compounds except p-cresol were removed to below detection levels. Degradation of the phenolics was faster by bacteria than by the fungi. Similar results were reported by Clemente *et al.* (1999). The degradation of phenolic compounds by *Fusarium oxysporum* has been reported previously (Madhosnigh, 1958; Anselmo *et al*., 1985, 1989). Different *Aspergillus* species have been reported by different workers to transform compounds derived from the parent benzene structure, including phenolics (Shailubhai and Sahasrabudhe, 1983; 1984; Sahasrabudhe *et al*., 1985, 1987). Seeholzer and Hock (1991) reported the degradation of phenolic compounds by *Pleurotus cornucopiae*. Similarly, *Cladosporium* has been known to occur in hydrocarbon-contaminated soils and to have the potential to oxidize a wide range of hydrocarbons (Bossert and Bartha, 1984). Complete mineralization of phenol by *Penicillium* was demonstrated by both Scow *et al.* (1990), Hofrichter *et al.* (1993) and Sack *et al.* (1997).

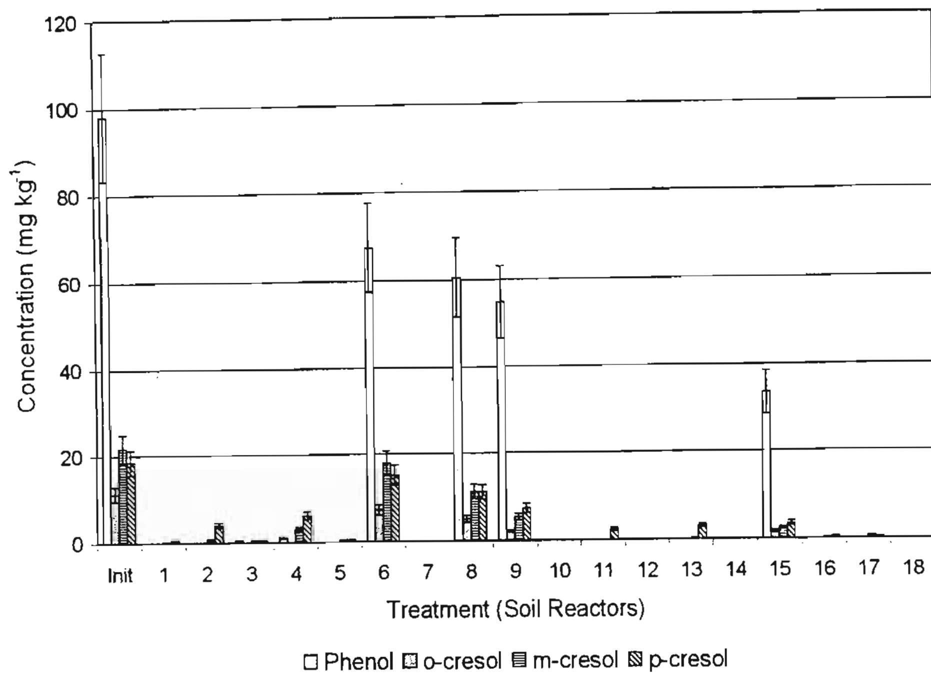



Fig.8.5 Concentrations of phenolic compounds in soil reactors after seventy days incubation at ambient temperature. Values are means of two ± 1 Standard Error.

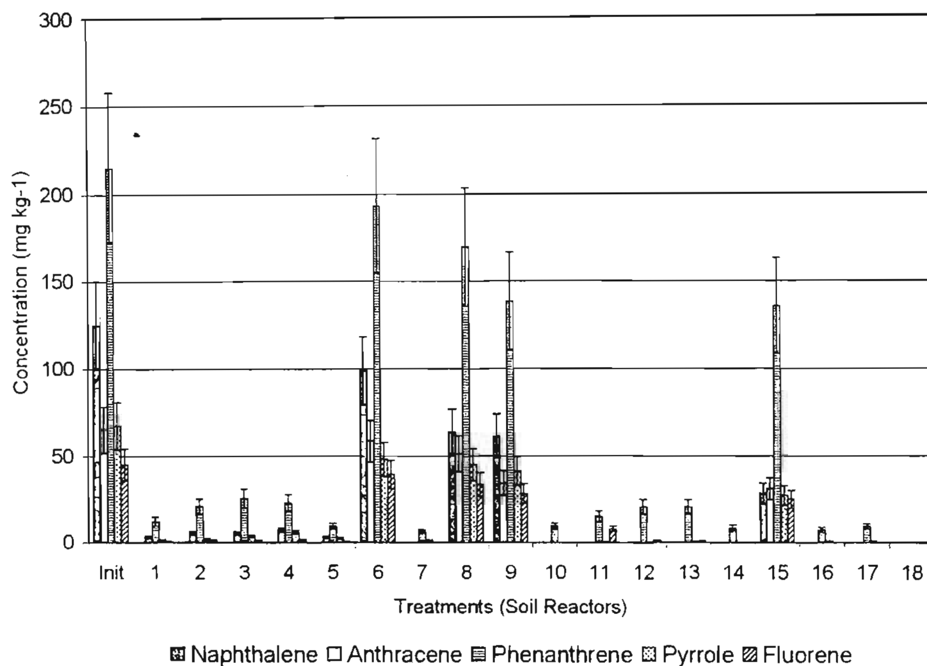



Fig.8.6 Concentrations of lower molecular mass PAHs in soil reactors after seventy days incubation at ambient temperature. Values are means of two ± 1 Standard Error.

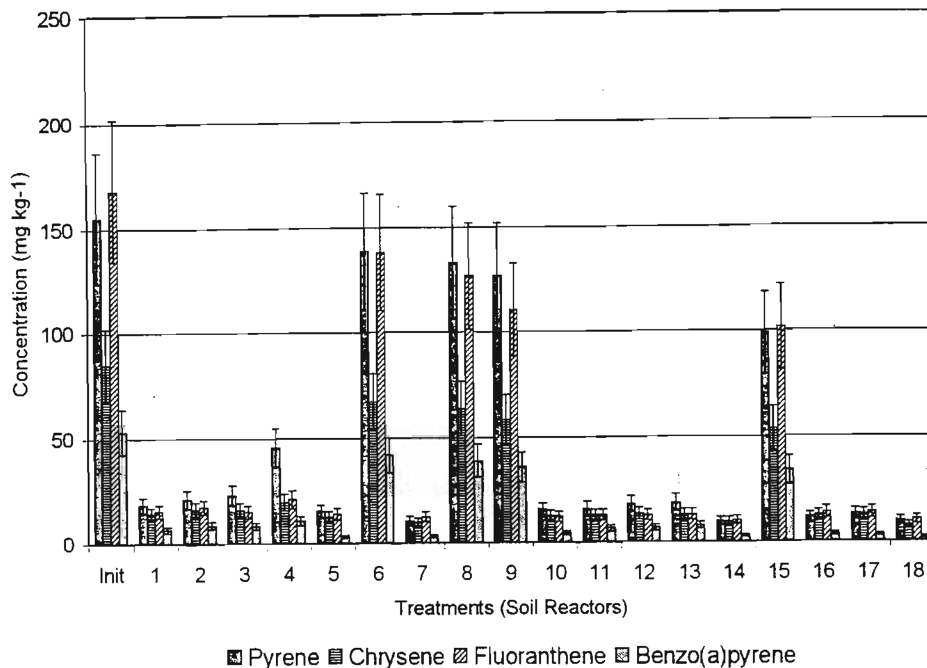



Fig.8.7 Concentrations of residual higher molecular mass PAHs in soil reactors after seventy days of incubation at ambient temperature. Values are means of two ± 1 Standard Error.

Overall, the lower molecular mass PAHs ( i.e. those with two and three benzene rings) were actively degraded. Naphthalene concentrations were reduced to below detectable limits in all the treated reactors (Fig. 8.6). Naphthalene has been reported to be degraded by many microorganisms, including bacteria and fungi. Earlier reports by Auret *et al.* (1971) and Ferris *et al.* (1973, 1976) showed that non-ligninolytic fungi can degrade naphthalene through a number of intermediates to form carbon dioxide and water. Other workers have reported the oxidation of naphthalene by both ligninolytic and non-ligninolytic fungi (Cerniglia and Gibson, 1977; Gibson and Subramanian, 1984). Naphthalene is more soluble in aqueous media than the other PAHs and is thus more available for microbial oxidation due to better desorption from the surfaces of soil particles. In the present study the degradation of naphthalene by the fungi was comparable with that by the bacteria,

both groups of organisms reducing the concentrations to below detectable levels.

The three-ringed PAHs ( anthracene, phenanthrene, pyrrrole and fluorene) were actively degraded in both the nutrient-supplemented and non-nutrient-supplemented treatments. Degradation was, however, greater in the soil supplemented with nutrients (Fig. 8.6). Anthracene was removed below detection limits in all treatments supplemented with nutrients ( Treatments 10, 11 12, 13, 14, and 16) and in all treatments that were inoculated with a mixture of the five fungal isolates ( Treatment 7 and 18). These results contrast with those obtained from the bacterial degradation experiments where anthracene was much less actively degraded. Novotný *et al.* (1999) reported a higher anthracene degradation by *Pleurotus ostreatus* than was reported by Andersson and Henrysson (1996) with the same organism. Phenanthrene removal reached 98% in the treatment supplemented with nutrients and inoculated with all five of the fungal isolates. Pyrrole and fluorene, both with similar heterocyclic structures, were degraded to below detection limits in most of the treatments supplemented with nutrients (Fig. 8.6).

Although there were differences in the oxidizing capacities of the different fungi investigated, the more prominent influencing factors in the oxidation of the creosote components were the presence of additional nutrients and an additional source of carbon. The enhancement of fungal PAH degradation by nutrient amendment has been reported previously by Rodriguez *et al.* ( 1999). The degradation of lower molecular mass PAHs in soil can be partially attributed to their relative solubility (Mueller *et al.*,1989 ; Eriksson *et al.*,2000). In addition, the ability of fungal mycelia to ramify through the soil to reach the substrate, even when it is strongly sorbed to soil surfaces, is

possibly another reason why the degradation of these compounds is successful. Degradation of these compounds by several fungi has been reported previously ( Bogan and Lamar 1995; Clemente *et al.*, 1999; Eggen *et al.*, 1999) and some white rot fungi, including *Pleurotus*, have been shown to degrade these compounds effectively (Bogan and Lamar 1999). This study has shown, however, that complete removal of these compounds is possible with both pure cultures of ligninolytic and non-ligninolytic fungi and mixed cultures of fungi, provided growth conditions are favourable.

The higher molecular mass compounds studied (i.e. those with four and five fused rings) were not completely oxidised at termination of the experiment. The recalcitrance of this group of compounds is due to their high molecular mass and relatively low water solubility. These properties contribute to their poor desorption from soil matrices, thus rendering them unavailable for microbial attack. Another reason for the incomplete removal of these compounds from the experimental soil is the age of the contamination, which has presumably resulted in strong sorption to the soil particles. Increasing duration of contamination has been reported to reduce biodegradation considerably (Field *et al.*, 1995; Eggen *et al.*, 1999), by reducing the bioavailability of the compounds. This occurs through immobilization in micropores or changes in binding forms such as oxidative coupling (Bollag *et al.*, 1992). However, the effective removal of higher molecular mass PAHs in this experiment (see Fig. 8.7) can be attributed to the ramification of the mycelia of the fungi tested and probably the secretion of non-specific extracellular enzymes. Fungi have been observed to degrade compounds such as lignin, DDT, TNT and PCBs by the same mechanism (Higson 1991; Barr and Aust, 1994; Eggen *et al.*, 1999). Some *Pleurotus* species have been demonstrated to degrade higher molecular mass PAHs in the environment (Eggen *et al.*, 1999; Rodriguez *et al.*, 1999). Rodriguez

*et al.* (1999), for example, demonstrated the degradation of benzo(a)pyrene by *Pleurotus ostreatus*. Bogan and Lamar (1999), Kotterman *et al.* (1999) and Novotný *et al.* (1999) have also demonstrated the degradation of a wide range of PAHs, including high molecular mass compounds, by the same organism.

The non-basidiomycete soil fungi ( *Penicillium, Cladosporium, Aspergillus* and *Fusarium* ) used in this study, along with *Pleurotus,* have been shown previously to degrade PAHs. Launen *et al.* (1994) showed the degradation of pyrene and benzo(a)pyrene by *Penicillium* and *Fusarium* species. *Aspergillus* has been reported to degrade pyrene and benzo(a)pyrene (Wunder *et al.*, 1994; Cerniglia 1997). The ubiquity and fast growth rate of *Aspergillus* species has made it possible for this fungus to adapt to a wide range of environments and to develop capacity to oxidize a broad spectrum of chemical substrates. However, the potential of members of this genus to oxidize pollutant compounds has not, as yet, been fully exploited.

From the results (Fig.8.7), pyrene, fluoranthene and benzo(a)pyrene were more actively degraded (from 155mg $kg^{-1}$, 168mg $kg^{-1}$ and 53mg $kg^{-1}$ to 13.2mg $kg^{-1}$, 13.8mg $kg^{-1}$ and 3.2mg $kg^{-1}$, respectively) by the organisms than chrysene ( from 85mg $kg^{-1}$ to 12.8mg $kg^{-1}$) (Figure 8.7). This difference in level of oxidation is a function of their initial concentration as well as their physical and chemical properties, which include their water solubility, ionization potential and adsorption and desorption properties (Alexander, 1999; Eriksson *et al.*, 2000). Differences in their molecular mass and the arrangement of their ring structures may also be implicated in their different susceptibilities to degradation by the same group of microorganisms under the same environmental conditions. To

a greater or lesser extent, higher molecular mass PAHs have been oxidised by lignolytic and non-lignolytic fungi in soils in both field and laboratory conditions (Wiseman and Wood,1979; Pothuluri *et al.*,1990;1992a & b;1993;1995; Wunder *et al.*,1994; Sack and Fritsche,1997; Sack *et al.*,1997). This potential remained poorly investigated, however, particularly in the areas of nutrient requirements of the fungi and hydrocarbon concentration in the environment.

### 8.3.5 Changes in pH in the soil reactors during treatment

The initial soil pH in all the reactors ranged between 3.8 and 4.9. Apart from occasional slight fluctuations, the pH generally tended to rise (Figure 8.8, 8.9 and 8.10). Changes in pH in Treatment 1 were erratic, measuring 5.3 on day 14, falling to 4.9 by day 28, rising again to 5.9 by day 56 and falling again to 5.7 on day 70 (Figure 8.8). These changes may be due to the production of acidic intermediary metabolites and their subsequent oxidation. They could also be due to changes in the concentration of enzyme produced during the course of the growth of the fungi (Clemente *et al.*, 1999). Treatments 6 and 15 showed a slight decrease in pH, followed by a gradual increase. These two treatments were not inoculated with any fungi, but fungal growth was detected in Treatment 15 during the later stages of the experiment. The initial fall in pH could be attributed to changes in the physical structure of the soil, release of the contaminant hydrocarbon and possibly solubilization of some of the hydrocarbons in the soil at the beginning of the incubation period. Treatments 4, 6, 10, 11, 12, 13, 15 and 18 showed increases in pH until the end of the incubation period. Treatments 1, 2, 5, 7, 8, 9, 14 and 16 decreased slightly between day 55 and 70. These decreases could be due to intermediary products and their oxidation, as discussed previously. The highest pH value reached was less than pH 6.6 and was observed in Treatment 11, which was treated with nutrients and

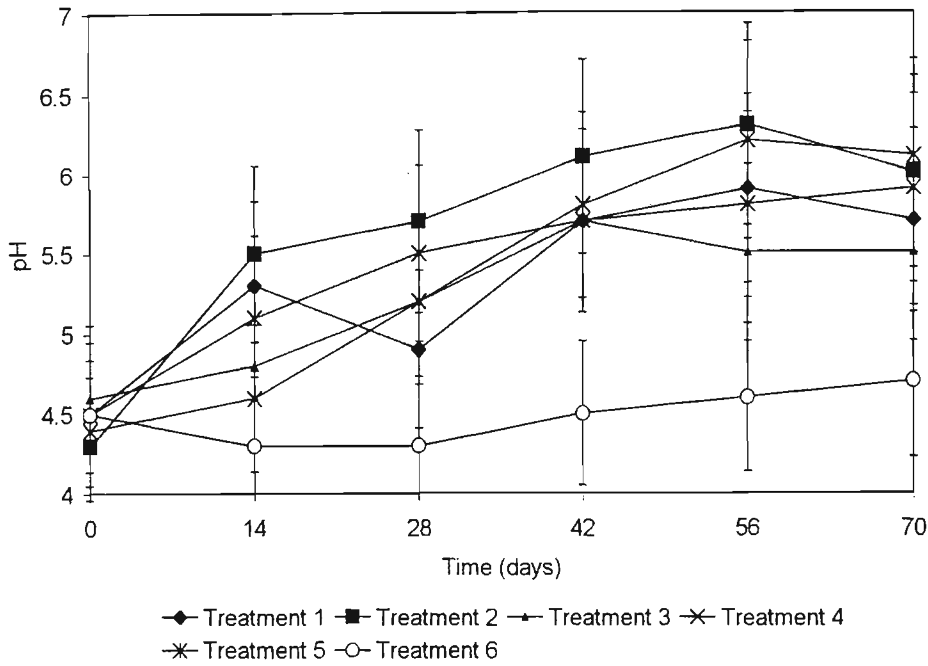



Fig.8.8 Changes in pH of soil in Treatments 1-6 (Treatments without nutrient supplementation). Values are means of two ± 1 Standard Error.

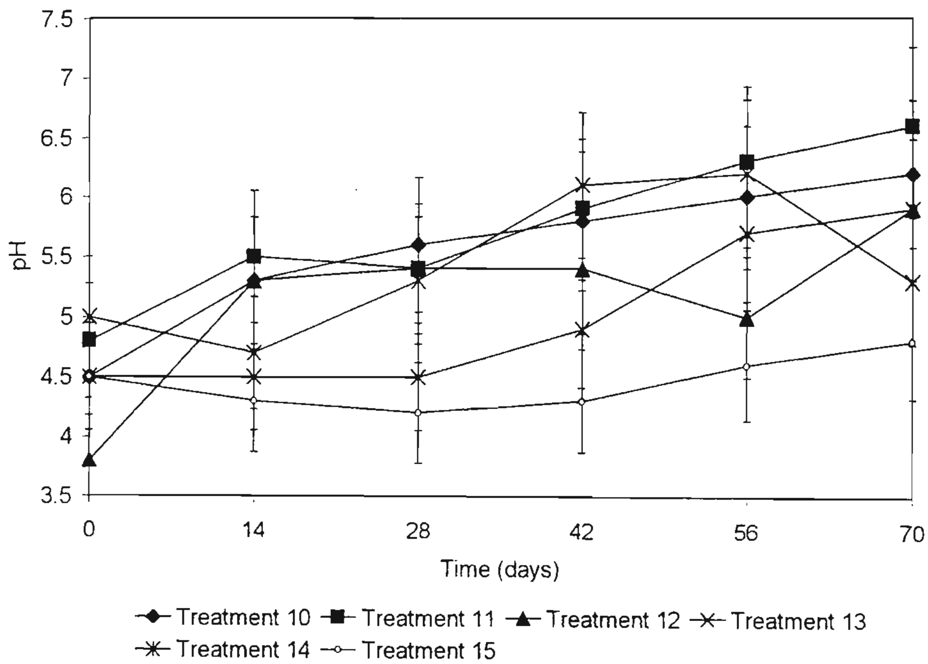



Fig.8.9 Changes in pH of soil in Treatments 10-15 (Treatments with nutrient supplementation). Values are means of two ± 1 Standard Error.

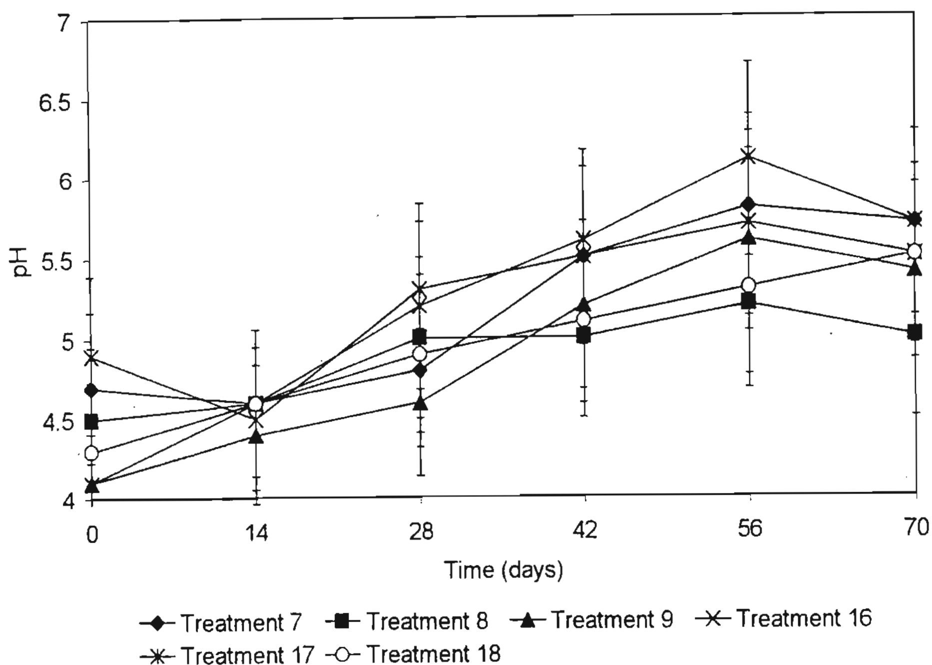



Fig.8.10 Changes in pH of soil in Treatments 7, 8, 9, 16, 17 & 18 (Treatments with no fungal inoculation and those with mixed fungal inoculation). Values are means of two ± 1 Standard Error.

inoculated with *Fusarium*. The general increase in pH is attributed to complete oxidation of some of the creosote components present in the soil leaving the soil, with a relatively less acidic medium than it was before treatment. Many fungi have been reported to thrive in acid media (Baker and Herson 1994). Thus the pH range observed in this study is within the pH limits for growth of many fungi. The pH fluctuations occurring during the study did not appear to affect the growth of the organisms and the degradation of creosote or its components. The effect of pH on the degradation of pollutant hydrocarbons has not been extensively studied. However, it is possible that soil type, the chemical(s) under survey and a number of other physico-chemical factors will influence the pH of the system over time.

ANOVA carried out on the changes in creosote concentration with time showed that Treatment 18

(which received barley and nutrient supplements and was inoculated with all the fungal isolates) was the most effective in removing creosote from the soil. There was no significant difference (p 0.05) between this treatment and Treatment12 (which received barley, *Penicillium* and nutrient). Treatments 10, 11 and 14 were ranked in the order as next best in the removal of creosote from the soil. There was no significant difference in the performance of these treatments, but they were found to be significantly different from Treatments 18 and 12, and from Treatments1, 2, 5, 7, 13, 16 and 17, which were all less effective. Treatments 3, 4 and 15 (control 4) were not significantly different from each other. The control reactors 6, 8, 9 and 15 were least efficient in the removal of creosote from the soil (Fig.8.1-7). However Control 4 (Treatment 15) was similar in performance to Treatments 3 and 4, which were inoculated with *Penicillium* and *Aspergillus* respectively, but did not receive any nutrient supplementation. This similarity in performance could be attributed to the fact that nutrient availability and the presence of barley as an additional carbon source in Control 4 could possibly have enhanced fungal growth and consequently enhanced the degradation of creosote in the soil.

Ranking carried out by the Duncan's New Multiple Range Test, at 0.05 significance, showed a similar result, except that most of the treatments were grouped together into a midrange and an upper and a lower performance group, with far fewer treatments. Treatments 18 and 6, however, were again shown to be the most and the least efficient, respectively.

## 8.5 CONCLUSION

Fungi have considerable potential for the degradation of creosote compounds in soil. It was established that the white rot fungus, *Pleurotus,* is a better creosote degrading organism than the non-basidiomycete fungi. Although all the pure cultures actively degraded creosote, mixed fungal cultures were more effective. The *Pleurotus* sp. isolate obtained from the creosote-contaminated environment showed stronger degradative capabilities than the isolate obtained from the uncontaminated forest soil. The *Aspergillus* isolate had the least degadative potential of all the fungi studied.

The provision of an additional carbon source, in the form of barley grains, effectively supported the growth of all the fungi with a concomitant increase in the degradation of the hydrocarbons. Soils not supplemented with barley grains supported less fungal growth and creosote degradation.

Nutrient supplementation was effective in enhancing both the growth of the fungi and the degradation of creosote. Although the difference between the nutrient-supplemented soil and the non-supplemented soil was not significant in some instances (for example, Treatments 4 & 13and 16 & 17), significant differences were observed in other treatments.

Soil tilling, which increased aeration, was also found to be advantageous to the growth of the inoculated fungi. This is important for the wide distribution of the enzymes responsible for the oxidation of the hydrocarbon substrate(s).

The higher molecular mass polycyclic aromatic hydrocarbons (PAHs) were more effectively degraded by the fungi (Fig.8.7) than by the bacteria (Fig.7.11, 12 and 15). Pyrene, fluoranthene and benzo(a)pyrene were more susceptible to fungal degradation (93.8, 93.9 and 96% removal respectively) than chrysene (91.2% removal), in spite of the fact that the latter compound was recovered at a lower concentration (7.5mg $kg^{-1}$) than pyrene and fluoranthene (9.6mg $kg^{-1}$and 10.2mg $kg^{-1}$ , respectively). The lower molecular mass PAHs were also found to be more susceptible to fungal than bacterial degradation. Anthracene, in spite of its relatively low water solubility, was well-degraded by all the fungi investigated. Apart from p-cresol, which was only poorly degraded, all the other phenolic compounds tested were degraded completely. Although there are no government-set standards for such compounds in soil in South Africa, many of the compounds have been removed to levels below the recommended standards of countries such as the USA and the Netherlands.

The low pH of the experimental soil inhibited neither the growth of the fungi nor the oxidation of the creosote compounds investigated. Although a general increase in pH was observed in all the treatments during the period of study, in no instance did the pH rise above 6.6. Thus low pH did not inhibit the growth of the organisms and did not appear to interfere with the degradation of the hydrocarbon substrate(s) tested.

### 8.6 Drawbacks and Recommendations

The major problems envisaged in the large-scale application of fungi for bioremediation is the production of large amounts of inocula and their long-term storage in viable conditions in order to ensure continuous spread of the fungi through the contaminated soil to be treated. It is therefore

recommended that methods for bulking and continued sustenance of fungal cultures on a large scale should be established before fungal bioremediation can be a viable option. In an outdoor system, the large-scale production of fungal spores may also present problems if not contained, since the presence of large numbers of spores in the atmosphere could constitute a health hazard. It will therefore be important to consider the control of sporulation and spore release during a large-scale bioremediation programme using fungi.

# CHAPTER 9

# FULL-SCALE LAND-FARMING OF CREOSOTE-CONTAMINATED SOIL

## 9.1 INTRODUCTION

The identification of 78 sites contaminated with chemical pollutants in South Africa in the early 1990s, many of which showed evidence of groundwater pollution, triggered research on techniques to remediate contaminated sites (Pearce *et al.*, 1995). Bioremediation techniques, particularly landfarming and vapour extraction, have been used successfully in South Africa and have been reported to be cost effective (Pearce *et al.*, 1995). In spite of the success of bioremediation in South Africa, Pearce *et al.* (1995) observed that degradation of pollutants in soil is generally slow and needs to be accelerated. They suggested that nutrient additions are likely to greatly enhance degradation. They noted that the establishment of a critical permissible level for various contaminants in South Africa would certainly promote the clean-up of contaminated sites.

The application of techniques to reclaim contaminated sites has developed differently in various parts of the world, depending on a range of factors influencing the economics and feasibility of clean-up. These include political, legislative, economic, social and environmental factors, all of which are interdependent to varying degrees. The integration of different technologies to optimize remediation programmes has become popular among remediation scientists and engineers (Ellis, 1992). Emphasis is, however, placed on tailoring a specific remedial scheme towards achieving a

cost-effective solution (Ellis, 1992).

A historical description and physical characterization of the site has been provided in Chapter 2. Based on reports from earlier laboratory and pilot-scale studies carried out on the contaminated site, and a cost analysis (as described in the materials and methods section of this chapter), an integrated bioremediation option, including *in situ* landfarming, bioaugmentation and biostimulation was embarked upon.

## 9.2 MATERIALS AND METHODS

### 9.2.1 Determination of regulatory clean-up standards in South Africa

There are no national standards defining acceptability levels for creosote compounds in soil or water in South Africa. The Oil Industry Environment Committee and the Department of Water Affairs and Forestry *do* periodically set localized standards based on information relating to specific contaminated sites, but these have been mainly for petroleum and its products. The standards used in this study are based on those recommended by the South African Department of Water Affairs and Forestry (DWAF) (1996) for other pollutants and standards set for creosote components by the United States of America Environmental Protection Agency (USEPA) (1995).

### 9.2.2 Cost analyses

Cost estimation was based on the area of contaminated land originally earmarked for treatment. The experimental site contained approximately 4219 $m^3$ of creosote-contaminated soil. This value was calculated as a land area 125 m by 75 m to a depth of 0.4 m. Cost estimation was carried out for three clean-up options to determine and justify the cost-effectiveness of a bioremediation option. The options included: (1) Excavation, disposal and backfilling with clean soil. (2) burial of contaminated soil on site; and (3) boiremediation by landfarming. The estimates included cost of labour, hiring of earth-moving equipment, acquisition of a landfill site, excavation of the landfill site, lining of landfill pits with protective materials to avoid leachates contaminating ground water, excavation and transport of contaminated soil, backfilling, post-filling monitoring, construction/preparation of landfarming treatment units, tilling, watering, fertilizers, laboratory analyses and administrative costs.

### 9.2.3 Extent of contamination

A preliminary tour of the facility, soil analyses and interviews with the factory operators indicated that contamination was limited to areas where wood treatment operations are, or had been, conducted. The preliminary survey of the factory site revealed three major areas of contamination. These included: (1) stacking lots for treated wood which covers a large proportion of the factory site; (2) an area of approximately 80$m^2$ surrounding the discharge systems for transferring creosote from tankers to the storage tanks inside the factory wood treatment building and (3) an area of

approximately 80m$^2$ surrounding an outside storage tank located about 150 m from the main factory building.

In each of the three areas, seven soil samples were taken randomly to a depth of approximately 35cm. The samples were bulked and thoroughly mixed in an electric concrete mixer before use. The bulk samples were taken to an independent analytical laboratory where they were analysed for total creosote and selected creosote component compounds by the EPA 418.1 method (USEPA, 1982).

### 9.2.4 Site preparation for the landfarming

Factory information revealed that the level of the groundwater at the site was below 300 m. Creosote contamination was found to be restricted to the top 30-40 cm of soil. Beneath this layer of topsoil a thick layer of shale and clay formed a continuous barrier to downward infiltration of substances. The upper layers of the shale (about 5-10 cm) were found to be impregnated with creosote in some areas where the soil layer was shallow. However, nowhere were the deeper layers of shale found to be contaminated. For this reason, the top 10cm of the shale layer was excavated and tilled along with the contaminated top soil. This had the advantage of providing the bulking needed to increase aeration. The risk of groundwater contamination was minimal and run-off was very unlikely, as the site was relatively flat. There was no body of surface water within five kilometres of the factory site. Thus it was decided to use an *in situ* landfarming/landtreatment approach, in which the shale-clay layer was prepared to prevent any further downward infiltration of creosote or leachate from the soil.

The soil was tilled with a ripper to a depth of 50cm to mix the contaminated top layer of the shale with the top soil. Samples were taken from below the tilled depth and analysed to ensure that the underlying layers of shale were not contaminated with creosote. The treatment bed was prepared by amending the exposed surface of the shale with more clay. The clay was mixed with water and allowed to dry before the excavated soil was spread over it for treatment. The soil was then ploughed repeatedly to ensure proper mixing of the soil.

A 5m x 20m section of the contaminated site was set aside as a control. This section was not ploughed nor treated with any nutrient supplement throughout the 11 months of the experiment. For logistic reasons, samples from this section were taken only at the beginning and at the end of the treatment period. The samples were collected and processed, as described for the treated plots, and used mainly for analysis of total creosote in the soil.

### 9.2.5 Bioremediation treatments

*i. Aeration*

Aeration was achieved by thorough ploughing the soil once every two weeks. Ploughing was done repeatedly each time to ensure proper mixing of the soil. Care was taken to avoid further ripping of the undisturbed underlying shale layer.

*ii Moisture*

Water was added to the soil by means of a PVC hose fitted with a spray nozzle. Watering was done weekly in the dryer months of the year (May to August). During the rainy months (September to March), watering occurred only during prolonged dry spells. Soil moisture was maintained at ± 70% of field capacity throughout the 11 months of landfarming.

*iii pH*

The pH of the soil was adjusted to approximately 7.0 by spreading dolomitic agricultural lime at a rate of 2 500 kg $ha^{-1}$ over the experimental plots and ploughing to a depth of ± 45 cm. Soil pH was measured monthly and lime was added to maintain the pH at ± 7.0.

*iv Nutrients*

Mono-ammonium phosphate (MAP) (11% (w/w) nitrogen and 22% (w/w) phosphate) was added to the soil at a rate of 2 000 kg $ha^{-1}$ at the onset of the landfarming and another 1000 kg $ha^{-1}$ was applied at the end the fifth month of treatment. After each application, the fertilizer was ploughed into the soil to depth of about 35 cm. Sewage sludge, 500 kg, was added to the soil as a carbon source at the end of the second month of treatment. The delayed addition of sewage sludge was to allow time to assess the effects of MAP on the growth of microorganisms and the degradation of creosote. The sludge was mixed thoroughly into the soil by ploughing. The sewage was obtained from the waste

water treatment plant in Hammarsdale, KwaZulu-Natal, South Africa. The same amount of sewage was again added during the seventh month of treatment.

*v. Sampling*

Samples were taken every 30 days to a depth of 30 cm using a cylindrical pipe with a diameter of 50 mm. The site was divided into a predetermined grid system and sampling points were predetermined within the units, that is the site was divided into six square plots designated 1,2,3,4,5 and 6, each measuring about 12.5 $m^2$. Each plot was further subdivided into smaller subunits measuring about 4 $m^2$ each. This pattern was used to facilitate uniform sampling across the study area. Samples were collected from each subunit and bulked to give a composite sample for the plot. This pattern was used for all six plots. The soil samples were transported to the laboratory and stored at $4^{O}C$ until required.

### 9.2.6 Analytical Methods

Plate counts of total heterotrophic microorganisms and hydrocarbon degraders were made on nutrient agar and oil agar, respectively, as described in Chapter 3. Total creosote concentration was determined by the EPA 418.1 method (USEPA, 1982), as described in Chapter 6. Determination of concentrations of selected creosote compounds was done by GC/FID. The same two temperature regimes described in Chapter 8 were used to facilitate the detection of all the compounds selected, including the low molecular mass compounds with low boiling points.

The pH of the soil was determined by weighing 10 g of air dried and sieved (<2 mm) soil into a beaker, adding 25 ml distilled water and stirring for about 1 min. The mixture was allowed to stand for 1 hour, after which it was stirred again for a few seconds and the pH measured (Foster, 1995) with a Crison Micro pH 2000 pH meter equipped with a glass electrode and a built-in reference electrode.

The water holding capacity of the soil was determined on duplicate 20 g field-moist soil samples in funnels lined with folded Whatman 2V filter paper on the inside and mounted on pre-weighed 250 ml flasks. Distilled water (100g) was added in small volumes to the soil. The funnels were covered with aluminum foil to prevent evaporation and allowed to stand overnight. Water adhering to the neck of the funnel was released into the flask by tapping the neck of the flask. The collecting flask was weighed to determine the mass of the collected water. Blanks containing only filter paper were run in duplicate to determine how much water was taken up by the filter paper. The water content of the soil was determined by transferring the soil to a pre-weighed wide-mouthed heat resistant beaker and drying in an oven at 105°C overnight. The beaker was cooled in a desiccator and reweighed to determine the dry mass of the soil. Percentage water holding capacity was calculated using the following formula:

$$\% \text{ water holding capacity} = [\,(100-Wp) + Wi]\,/dwt \times 100$$

where $Wp$ is the mass of the percolated water in grams, $Wi$ is the initial amount of water in grams contained in the sample and $dwt$ is the soil dry mass in grams (Foster 1995).

Carbon dioxide evolution and oxygen consumption were determined by soil respiration experiments.

Duplicate 30 g field-moist soil samples from the experimental site were placed in beakers inside glass jars. A plastic vial containing 20 ml of a 0.1 M sodium hydroxide solution was suspended from a tripod in each jar. The jars were tightly closed and incubated for 3 days at room temperature. The vials' contents were transferred to flasks and 2 ml of barium chloride solution added. To each flask was added 3-4 drops of indicator (phenolphthalein) solution and the mixture titrated with 2 M HCl. Controls were set up identically but without soil. Results were calculated as follows:

$$\mu g\ CO2 - C/\ g/\ day = V_{sample} - V_{blank} \times 2.2 \times 0.27/\ dw \times day \times 1000$$

Where $V_{blank}$ is the volume of HCl for the blank, $V_{sample}$ is the volume of HCl for the sample, 2.2 is the conversion factor (1 ml 0.1 M NaOH = 2.2 mg $CO_2$); 0.27 is mg $CO_2$ - C and *dw* is dry mass.

## 9.3 RESULTS AND DISCUSSION

### 9.3.1 Determination of regulatory clean-up standards

After discussions with the factory owners and officials of the DWAF failed to come up with an officially permissible level for creosote contamination, a clean-up target level of 2 000mg $kg^{-1}$ was established, based on the fact that the site was classified as industrial and was unlikely to be required for agricultural purposes or human occupation in the foreseeable future. The decision was also based on the fact that there was no groundwater or surface water contamination risk in the immediate vicinity of the facility. A third factor in selecting this clean-up target is the fact that DWAF had previously recommended a similar target concentration for similar hydrocarbons in an industrial area (Snyman, 1996). No published set standards for the selected creosote compounds that were studied

are available. However, site remediation levels for individual PAHs have been set previously at between 2 000 mg $kg^{-1}$ for light contaminations and 5 000 mg $kg^{-1}$ for heavy contaminations in industrial soils, depending on the region and the local authorities involved (Di Leo *et al.*, 1999).

### 9.3.2 Cost analysis

The results of cost analyses showed that bioremediation was the most cost-effective method of remediation considered. The operational costs of implementing the different remediation technologies considered are as follows:

(1) Excavation, disposal and backfilling: R550 000

(2) Burial on site: R280 000

(3) Bioremediation: R57 000

### 9.3.3 Extent of contamination

The three major areas from which soil samples were taken were polluted with different concentrations of creosote: (1) Contaminations of the stacking lots for treated wood (Plate 2.1 & 2.2; See Chapter 2) were mainly due to the creosote dripping from the treated wood which was spread out in rows to dry. Contamination in these areas averaged about 250 000mg $kg^{-1}$ ; (2) The area surrounding the discharge systems for transferring creosote from tankers to storage tanks inside the factory wood treatment building was contaminated mainly through direct leakage from the discharge systems. At this site creosote contamination exceeded 300 000mg $kg^{-1}$ ; (3) Examination of the area

surrounding an outside storage tank, located about 150m from the main factory building (Plate 1a ), showed that the tank had a containment area of about 1 m around it, within which unweathered creosote could be seen in a large pool. Large amounts of congealed creosote were found within a radius of 5m of the tank (Plate 1b). This waste is believed to have been removed from the containment area and spread out on the surrounding land. Creosote concentration in the soil in this area averaged more than 380 000mg $kg^{-1}$ .

The area originally chosen for the landfarming operation was an expanse of land measuring 125 m by 75m which had been used for stacking the treated wood. However, while laboratory experiments were still under way, the factory changed hands and full operation was resumed. Thus by the time the laboratory-based results had been analysed the land earmarked for the landfarming was no longer available.

The replacement site selected for the landfarming operation was less than half the size of the original site and was located at another section of the premises dedicated to the stacking of treated wood. The creosote levels thus had to be determined again to measure the extent to which they had increased over the period of renewed activity. Creosote concentration levels had risen to 310 000mg $kg^{-1}$ in the top 30 cm of the soil before treated wood was removed from the plot, which measured about 40m by 25m.

### 9.3.4 Landfarming

*i. Moisture*

The mean moisture content (m/m) of the soil varied from 16.9% to 24.0% (Fig.9.1). This represented 52.8% to 75% of the water holding capacity (field capacity)of the soil, which was determined to be 32 % (m/m). This relatively high water retention capacity is attributed to the clay content of the soil (18.75%). The water was advantageous to the activity of microorganisms in the soil as microbial counts were seen to increase rapidly during the treatment period.

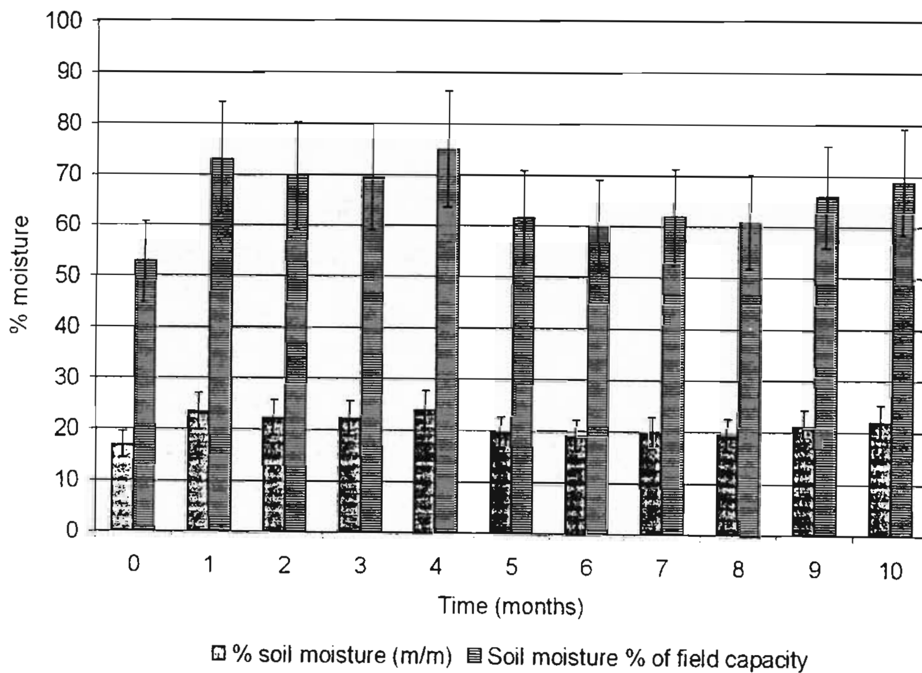



Fig.9.1 Soil moisture during full-scale landfarming. Error bars indicate ± 1 Standard Error.

Biodegradation of contaminants is dependent on an adequate supply of water within the soil environment. Water is necessary not only to meet the physiological requirements of microorganisms

but also for the transport of nutrients and metabolic by-products to and from the microorganisms and to determine the oxygen status of the soil micro-environment (Baker and Herson, 1995).

A water content of between 50 and 80 % of field capacity has been reported to be favourable for biodegradation of organic contaminants in soil (Alexander, 1999; Tate, 1995; Dibble and Bartha, 1979; Riser-Roberts, 1992). Optimal moisture levels for degradation of organic contaminants in the soil, however, vary from one soil type to another (Baker and Herson, 1995)

In the landfarming operation in the present study, the water content of the soil was measured regularly and over watering was avoided, because excess moisture can lead to the development of anoxic conditions which can be limiting for creosote biodegradation

*ii. Aeration*

Aeration through tilling enhanced microbial growth, as evidenced by increased microbial counts following ploughing, excavation and spreading (Fig.9.2). The increase in microbial activity was reflected as an increase in respiration rate (Fig.9.3) and a consequent decrease in concentration of the contaminant hydrocarbons (Fig.9.4). Oxidation of the contaminant hydrocarbon molecules requires oxygen (Gibson and Subramanian, 1984). The presence of adequate amounts of oxygen within the soil is therefore essential for bioremediation. The aerobic microorganisms responsible for degradation of organic contaminants in the soil require oxygen as their electron acceptors. In general, a minimum of 10 % air-filled pore spaces is necessary to maintain adequate aeration for aerobic microbial activity (Paul and Clark, 1989; Forth, 1984).

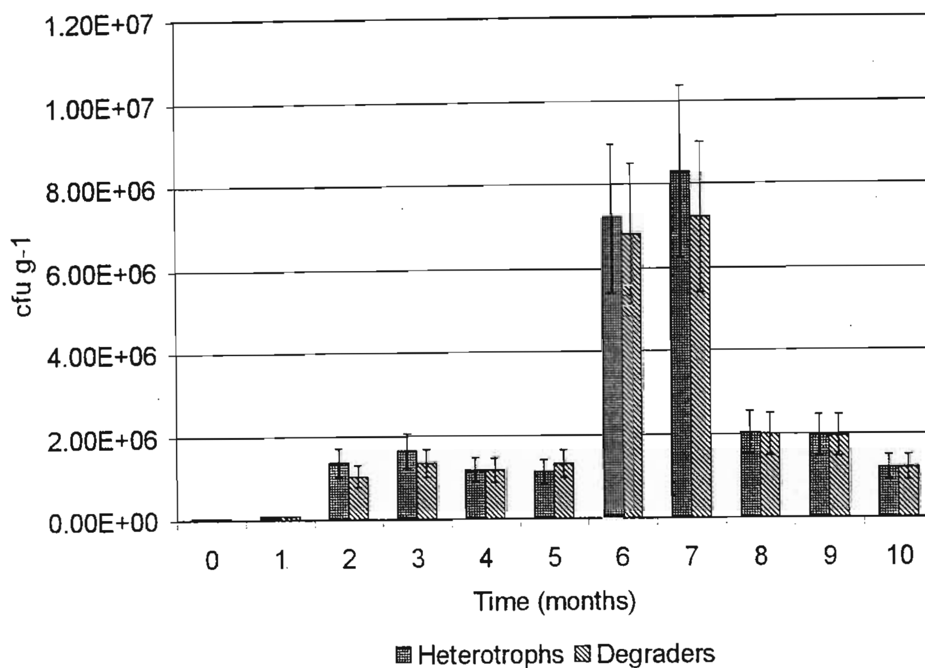



Fig.9.2 Counts of microorganisms in soil during full-scale landfarming. Error bars indicate ± 1 Standard Error.

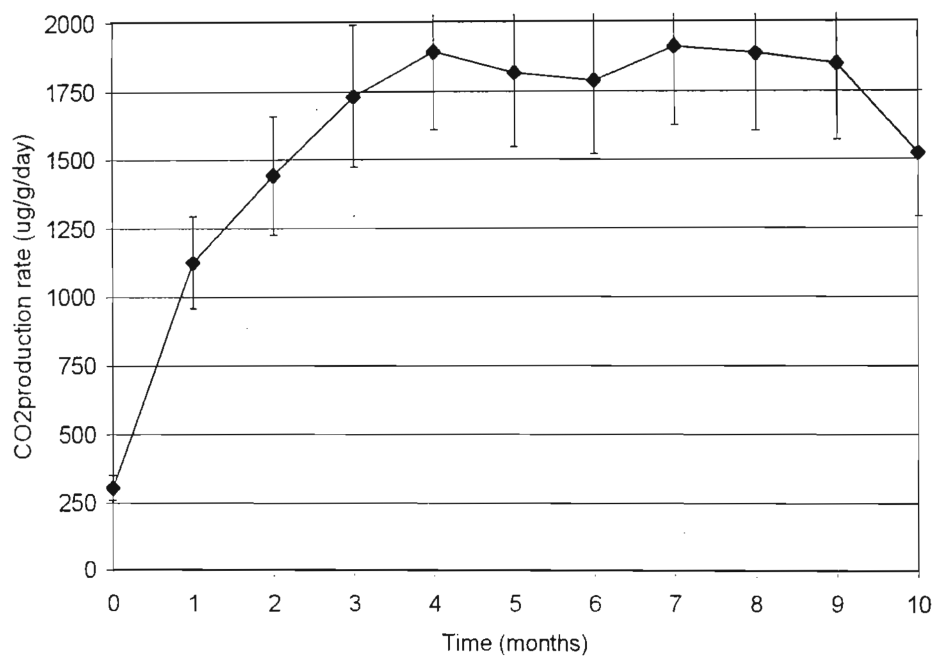



Fig.9.3 Monthly respiration rates of soil organisms during full-scale landfarming. Error bars indicate ± 1 Standard Error.

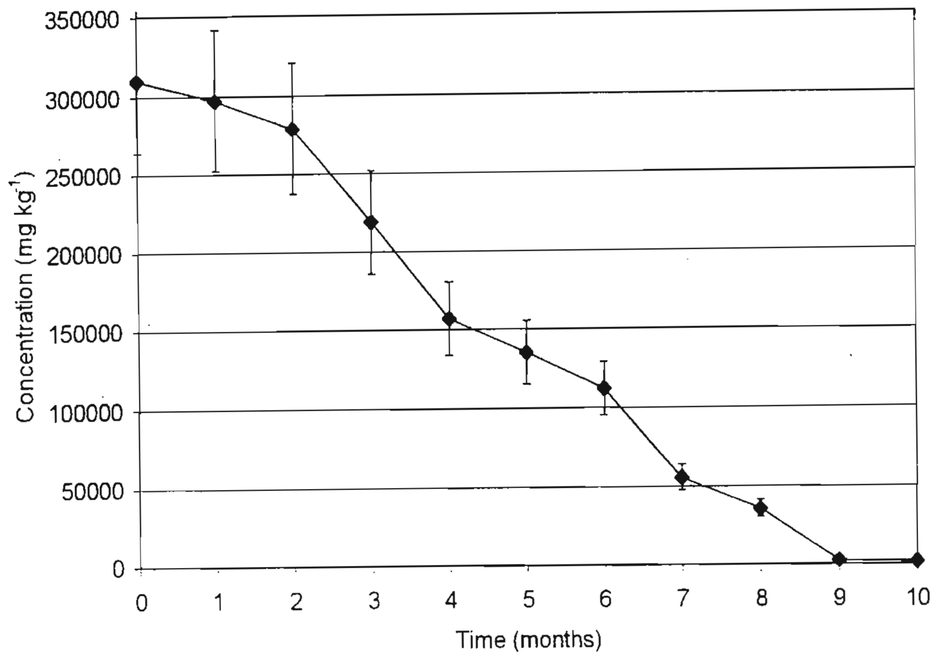



Fig.9.4 Changes in creosote concentration during full-scale landfarming.
Error bars indicate ± 1 Standard Error.

*iii. Soil pH*

The soil pH before treatment started was about 5.2 (Fig. 9.5). The pH was raised to 7.4 through the application of agricultural lime. Biodegradation of contaminants in the soil has been reported to be fastest at neutral or near neutral pH (Fu and Alexander, 1992; Dibble and Bartha, 1979; Verstrate *et al.*, 1976). As most soils in nature have an acid pH ( Baker and Herson, 1994) it has become customary in bioremediation projects to raise the pH to near neutral values (Baker and Herson, 1994). Verstrate *et al.*(1976) found that the adjustment of soil pH from acid conditions (pH 4.5) to near neutral conditions (pH 7.4) resulted in a doubling of the rate of biodegradation of gasoline in soil.

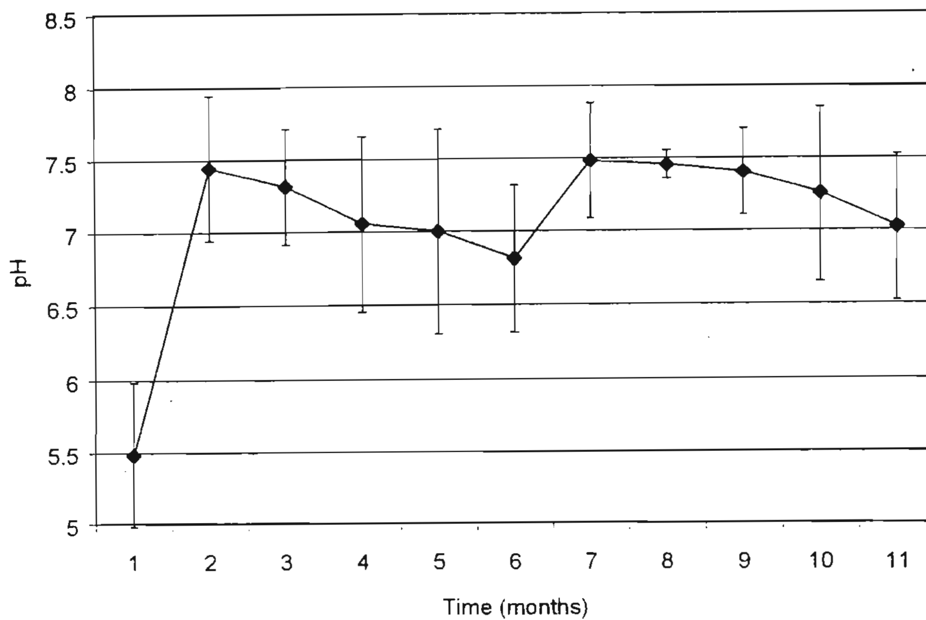



Fig. 9.5 Changes in soil pH during full-scale landfarming. Error bars indicate ± 1 Standard Error.

Apart from influencing the concentration of hydrogen and hydroxyl ions in the soil, the chemical form, solubility and bioavailability of essential soil nutrients such as N and P are also influenced by pH. Substances such as aluminium and manganese are less soluble at higher pH values, thus reducing the potential toxicity caused by these elements to the soil microbial community (Baker and Herson, 1994; Winningham *et al.*, 1999). This reduction in toxicity allows proliferation of organisms that would have otherwise been inhibited in such soils.

The pH of the soil during the period of treatment was maintained at close to neutral as this is widely reported to enhance biodegradation (Findley *et al.*, 1995; USEPA, 1998; Connolly *et al.*, 1999). The slight decreases in pH (Fig.9.5) observed during the treatment had no noticeable effect on the rate of creosote biodegradation.

*iv. Nutrients amendments*

Table 9.1 Nutrient concentrations before and after the addition of monoammonium phosphate (MAP) during the bioremediation period. Values are means ± 1 Standard Deviation.

| Time | Organic Carbon ($mg\ kg^{-1}$) | Total Nitrogen ($mg\ kg^{-1}$) | Extractable Phosphorus ($mg\ kg^{-1}$) |
|---|---|---|---|
| Before first nutrient addition | 45,000 | 600 | 5.5 |
| After first nutrient addition | 48,050 | 1,100 | 96.5 |
| Before second nutrient addition | 18,100 | 900 | 41.5 |
| After second nutrient addition | 21,000 | 1,600 | 88.6 |

These results show that the addition of MAP enhanced the nutrient concentration of the soil. The depletion of the nutrients between the first and second application of MAP was attributed to microbial utilization. These nutrients boosted the microbial population of the soil and concomitantly enhanced the utilization of the contaminant hydrocarbons (e.g. total creosote from 310 000mg $kg^{-1}$ to1762mg $kg^{-1}$). Nitrogen and phosphorus are required for growth of microorganisms. Essentially, N is needed for cellular protein and cell wall formation and P is needed for nucleic acid, cell membrane and ATP formation. Thus, for active growth of these microorganisms, an adequate supply of these elements is required.

Ammonia is readily assimilated by microorganisms (Hadas *et al.*, 1992). Even when other forms of

nitrogen are present, they are converted to ammonia or to ammonia ions before assimilation. Hadas *et al.* (1992), however, showed that more than one form of nitrogen can be assimilated concurrently. The soil pH range during the project also allowed for the solubilization of phosphorus (Baker and Herson, 1994).

Reports of the enhancement of biodegradation of xenobiotic compounds by amendment of soil with supplemental N and P abound in literature and biodegradation rates in soil have been shown to be stimulated by addition of N and P in different forms (Jamison *et al.*, 1975; Jobson *et al.*, 1974; Verstrate *et al.*, 1976; Thornton-Manning *et al.*, 1987; Harmsen, 1991; Song *et al.*, 1990; Block *et al.*, 1990). Leavitt *et al.* (1991), reported that even in the presence of significant initial concentrations of inorganic nutrients ($NH_4^+$ and P), microbial degradation of coal-coking wastes could be stimulated by supplementation with readily available nutrients such as N and P.

v. *Addition of sewage sludge*

The addition of sewage sludge in the third month of treatment resulted in an increase in the soil microbial population (Fig.9.2). There was a significant decrease in the concentration of creosote in the two months following addition of the sludge (Fig. 9.4). Although there was no significant decline in the microbial population, the rate of reduction in creosote concentration decreased from about 19 % over the first month to 7 % in the second month following the application of the sewage sludge (Fig.9.4). A second addition of sewage sludge in the sixth month again resulted in an increase in microbial population and a consequent enhancement of the rate of creosote degradation (Fig.9.4).

Sewage sludge has a high concentration of microorganisms adapted to growing on substrates of diverse origin (Vipulanandan *et al.*, 1994). The Hammersdale waste water treatment plant handles both industrial and domestic sewage. This sewage includes waste waters from factories handling dyes, fats and hydrocarbons. Microorganisms from the sludge were found to adapt readily to the creosote-contaminated soil during the pilot-scale experiment (Chapter 7). The addition of sewage as a nonspecific ancillary carbon source has been known to enhance biodegradation of organic pollutants in soil (Linkenheil, 1988; Stegmann *et al.*, 1991). It is not very obvious whether the enhanced biodegradation is a result of the additional carbon, microbial biomass or alterations in the properties of the soil. The effect of such additional carbon from an extraneous source can be explained when the carbon concentration of the contaminant is below the threshold level at which microbial growth can occur, thus encouraging mixed substrate utilization and biodegradation (Schmidth *et al.*, 1987).

On the other hand, supplemental carbon could reduce the per-microbe toxin concentration in the presence of high levels of toxic organic compounds by increasing the overall biomass (Baker and Herson, 1994). Brown *et al.* (1986) showed that the presence of a supplemental carbon source could increase degradation of hydrocarbons even at high autochthonous carbon levels. The addition of organic matter to soil promotes co-metabolism through analogue enrichment (Baker and Herson, 1994; Spain *et al.*, 1991; Keck *et al.*, 1989; Sims and Overcash, 1981), thus encouraging greater degradation hydrocarbons as a result of the multiple utilization of different substrates. It is also believed that non-acclimated sewage added to an organic medium will accelerate substrate degradation because the microbes have apparently been starved of such organic substrate (Vipulanandan *et al.*, 1994).

*vi.* *Contaminant reduction and microbial action*

Total creosote concentration in the soil and microbial plate counts during the full-scale landfarming are shown in Fig.9.2 & 9.4. The mean monthly decrease in concentration of total creosote was observed to accelerate slowly over the first two months of treatment (Fig.9.6). This was believed to be due to physical disruption of the soil, which released more of the contaminant hydrocarbon from the soil matrix and also to the acclimation of the soil organisms to the new oxygen environment. Although the increase in porosity increased aeration and moisture availability in the soil, the organisms needed some period to adjust to the new soil climate (Tate,1995; Alexander,1999).

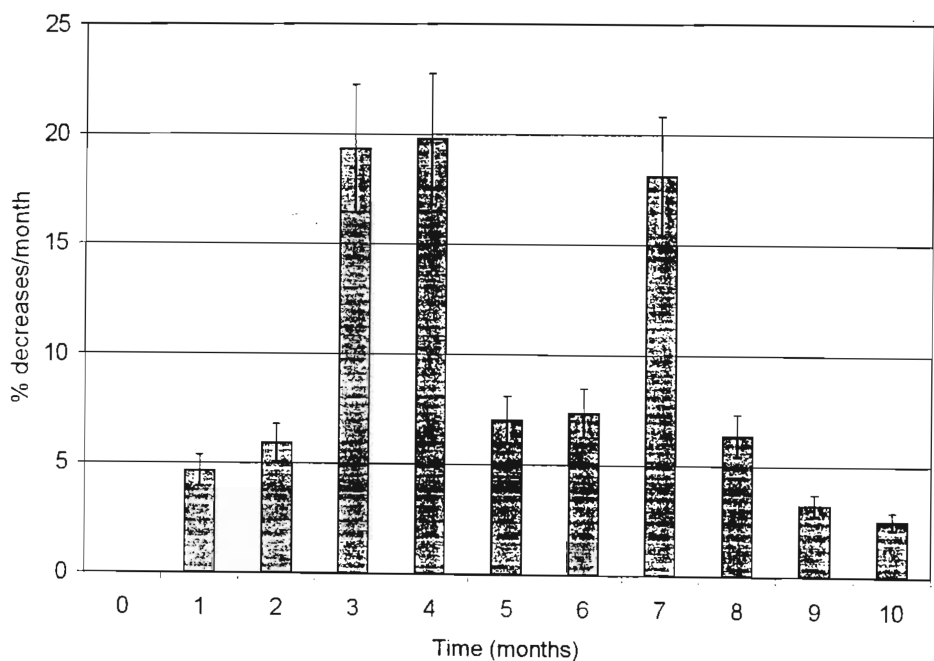



Fig.9.6 Monthly decreases in creosote concentration during full-scale landfarming. Error bars indicate ± 1 Standard Error.

The higher reduction in creosote concentration observed in the third and fourth months of treatment (Fig.9.6) was believed to be due to a number of reasons, including: (1) the soil organisms had by this time adapted to the new soil environment, as suggested earlier; (2) the microbial growth-enhancing effect of the fertilizer had stabilized; and (3) the supplemental carbon and additional biomass introduced in the form of sewage sludge combined to boost the population of microorganisms in the soil and hence increase the rate of utilization of the pollutant carbon substrate. As explained earlier in this chapter, the presence of supplemental carbon enhances the degradation of the pollutant organic carbon.

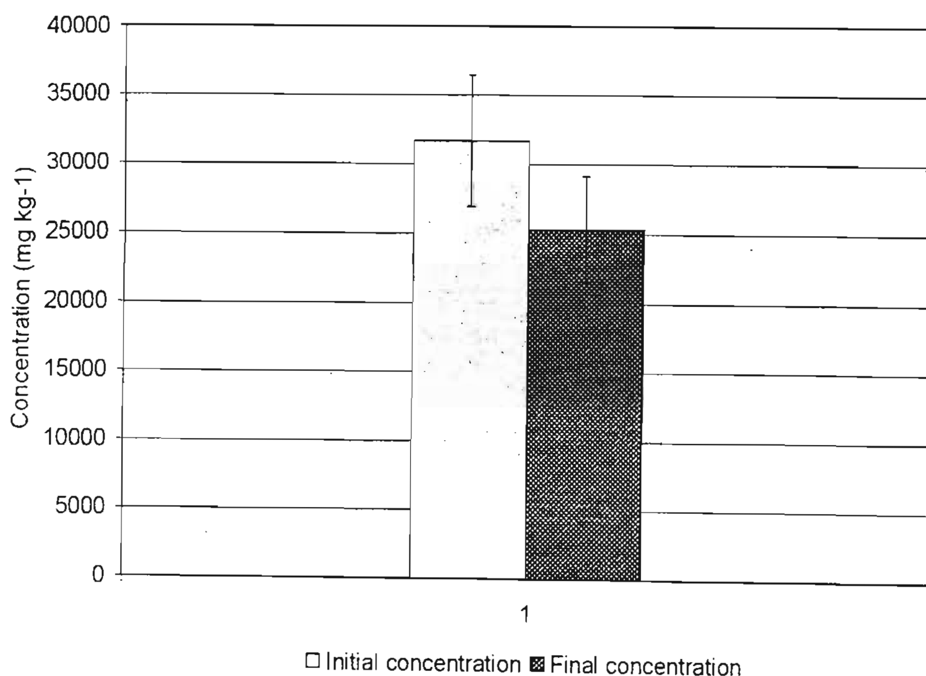


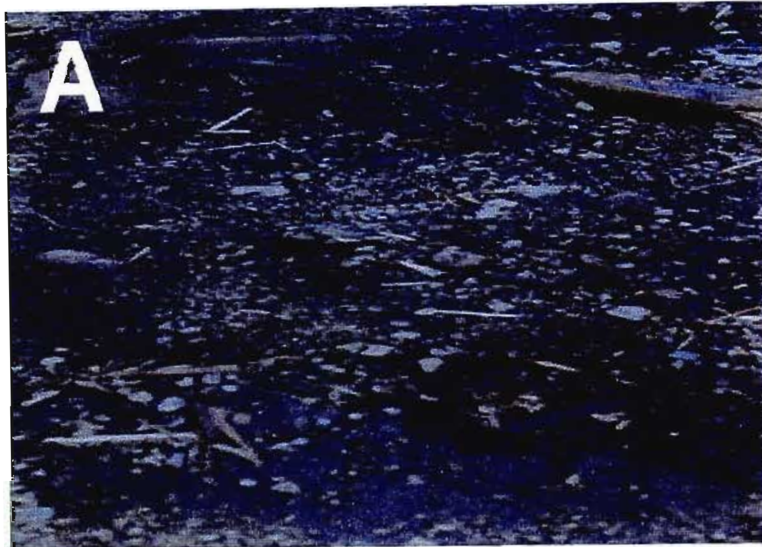

Fig.9.7 Change in creosote concentration in the control plot. Error bars indicate ± 1 Standard Error.

A comparison between the decreases in creosote concentration in the control and in the treated plots shows that these were significantly different. In the treated plots more than 90% of the creosote was degraded during the treatment period (Fig.9.4), whereas in the control plots just over 20% was degraded in the same period of time (Fig.9.7). This is a clear indication that the treatments applied to the soil were responsible for the faster rate of creosote degradation. It also shows how manipulation of creosote-contaminated soil can bring about faster restoration of the contaminated soil than is possible through natural attenuation. Plates 9.1 A, B & C shows the contaminated soil before, during and after landfarming for seven months. The growth of weeds in the soil by the seventh month is an indication of large reduction in the concentration of creosote in the soil. At this stage, a total reduction of >80% in creosote concentration had been achieved by the modified landfarming process.

Reduction in the concentrations of the creosote compounds studied is shown in Fig.9.8, 9.9 and 9.10. Reduction in concentration of phenol, o-cresol, m-cresol and naphthalene proceeded effectively, with the addition of only mono-ammonium phosphate (MAP), together with tilling and watering. These compounds were degraded to below detectable limits by the end of the third month, without the addition of sewage sludge. Without the addition of fertilizer (MAP) it is possible that some of these compounds would have degraded, but very slowly, as was reported by Findley *et al.* (1995). Thus the addition of MAP speeded up the degradation rate of these compounds.

As stated in Chapters 6, 7 and 8, the 1- and 2-ring compounds were readily degradable following addition of fertilizer. The removal of these compounds from the soil within relatively short periods

Plate 9.1 Creosote-contaminated soil at lanfarming site: (A) Before landfarming, (B) During landfarming, (C) After seven months of landfarming.



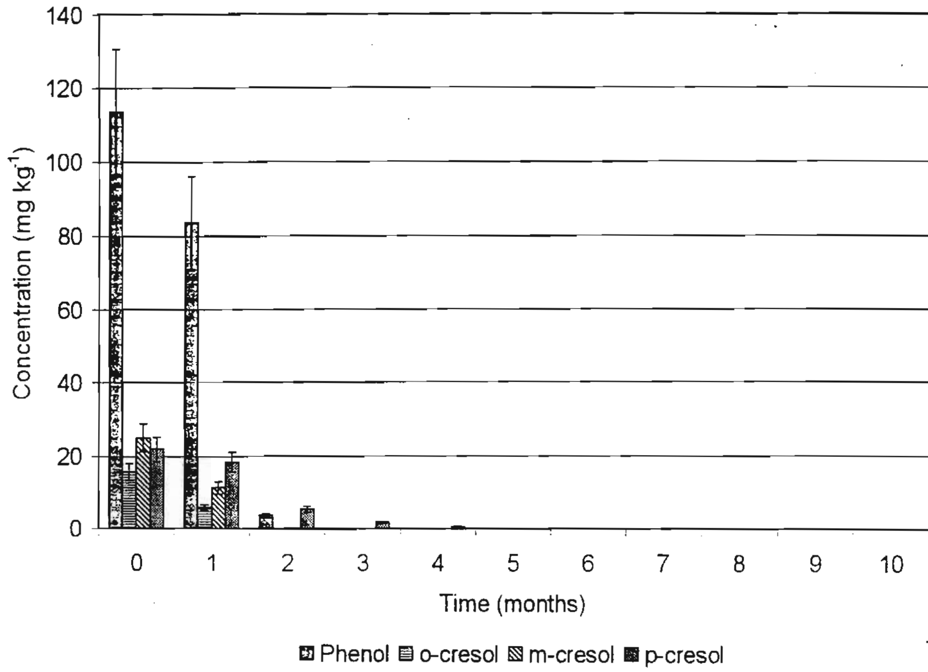



Fig.9.8 Changes in concentration of phenolic compounds during full-scale landfarming. Error bars indicate ± 1 Standard Error.

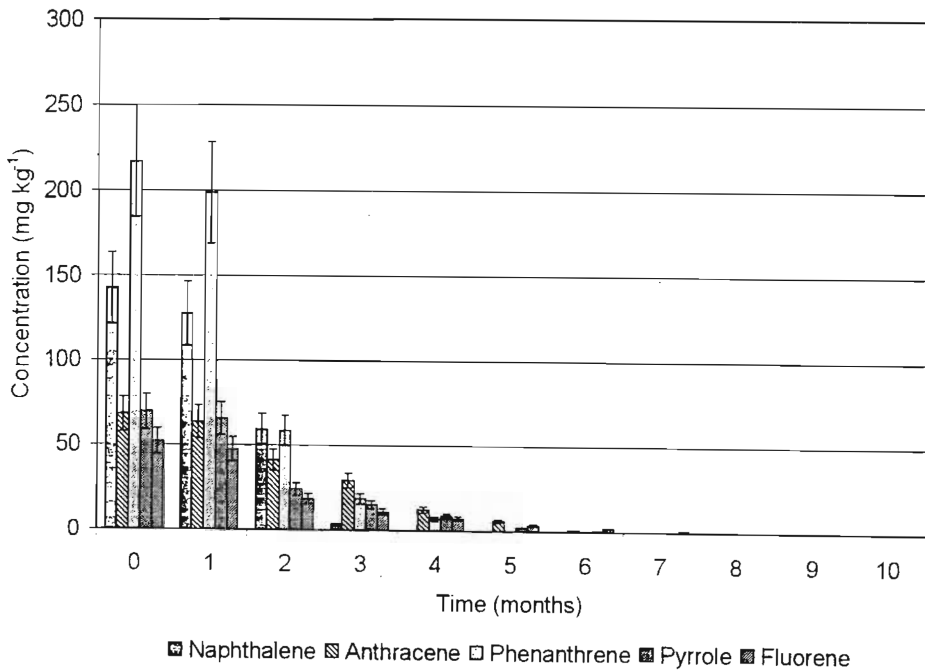



Fig.9.9 Changes in concentrations of lower molecular mass PAHs during full-scale landfarming. Error bars indicate ± 1 Standard Error.

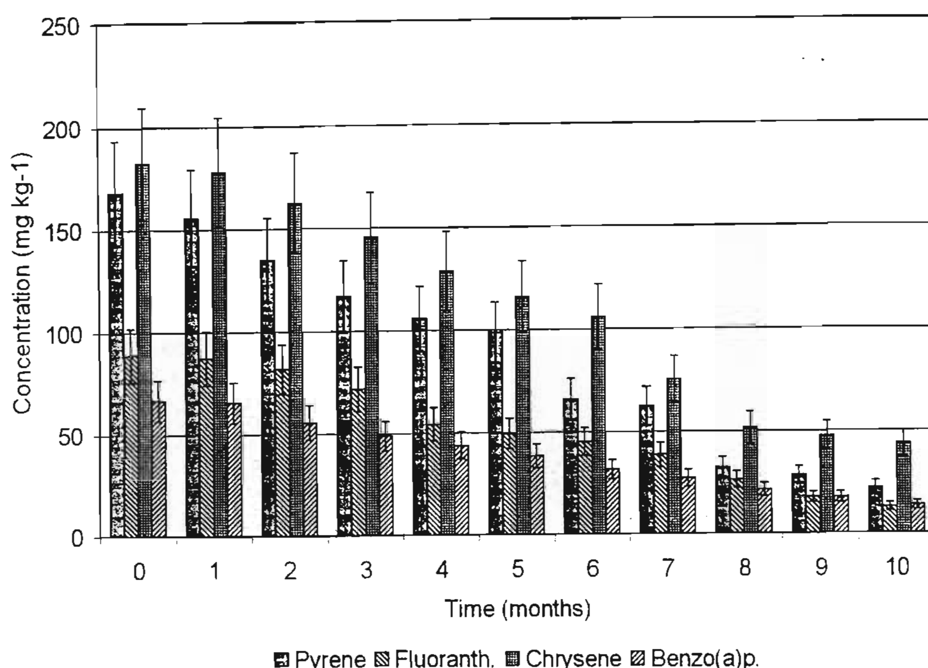



Fig.9.10 Changes in concentrations of higher molecular mass PAHs during full-scale landfarming. Error bars indicate ± 1 Standard Error.

(o-cresol and m-cresol in 2 months, phenol and p-cresol in 3 months and naphthalene in 4 months) (Fig.9.8 & 9.9) shows that soils contaminated by such compounds can readily be treated by landfarming. The complete removal of the 3-ringed PAHs from the soil varied according to the compounds. Phenanthrene was completely removed by the end of the fifth month. Anthracene and pyrrole were completely removed at the end of the seventh month, while fluorene was completely removed at the end of the eighth month (Fig.9.9). Although the addition of MAP accelerated the degradation of these compounds, the addition of sewage sludge had a greater enhancing effect on their degradation (Fig.9.6). Among this group of compounds phenanthrene was degraded most rapidly and was below detection limits by the end of the fifth month. The remaining 3-ring compounds were degraded below detectable limits by the end of the seventh and eighth months. High

levels of degradation of 3-ring PAHs in soil have been reported in the literature (Brown *et al.*, 1999; Eggen *et al.*,1999; Connolly *et al.*,1999). However, reports of complete degradation of high concentrations of these compounds, as observed in the present study are rare in the literature.

The higher molecular mass PAHs were generally more recalcitrant, persisting until the end of the eleventh month (Fig.9.10). As mentioned previously, molecular mass and the number of rings (which tend to determine solubility and bioavailability) affected the biodegradability of these compounds in the experimental soil. Although great levels of reduction in concentrations of the higher molecular mass compounds were achieved at the end of the landfarming, none of the compounds was completely removed from the experimental soil.

The microbial population was enhanced by tilling and liming, as can be seen in Fig. 9.2. Addition of MAP also increased the microbial population. However, the highest increase in microbial population was recorded after the addition of sewage sludge to the soil. Sewage sludge has been used by several workers as a source of carbon and microbial supplement for the bioremediation of contaminated soil (Stegmann *et al.*, 1991; Baker and Herson,1994; Vipulanandan *et al.*, 1994). In the present investigation, addition of sewage sludge raised the microbial population by several millions and these population increases corresponded with periods of high percentage decreases in the concentration of the target substrate.

Statistical analysis of the mean monthly reduction in total creosote concentration showed that there was no significant change between the degradation rates in the first and second months of the

experiment. This period corresponds to the period when only tilling, liming and addition of MAP was carried out. There was, however, a significant change in the degradation rates between the first two months and the next two months. Month 3 corresponds to the period when the first addition of sewage sludge took place. The fall in rate of creosote disappearance during months 5 and 6 is possibly due to depletion of the alternative carbon source derived from the sludge, thus leaving the organisms solely dependent on the contaminant for their carbon needs. Although acclimation to the contaminant carbon source was seen to have taken place, the depletion of the supplemental carbon source (sewage sludge) still presented a negative impact on the population. This decrease in microbial population negatively affected the utilization of the substrate. Further addition of sewage sludge resulted in an increase in the population of microorganisms and consequent increase in percentage monthly loss of contaminant carbon. This was quickly followed by a concomitant decline in microbial population and degradation rate of the substrate. This further decline in activity of the microorganisms and rate of creosote degradation is attributable possibly to a low level of substrate in the soil which reduced the overall availability of carbon to the microorganisms (Baker and Herson, 1994; Sutherland *et al.*, 1995).

### 9.3.5 Respiration of soil microorganisms during full-scale landfarming

Respiration rates of soil microorganisms during the full-scale landfarming operation are shown in Fig.9.3. In the first month, carbon dioxide evolution increased by more than four times the initial values recorded before the soil was tilled and limed. This increase, which correlated positively with the more than doubled microbial population size over the same period, was, however, parallelled by

only a slight decrease (4.65%) in the creosote concentration. This, as suggested earlier in this chapter, may have been due to the release of more creosote from the soil matrix, as a result of tilling, thus increasing the concentration of the contaminant in the soil. The concentration of contaminants in the soil are not generally uniform throughout the soil matrix as may be seen in water. Substances are known to be locked in the interstitial spaces of the soil (Tate,1995; Alexander,1999) and are only released as a result of activities such as tilling.

Subsequent increases in microbial respiration rate were of a relatively smaller magnitude and corresponded to the addition of MAP and sewage sludge. The corresponding increase in microbial counts and decrease in creosote concentration are shown in Fig.9.2 & 4, respectively. The decline in respiration observed in the fifth and sixth months is believed to be due to the depletion of carbon from the sewage sludge, which reduced the population of the non-creosote-degrading organisms present in the system. This is reflected in the drop in the counts of heterotrophic organisms over this period. Further addition of sewage sludge resulted in an increase in respiration rate and renewed loss of creosote. This was, however, not sustained for long, as a slight decline in respiration occurred over subsequent months.

Measurements of the respiration rates of soil microorganisms have been used in the study of aerobic biodegradation of contaminants in the soil, including the determination of biological oxygen demand (BOD), biokinetic rate constants, and treatability tests (Barbeau *et al.*,1995; Li and Zhang,1996). Biodegradation is a slow process in soil. The objective of bioremediation is thus the enhancement of microbial activity to such an extent that contaminants are degraded within the shortest possible

time. The correlation between respiration rate and contaminant removal rates will enable the quantification of the effects of nutrient amendment and bioaugmentation in the bioremediation process. From the results obtained, the effects of amendments and bioaugmentation can readily be estimated. Effects of autotrophic ammonium oxidation should, however, be taken into account when interpreting results (Snyman, 1996).

Statistical analysis of the results from the current experiments shows that the rate of creosote removal from the soil remained virtually constant during the last two months of treatment. There was, however, a significant difference (p=0.05) in the rate of creosote lost from the soil in successive months from the first month to the last month of treatment. For the first six months the respiration rate of the soil microorganisms during successive months showed significant differences. However, the respiration rates measured during months 5, 9 and10 were not significantly different from each other, and likewise the rates measured in months 6 and 7 were also not significantly different from each other. These periods correspond with periods of diminished microbial numbers (Fig.9.2). Microbial counts for months 1 and 2 were statistically similar but different from months 3 and 4, which were again similar to each other. Other months showed significantly different microbial counts, except months 8 and 9, which were different from the other months but similar to each other. This correlates well (p=0.05)with the creosote removal rates in which there was no significant difference in months 8 and 9 of treatment. The monthly rate of decrease in creosote concentration and microbial activity became relatively lower in the last three months of treatment (Fig.9.2 and 9.6).

### 9.3.6 Comparison of the results of the pilot and full-scale landfarming experiments

As discussed in Chapter 3, a number of reasons are responsible for the non-duplicability in the field of laboratory experiments. Most of these reasons could be related to the failure of laboratory-based experiments to fully account for the rate-limiting factors prevalent in natural soil systems (Autry and Ellis, 1992). In bioremediation it is always recommended that some treatability test be conducted before commencing with the actual bioremediation operation, to ensure that the unique conditions that would hinder the remediation operation do not exist (Block *et al.*, 1992). It is therefore important to take every possible condition into consideration when planning a laboratory or pre-treatment study for a bioremediation project.

In the present research the soil samples used for the pilot-scale study were obtained from the actual contaminated site to be treated and the creosote concentration in the soil was 258 257.4 mg $kg^{-1}$. The plot eventually used for the full-scale operation had an average creosote concentration of 310 000 mg $kg^{-1}$. This disparity results from the renewed use of the plot for drying creosote-treated wood before results of laboratory experiments became available, as was earlier explained. Although there was a considerable difference in the creosote concentration of both soils, it was believed that the concentration in the pilot-scale experiment was high enough to have a similar effect to that of the full-scale operation, which had already become higher by the time the full-scale landfarming began.

Comparatively, however, the contaminant hydrocarbons in the field study degraded more slowly than in the pilot-scale operation. This was expected, considering the fact that the concentration in the

pilot-scale experiment was very much lower than that in the full-scale operation. This difference in the degradation rates is also attributed to the fact that the pilot-scale samples contained only completely weathered creosote, whereas for the full-scale field experiment the soil contained a mixture of weathered and completely unweathered creosote.

Notwithstanding all the variables in the field, which could not be simulated completely in the pilot-scale operation, the full-scale landfarming project was considered to have been successful. Although creosote degradation was faster in the pilot-scale project, the rate at which the contaminant was removed in the field, given the existing limitations, was considered to be acceptable.

## 9.4 CONCLUSIONS

From the results obtained for the full-scale landfarming project, and from the analyses made thereof, the following conclusions may be drawn:

- the full-scale landfarming process reduced the concentration of total creosote hydrocarbons from 310 186.8mg $kg^{-1}$ to1 762.5mg $kg^{-1}$ over a period of 10 months;
- sewage sludge enhanced microbial growth in the soil and consequently accelerated creosote degradation;
- the clean-up criteria set for the bioremediation project were achieved by the end of the treatment period;
- full-scale landfarming did not remove all the higher molecular mass PAHs present in creosote during the treatment period;

- there was a positive correlation between soil microbial respiration rate, microbial population size and degradation rate of the contaminant creosote.
- the effect of nutrient amendments (biostimulation) and bioaugmentation could be measured as can seen from the monthly results of creosote degradation, microbial respiration and microbial counts.
- the results of the treatments are reproducible, as shown by the results from both the pilot-scale and full-scale landfarming operations. The combined biostimulation and bioaugmentation produced similar effects in both experiment.

# CHAPTER 10

# BIOREMEDIATION BY CO-COMPOSTING OF SOILS HEAVILY CONTAMINATED WITH CREOSOTE HYDROCARBONS

## 10.1 INTRODUCTION

Composting (i.e. the process by which compost is produced from raw materials) is an above-ground process in which organic waste materials are biologically converted to a humus-like end-product. Composting systems are used to degrade and stabilize organics such as manure, municipal sewage sludge, municipal refuse, yard waste and food processing wastes (Fan and Tafuri, 1994; Potter *et al.*, 1999). Composting relies on the actions and interactions of a consortium of microorganisms which includes bacteria, protozoa, actinomycetes and fungi that thrive within different temperature ranges, to achieve the stabilization and minimization of wastes (Fan and Tafuri, 1994; Borazjani and Diehl, 1998). Composting has become generally accepted in the treatment of agricultural and municipal wastes (Baker and Herson, 1994; Gray *et al.*, 2000). Compost systems range from relatively simple compost piles (windrows) to highly engineered and controlled continuous-feed reactors (Atlas and Barther, 1987). Composting is an obligately aerobic and thermophilic process and, as a result, the maintenance of adequate oxygen levels and appropriate temperature is paramount when designing an effective system (Baker and Herson, 1994).

The ever-increasing quest for remediation of environments contaminated with chemical pollutants

has given rise to a new compost technology known as compost bioremediation ( USEPA, 1997; Barnes *et al.*, 2000). This technology is being used to restore contaminated soils, manage storm water, control odours and degrade volatile organic compounds (VOC) (USEPA, 1997). Compost bioremediation refers to the use of a biological system of microorganisms in a mature, cured compost to sequester or breakdown contaminants in water or soil. The microorganisms consume the contaminants which are in turn digested, metabolized and transformed into humus and inert products such as carbon dioxide, water and salts (USEPA, 1997). Compost bioremediation has been used to treat soils contaminated with chlorinated and non-chlorinated hydrocarbons, wood-preserving chemicals, explosives, heavy oils, heavy metals, solvents and pesticides (Ziegenfuss *et al.*, 1991; Williams *et al.*, 1992; Baker and Herson, 1994; Fan and Tafuri, 1994; USEPA, 1997; Potter *et al.*, 1999).

Compost used in bioremediation is referred to as tailored or designed compost, in that it is specially made to treat specific contaminants at specific sites (USEPA, 1997). Soil remediation carried out by composting decontaminates the soil as well as providing the nutrients required for revegetation. Compost bioremediation, more than any other soil clean-up technology, results in an enriched soil as end-product and can leave the soil in better condition than before it was contaminated (Cole *et al.*, 1995).

Composting is characterized by a variable temperature range during degradation of organic compounds. In the early stages of composting, the temperature increases from ambient to approximately 40$^{0}$C. With continued degradation of organic matter, more heat is evolved, raising

the temperature to thermophilic ranges (40 - 70°C ) (Fan and Tafuri, 1994). It has been suggested that optimal organic decomposition takes place in the thermophilic range 55 to 60°C ( Fan and Tafuri, 1994). Further increase in temperature results in a decrease in decomposition rate and consequent cooling of the compost. This decrease is due to loss of water from the compost as a result of high temperatures in the system (Fan and Tafuri, 1994).

For effective composting, various operational procedures are recommended. In aerated windrow and in-vessel composting, these may include amendments of the organic matter with a bulking agent such as wood chips, aeration of the compost pile, recovery of the bulking agent, further curing of the compost and product utilization and disposal. Aerated static pile-composting consists of a pile of organic-laden material such as sludge, amended with a bulking agent, covered with compost for insulation and piled on top of a ventilation system. Volatile emissions are usually controlled by covering or enclosing the pile and using the ventilation system to discharge the off-gas to a volatile organic compound emission control unit (McGugan, 1997).

Compost bioremediation is carried out by co-composting the contaminated soil with suitable compost materials to effect biodegradation of the contaminant. Previous studies have examined the degradation of organic pollutants in composts (Reid *et al.*, 1999). For example, it has been shown that the microbes present in windrow composts are capable of mineralizing pentachlorophenol (Valo and Slakinoja-Salonen, 1986). Although composting has been used in the remediation of soils contaminated with a number of organic compounds, including PAHs, the use of composting as a bioremediation technology has been given very little attention (Potter *et al.*, 1999). Much of the work

on treatment of contaminated soils by composting (Valo and Slakinoja-Salonen, 1986; Potter *et al.*, 1999; Reid *et al.*, 1999) has been done on soils with lower concentrations of the contaminating substance than were present in the present study, in spite of the fact that composts have been reported to have good potential for remediation of heavily contaminated sites (Reid *et al.*, 1999).

As indicated earlier, the present experimental site had locations contaminated with >380 000mg $kg^{-1}$ of creosote. These high levels of creosote provided a good opportunity to study and further understand the potentials of composting in soil bioremediation.

The aim of this research is, therefore, to study the effects of co-composting different compost materials with soil contaminated with very high concentrations of creosote, on the degradation of creosote in a static-pile compost system. It is also aimed at investigating the temperature profiles of the different composts and the changes in nutrient composition, and moisture occurring therein, during the treatment period. This will help determine the requirements of each compost type and their practical application on large-scale treatment of contaminated soils.

## 10.2 MATERIALS AND METHODS

### 10.2.1 Soil samples

About 2 000kg of creosote-contaminated soil were excavated from the experimental site using a spade. The soil was excavated to a depth of approximately 50 cm. The soil was placed in clean nylon

fibre bags for transporting to the laboratory, where it was stored in a cold-room at 4 $^{O}$ C. Three randomly selected samples of the soil (250g each) were taken to an independent laboratory for determination of the creosote concentration, using the methods described in Chapter 6 (section 6.2.8.1). The bulk soil was homogenized in an electric concrete mixer before being used to make the soil compost mixture.

### 10.2.2 Compost materials

Sewage sludge was collected from the wastewater treatment plant in Hammarsdale in KwaZulu-Natal, South Africa. The sludge was collected from the sludge drying beds at the plant and was moderately dry (approximately 25% water content) at the time of collection. Poultry manure, cow dung and hay for covering the compost heaps were obtained from the University of Natal's Experimental Farm at Ukulinga. Mixed vegetable waste was obtained from a fresh produce market at Nkondeni in Pietermaritzburg. Compost heaps were made on slatted, sawn wood pallets overlaid with nylon fibre bags, which allowed passage of air and excess water in and out of the compost heaps.

### 10.2.3 Preparation of contaminated soil-compost mixture

Three hundred and fifty kilograms of homogenized contaminated soil were mixed with wood chips (20cm x 4cm) in a ratio of 1:1 (v/v). The mixture was then mixed with one of the compost materials described in section 10.2.2 in a ratio of 4:1(contaminated soil + wood chips : compost material)

(v/v). This procedure was repeated for each type of compost material, which were regarded as different treatments. The control was a mixture of contaminated soil and wood chips without any compost material added. Each treatment was placed on a separate palette and covered with hay for insulation.

### 10.2.4 Temperature measurement

Because the only temperature data logger available had a limited number of terminals, only one thermocouple could be inserted into each compost heap. The thermocouples were located in the middle of the heap about 45 cm from the top. Because of the deficiency in the availability of thermocouples, and the threat of periodic malfunctioning of the system, the temperature was measured manually at three depths (15cm, 35cm and 50cm) in each of the compost heaps. Measurements were taken biweekly at noon.

### 10.2.5 Determination of moisture content

The moisture content of the compost was determined weekly during the dry months and, when necessary, water was added using a garden water sprinkler, to maintain a water content of about 70% field capacity. The water holding capacity was determined, as described in Chapter 6 (section 6.2.4).

### 10.2.6 Measurement of pH

The pH of each compost heap was measured in triplicate at monthly intervals, using the same procedures described in Chapter 6 (section 6.2.7).

### 10.2.7 Measurement of ash content

The ash content of each compost type and the control was measured by weighing 10 g of each treatment mixture separately into a preweighed crucible and reweighing before heating in a furnace at 400$^{0}$C for 6 hours. The crucible and ash were cooled to a constant temperature in a desiccator before being weighed again. These measurements were carried out at the start and at the end of the experiment.

### 10.2.8 Respiration of compost microorganisms

Determination of the respiration rates of the compost-inhabiting microorganisms was carried out as described in Chapter 9 (section 9.2.6). Duplicate samples for each treatment type were analysed and the mean values were used to plot the graphs showing carbon dioxide evolution against time.

### 10.2.9 Microbial plate counts

Three samples (about100g each) were taken from each heap every month by inserting a glass tube

of about 3 mm diameter horizontally into the middle of the heap. The samples were taken at about 20cm, 35cm and 50cm from the top of each heap. Plate counts of total heterotrophic microorganisms present in the compost mixture were determined by weighing 1 g samples of the soil-compost mixtures into separate beakers and preparing a serial dilution, before inoculating onto solidified nutrient agar. The plates were incubated in an inverted position for 48 hours at 30°C. The results are represented as colony forming units per gram (cfu $g^{-1}$).

### 10.2.10 Nutrients

Total organic carbon, extractable phosphorus and total nitrogen were determined, as described in Chapter 6 (section 6.2.2).

### 10.2.11 Determination of concentration of residual creosote and selected creosote components

Analyses for total residual creosote and selected creosote components were carried out monthly for nineteen months by EPA method 418.1 (USEPA, 1982) and gas chromatography (GC/FID) (Eriksson *et al.*,2000), as described in Chapter 6 (section 6.2.8.1).

## 10.3 RESULTS AND DISCUSSION

### 10.3.1 pH of compost

The pH of all the treatments, including the control, was found to increase in the first five months of composting. The highest values reached for the different treatments were pH 8.4 in the poultry manure in the fourth month, 8.2 in the sewage sludge in the fifth month, 7.9 in the vegetable waste in the fourth month, 7.7 in cow manure in the fifth month and 7.6 in the control in the thirteenth month. Apart from the control, which showed a slow but continuous increase in pH up to the ninth

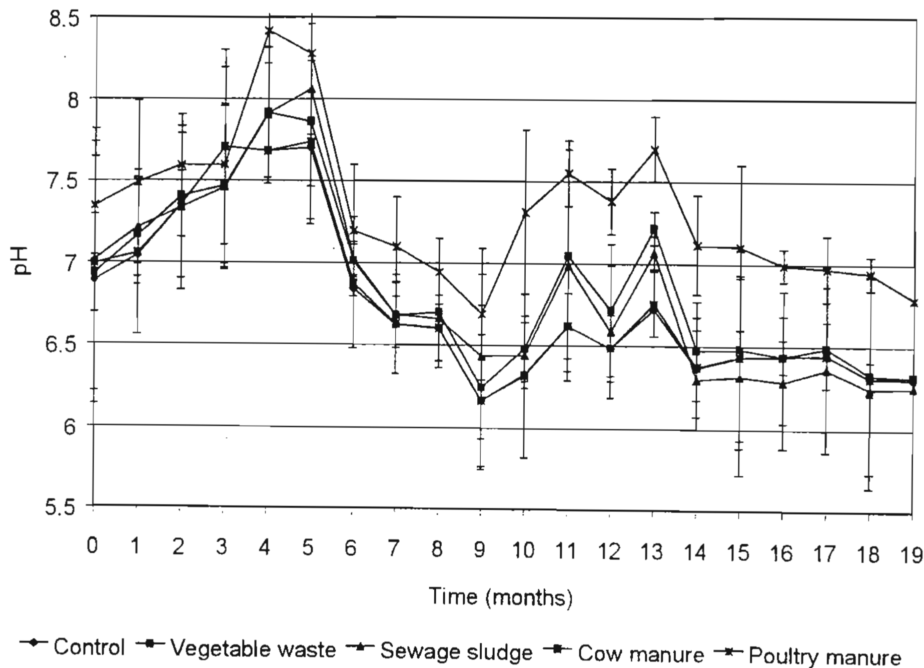



Fig. 10.1 The pH of compost during the period of incubation. Values are means of three ± 1 Standard Error.

month, with a decrease over a short period that was followed by a slight increase, pH in all other

treatments increased rapidly up until the fifth month, reaching peaks between 7.7 and 8.4, before decreasing sharply to between 6.8 and 7.1 in the sixth month, and then becoming relatively stable, with some fluctuations for the remaining time of the treatment (Fig.10.1). For most of the experimental period, the pH remained between 6.2 and 7.6 in all the treatments. This pH range is well within the recommended range for composting of organic materials (Hunter *et al.*, 1981; Kubota and Nakasaki, 1991). The pH of the poultry manure treatment increased above that of the other treatments during the first five months of incubation, probably as a result of the high ammonia content of the manure (Fig.10.1). The subsequent decreases observed after the sixth month are attributed to the degradation of the compost and the pollutant hydrocarbons, which resulted in the release of probably intermediate and final products that have a lowering effect on the pH of the mixture (Alexander, 1999; Lee and Lee, 2001, Fava and Piccolo, 2002).

### 10.3.2 Nutrients

The recommended C:N ratio for effective composting is in the range 25:1 to 35:1 (Anderson, 1991; Kubota and Nakasaki, 1991). A relatively high nitrogen concentration is thus required to facilitate effective compost bioremediation. Table 10.1 shows the C:N ratio of the compost materials used in the present study. The results show a high nitrogen content in the compost materials.

The initial soil C:N ratio was 200:1. This ratio was considerably reduced on addition of compost, as shown in Table 10.2. Although the nitrogen content in these C:N ratios are higher than those recommended by Anderson (1991), they are considered adequate for composting organic materials, considering the amount of compost materials used.

Table 10.1. Result of analyses of compost materials. Values are means of three ± 1 Standard Deviation

| Compost | C (g kg$^{-1}$) | N (g kg$^{-1}$) | C:N |
|---|---|---|---|
| Vegetable waste | 41.5 x 10 | 0.9 x 10 | ≈46:1 |
| Sewage sludge | 30.13 x 10 | 3.37 x 10 | ≈9:1 |
| Cow manure | 42.03 x 10 | 1.73 x 10 | ≈24:1 |
| Poultry manure | 36.03 x 10 | 3.63 x 10 | ≈10:1 |
| Contaminated soil | 24.53 x 10 | 0.08 x 10 | ≈306:1 |

The C:N ratios in the different treatments were observed to change over time as the incubation progressed. The ratio was observed to decrease in the vegetable waste compost in the first six months but then increased during the remaining period of incubation. The other treatments showed increases in their C:N ratios until the end of the incubation period, except for the cow manure compost, which showed a decrease in the first six months (Table 10.2). The faster depletion of the nitrogen content in the poultry manure can be attributed to microbial activity and the release of ammonia. There was a relatively low loss of nitrogen from the controls over the 19 months of incubation. This is believed to be due to the low microbial activity in the soil and the consequent slow breakdown of the organic substrate which would have resulted in the depletion of the nitrogen content.

Table 10.2. Changes in C:N ratio of the treatments during the incubation period. Values are means of three ± 1 Standard Deviation.

| Compost-soil mixture | 0 Time | 6 Months | 12 Months | 18 Months |
|---|---|---|---|---|
| Control 1 (without compost) | 200 : 1 | 126 : 1 | 225 : 1 | 189 : 1 |
| Vegetable waste | 12 : 1 | 6 : 1 | 8 : 1 | 11 : 1 |
| Sewage sludge | 3 : 1 | 5 : 1 | 5 : 1 | 7 : 1 |
| Cow manure | 7 : 1 | 6 : 1 | 8 : 1 | 14 : 1 |
| Poultry manure | 2 : 1 | 3 : 1 | 5 : 1 | 8 : 1 |

### 10.3.3 Ash content of compost

The ash components of the compost mixtures and the controls show that there were no significant differences between the ash contents of the initial soil-compost mixture and those of the mixture at the end of the incubation period (Table 10.3). This is an indication that there was no significant change in the mineral components of the soil at the end of the treatment.

Table 10.3. Ash mass (g)of compost-soil mixture at the start and end of experiment (19 months). Values are means of three ± 1 Standard Deviation.

| compost-soil mixture | Initial | Final |
|---|---|---|
| Control | 6.37 | 6.41 |
| Vegetable waste | 5.27 | 5.27 |
| Sewage sludge | 4.62 | 4.60 |
| Cow manure | 5.26 | 5.27 |
| Poultry manure | 4.1 | 4.15 |

**10.3.4 Residual concentrations of creosote and selected creosote components**

Results from the composting experiment show that there was a significant difference in the degradation of creosote in the four treatments (97%-98%) and the control (17%) during most part of the treatment period. The sewage sludge microflora were observed to remove the creosote faster than microbial associations in the other compost materials (Fig.10.2). The rate of creosote degradation follows the order: sewage sludge > cow manure > vegetable waste > poultry manure > control. Sewage sludge, cow manure, vegetable waste and poultry manure have been reported by other researchers to enhance the degradation of hydrocarbons in soil-compost mixtures (Hill and McCarthy,1967; Deever and White,1978). The rapid degradation of creosote in the sewage sludge-soil mixture was not unexpected, since sewage sludge is known to be rich in carbon and mineral nutrients, particularly nitrogen (Williams and Packer,1979; Hall and Vigerust,1984; Kellog-Johnson,1996). The organisms growing on the nutrient present in the sewage sludge were found to

readily metabolize the contaminant creosote in the mixture while still growing on the sewage substrate.

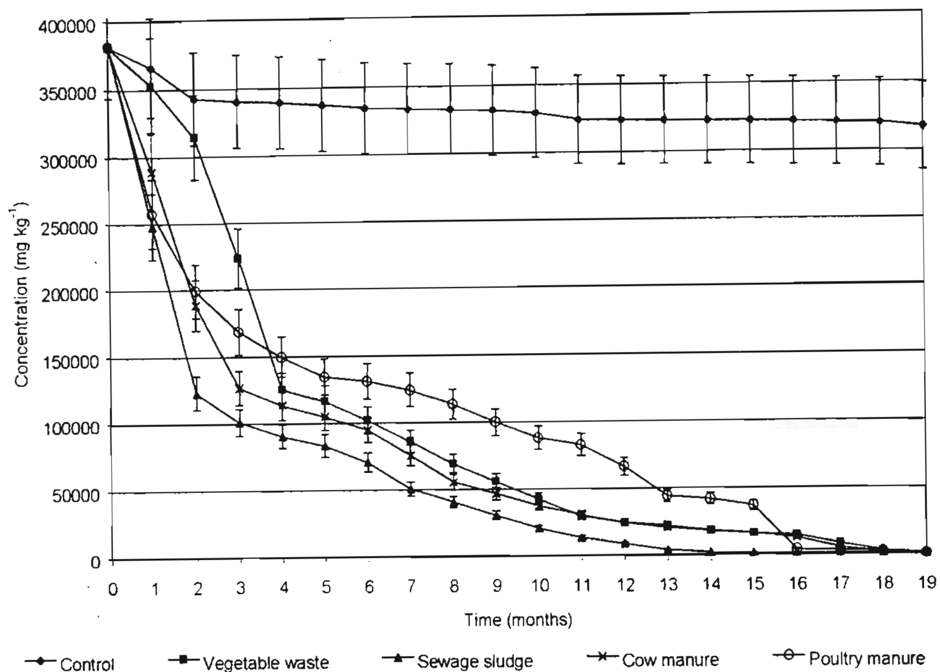



Fig. 10.2 Changes in creosote concentration during composting. Values are means of three ± 1 Standard Error.

The sewage substrate afforded the organisms the opportunity to grow while adapting to the creosote hydrocarbons. It also afforded the organisms the opportunity to produce enzymes that are required to metabolize the creosote in the compost matrix (Fritsche,1992; Sutherland,1995; Bardos *et al.*, 1996; Civilini *et al.*,1996; Diaz *et al.*,1996). The fast adaptation of the organisms to the creosote contaminated soil environment could be attributed to the heterogeneity of the substrate from which the sewage was generated and which included dyes, oils, fats and hydrocarbons. The high microbial load (1.28E+07) at the beginning of the treatment period also afforded the population the opportunity to remain high while adapting to and attacking the compost-soil mixture (Baker and Herson,1994,

Diaz *et al.*, 1996).

At the high concentration of creosote (>380 000mg $kg^{-1}$) used in this experiment, it was anticipated that the organisms would have difficulty in establishing in the contaminated soil and in degrading the pollutant creosote ( Baker and Herson, 1994; Alexander, 1999). Thus the time taken to reduce the concentration of the creosote to <1 000mg $kg^{-1}$ (i.e. about 19 months) was considered to be relatively rapid, since aeration and temperature in the compost-soil mixture was not controlled to increase the performance of the system.

### 10.3.5 Microbial population and creosote degradation

The microbial populations in the treatments with sewage sludge (1.28E+07) and poultry manure (7.39E+05) were observed to be high at the start of the treatment, when compared to vegetable waste (1.05E+04) and cow manure (1.04E+05). Total reductions and the reduction rates in the concentrations of the contaminating creosote in the vegetable waste and cow manure treatments were also found to be similar (Fig.10.2). Although the degradation rates differed in the beginning, the rates were not significantly different at the end of the treatment period. The difference in the performance of the two at the beginning could be attributed to the nature of the vegetable waste and the time taken to break down the waste to allow the establishment of the large microbial population required to metabolize the pollutant effectively. The vegetable waste was chosen because of its slow breakdown which would ensure a continuous supply of carbon for microbial growth until the organisms had adapted to the pollutant hydrocarbon. The initial creosote concentration was also probably inhibitory

to microbial growth (Baker and Herson, 1994), which would explain the non-degradation of the vegetable waste until the end of the first month. In contrast, cow manure contained large microbial populations capable of degrading cellulose and lignin ( Alexander, 1977; Atlas and Bartha, 1987; Criddle, 1993). The cellulose base of the cow manure afforded the organisms the opportunity to adapt to the creosote while growing on the manure, with corresponding degradation of the pollutant hydrocarbons taking place (Bardos, 1996). The initial fast degradation observed in cow manure may also be due to the heterogeneity of the organisms present, which included bacteria, fungi and actinomycetes.

The microflora in the cow manure differed from those in the sewage sludge because of the differences in their major organic constituents. Thus the observed difference in rate and extent of creosote removal during the treatment period was possibly due to differences in the degradative capabilities of the different microbial populations present and the time required for the adaptation of these different populations to creosote as their substrate.

Degradation of creosote in the vegetable waste compost was rather slow initially, but accelerated during the third month of composting. This is attributed to the nature of the mixed vegetable waste, which was specially selected for its slow decomposition rate so as to facilitate continued microbial growth over a protracted period of time, thus allowing for adaptation of the organisms to the compost-creosote-contaminated soil mixture, as was discussed earlier. The high concentration of creosote in the mixture may also have partially inhibited microbial proliferation at the beginning of the process.

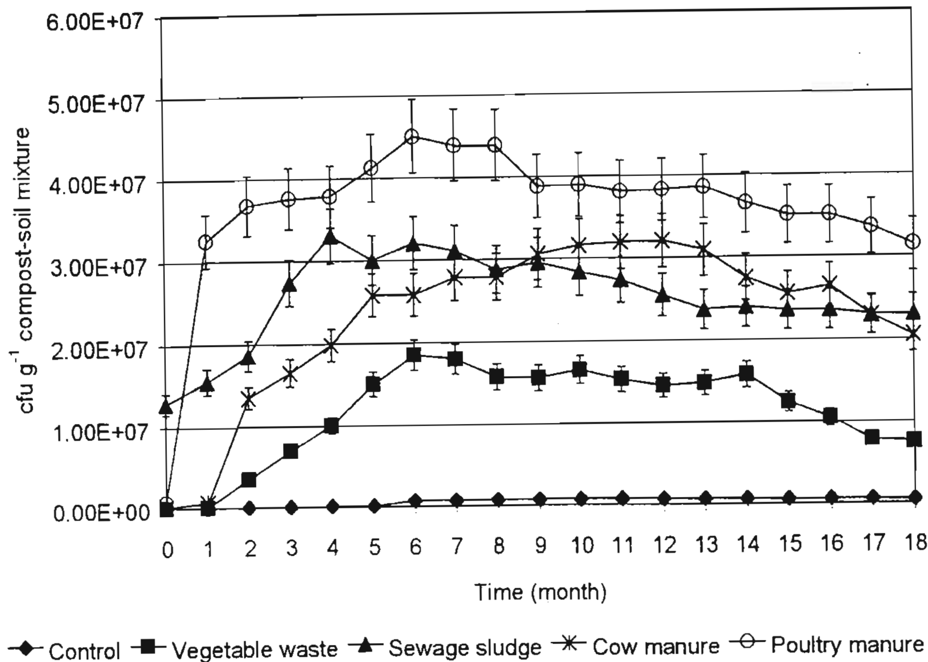



Fig. 10.3 Counts of total heterotrophic microorganisms during co-composting of creosote-contaminated soil. Values are means of three ± 1 Standard Error.

During the first two months of the experiment, the counts of total heterotrophic organisms increased greatly in the polluted soil treated with poultry manure compost (Fig.10.3). The microbial population was, however, observed to decrease after the second month. The initial increase is probably attributable to the abundance of readily available microbial nutrients in the manure. The high nitrogen and ammonia content of poultry manure can, however, be inhibitory to microbial growth (Baker and Herson, 1994; Snyman, 1996). As ammonia was increasingly released during decomposition of the manure it may have eventually become toxic to the microorganisms, which would explain the decline in the microbial population after eight months. High nitrogen contents in soils have sometimes been implicated in a slow rate of hydrocarbon decomposition as a result of their inhibitory effects on microbial growth (Alexander, 1999). The decrease after eight months

could also be due to very high temperatures (Fig.10.5), which resulted in an increase in the loss of water from the soil-compost matrix, thus reducing the activity of the microbial population (Fan and Tafuri, 1994). Regular monitoring of the water content, and watering when necessary, kept the temperature from increasing higher than it did. Degradation of creosote in this treatment improved in later months, possibly due to loss of some of the nitrogen as gaseous ammonia (Piccinini *et al.*, 1996). This loss may be the result of the initial microbial activity and high temperatures reached at the start of the treatment (Piccinini *et al.*, 1996). The high temperature usually associated with composting of poultry manure was also implicated in the decrease in microbial population of the manure observed during the later stages of the experiment. Although poultry manure has been found to accelerate the composting of garden refuse, its performance in the present experiment fell off after two months. The decrease in the temperature (Fig.10.5) in the poultry manure-creosote-contaminated soil mixture in the ninth and fifteenth months resulted in an increase in microbial population at those times (Fig.10.3) and consequently an increase in the rate and extent of creosote removal (Fig.10.2).

Over the last three months of the experiment there was relatively little difference in the degradation rate of creosote for all four treatments (Fig. 10.2). This is due to the composting process being completed as shown by the stabilized pH (Fig. 10.1) and temperature (Fig.10. 5) in all the treatments at this stage of the process. It may also be due to the fact that most of the creosote has been utilized by the degrading microorganisms resulting in low residual concentrations which are unavailable to the microorganisms. This condition explains the decrease in microbial population during these months.

Result of the analyses for residual concentrations of selected creosote components at the end of the composting period showed that all the phenolic compounds and the 2 and 3 ring PAH compounds were removed to below detectable limits in all the treatments, but not in the control, where significant levels of anthracene, phenanthrene, pyrrole and fluorene were still present (Fig.10.4). The 4 and 5 ring compounds were also removed to below 1 mg $kg^{-1}$ in all the treatments except the vegetable waste, in which up to 1.6 mg $kg^{-1}$ of chrysene was detected. The continued presence of the 4 and 5 ring PAHs in significant quantities after most of the other compounds had disappeared was the reason for continuing the composting for a further six months after the initially intended twelve months had expired. In all, benzo(a)pyrene and chrysene were the most recalcitrant of all the PAHs studied in the creosote complex, as these continued to remain in significant quantities during most of the experimental period.

### 10.3.6 Changes in temperature during composting

Temperatures were observed to rise into the thermophilic range in the second month of incubation in the sewage sludge, cow manure, and poultry manure compost-creosote-contaminated soil mixtures. The vegetable waste-soil mixture only became thermophilic in the fourth month of incubation. This is an indication of the long period of time required to break down the mixed vegetable waste to enable

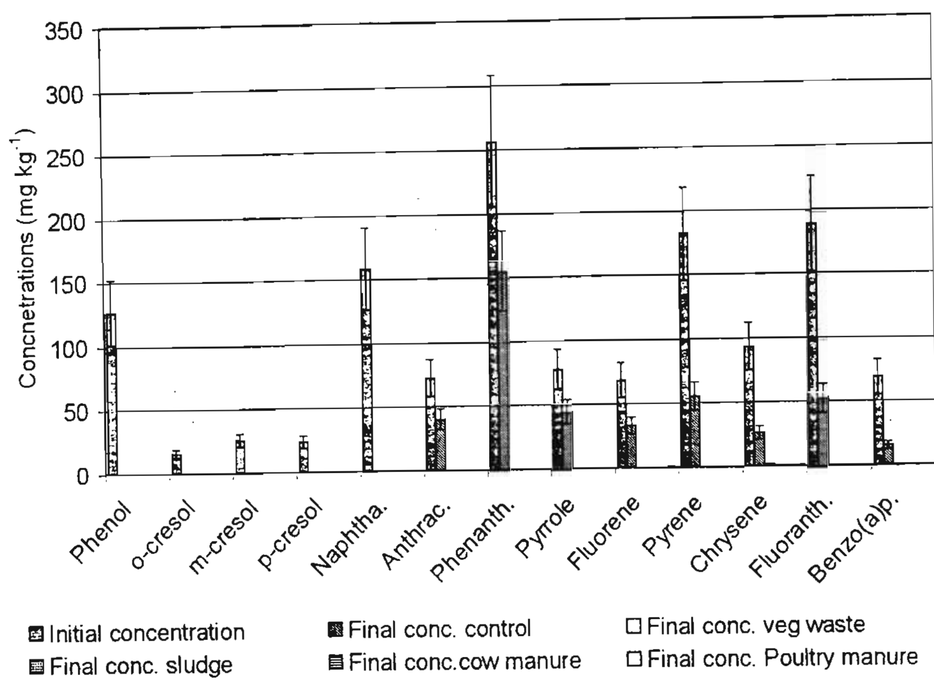



Fig.10.4 Residual concentrations of selected creosote components after 19 months of co-composting. Values are means of three ± 1 Standard Error.

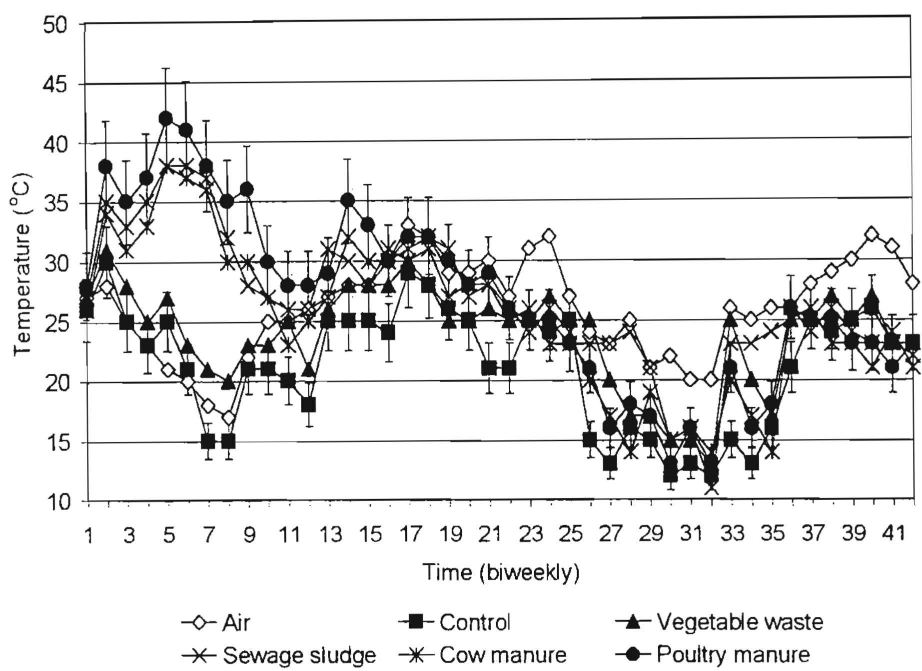



Fig.10.5 Noon day temperature of air and composts during co-composting. Values are means of three ± 1 Standard Error for the compost heaps. Only one measurement was taken for air on each occasion.

the establishment of a large, actively metabolizing microbial population. The temperatures in all four treatments continued to fluctuated widely (13$^{O}$- 42$^{O}$C) during the treatment period. On the average, most of the treatments ran on a mesophilic temperature range of between 20$^{O}$ and 35$^{O}$ C for most of the treatment period. This is an indication of the high level of microbial activity in all the treatments. Although below the temperature range (55-60$^{O}$ C) suggested by Fan and Tafuri (1994) as optimal for organic matter decomposition, the observed temperature range ensured that greater microbial diversity in the soil-compost mixture is maintained (Potter *et al.*,1999). This period was characterised by a gradual but continuous decrease in creosote concentration. The daily diurnal temperature of the outside air influenced the temperature of the control throughout most of the treatment period. This effect is due to the low microbial activity in the control, which is attributed to the inhibitory concentration of creosote (>380 000mg $kg^{-1}$) in the soil and the lack of compostable materials needed to speed up microbial proliferation (Finstein and Morris,1975; Bertoldi *et al.*,1982) to produce the subsequent increase in temperature. The lack of compost also caused the creosote concentration of the control to remain very high and hence toxic to microorganisms. Composts have been reported to render contaminants less toxic to microbial populations through dilution effects, thus affording them the opportunity to grow (Stegmann *et al.*, 1991). The vegetable waste-contaminated soil mixture was also influenced by the diurnal temperature in the first month until the microbial population began to increase. The effect of diurnal temperature fluctuations was less pronounced in the other treatments, although outside air temperatures *did* affect the compost temperature more significantly towards the end of the treatment period. This was attributed to the reduced microbial activity within the heaps at this stage. Without biological heat generation the outside temperature would tend to affect the compost temperature significantly (Fig.10.5).

Despite occasional malfunctioning of the data logger, the temperatures within the compost heaps that were successfully recorded showed that these temperatures did fluctuate in synchrony with the diurnal temperature on many occasions (See data logger temperature graphs in appendix). Although creosote degradation rates within the compost heaps could not be indisputably related to diurnal temperature changes in the earlier parts of the treatment, such a relationship was clearly evident during the later stages of the experiment. Temperatures within the control and poultry and cow manure-supplemented compost heaps were observed to increase in the summer months at the end of the treatment period (Fig.10.5), with parallel increases in the rate of creosote degradation (Fig.10.2). This development at the end of the treatment period showed that microbial activity was responsible for the prevailing temperatures and the hydrocarbon degradation rates within the compost-contaminated soil mixtures.

The temperature regime in the compost heaps was closely related to creosote degradation rates. The rapid degradation rates maintained over most of the treatment period in the compost-contaminated soil mixtures occurred in the temperature range $35^0$ to $43^0$ C (Fig. 10.5). This was clearly observed in the degradation pattern of the vegetable waste compost, where the concentration of the creosote began to decrease appreciably as the temperature began to increase towards the end of the third month (Fig. 10.2 &10.5).

The initial increase in temperature observed in the poultry manure-contaminated soil mixture was parallelled by a corresponding increase in creosote degradation-rate as was earlier stated (Fig.10.2 &10.5). However, the increase in temperature became inhibitory to continued microbial growth after two months, as reflected by a decrease in microbial respiration (Fig.10.6) and a corresponding

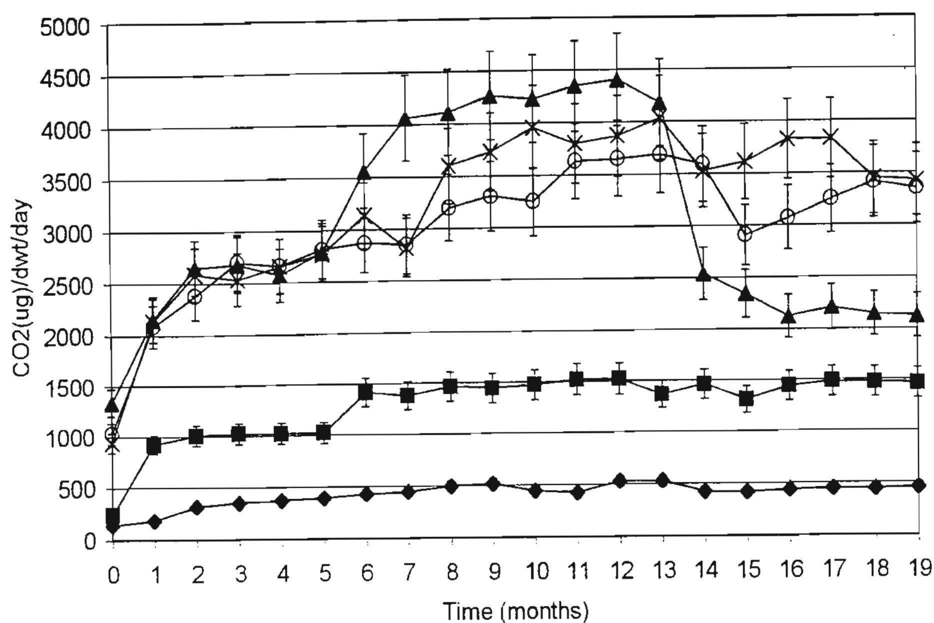



Fig.10.6 Respiration rates of soil microorganisms during co-composting of creosote contaminated soil. Values are means of three ± 1 Standard Error.

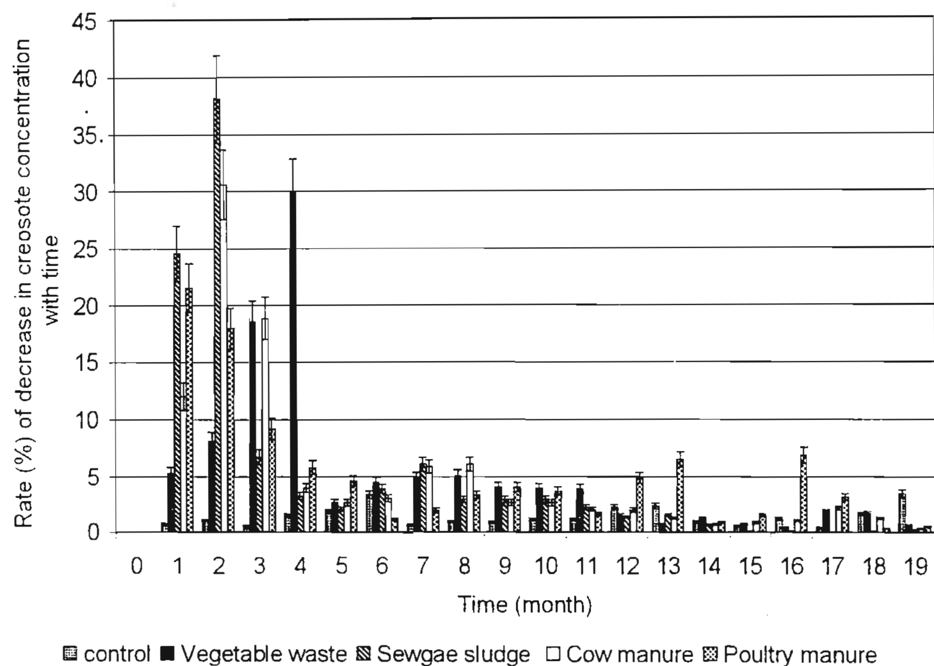



Fig.10.7 Rate (%) of decrease per month in creosote concentration during co-composting of creosote-contaminated soil. Values are means of three ± 1 Standard Error.

decrease in rate of creosote degradation (Fig.10.7). Although temperature increases were expected to increase microbial activity and hence creosote degradation, increases as large as these recorded in the poultry manure-soil mixture can inhibit microbial activity and creosote degradation (Potter *et al.*, 1999). If contained within the tolerance level of the prevailing population, as observed in the cow manure and sewage sludge composts, temperature increases should be beneficial. The maintenance of temperatures within the mesophilic range ($30^{O}$-$45^{O}$C) in these two compost treatments for a long period of time shows that microbial activity remained high for a long time. This is attributed to the continued availability of the creosote hydrocarbons. The period of visible decrease in creosote concentration coincides with the period of decrease in temperature (Fig.10.2 & 10.5).

Results from the microbial respiration experiments and plate counts of microorganisms show that microbial activity was directly related to creosote degradation. As the microbial populations and respiration rates decreased, the percentage reduction in creosote concentrations also decreased (Fig. 10.3, 10.6 & 10.7). This is an indication that metabolic activity of the microorganisms is responsible for the reduction in concentration of creosote in the soil-compost matrix. Respiration rates in sewage sludge and cow and poultry manures were observed to be significantly higher than those in vegetable waste, throughout the treatment period. This is believed to be due to differences in the size and composition of the initial microbial population present in the respective compost mixtures. Respiration in the vegetable waste-supplemented soil was, however, much higher than in the control soil. Respiration rates continued to rise until about the fourteenth month in all the treatments, after which they declined significantly in the sewage sludge and cow and poultry manures. In the vegetable waste treatment, however, a slow but continuous increase in respiration rate was

maintained throughout the experimental period.

Notwithstanding the differences in microbial biomass and respiratory activity, substantial degradation of creosote occurred in all the compost-supplemented treatments. This suggests that the level of creosote degradation observed in the compost systems may not all be directly due to biological attack. Chemical and physical conditions established by the process may exert an important influence on the breakdown of certain organic compounds which are known to be affected by changes in conditions such as UV light, temperature and pH (Diaz *et al.*, 1995). Hence, the overall results observed are an aggregate of the effects of physical, chemical and biological destructive agents, combined to degrade the pollutant, thus magnifying the effectiveness of composting (Diaz *et al.*, 1995).

The means of the results obtained from the degradation study show that there was no significant difference in the degradation capabilities of the microorganisms present in the vegetable waste and cow manure composts at a Standard Error of 1. This is believed to be due to the similarity in their microbial complement, dictated by their common substrate, plant cellulose, as was previously explained. The performances of sewage sludge and poultry manure in creosote degradation were significantly different from each other and from the other compost materials investigated. The control was statistically rated the least effective in creosote degradation. The means of the results obtained from the respiration experiment (Fig.10.6) showed that there was no significant difference in the microbial activity in sewage sludge, cow manure and poultry manure. However, respiration of the microorganisms in the vegetable waste compost was significantly different from the above

three and from the control. This was largely due to the difficulty experienced in establishing a microbial population in the material and the large difference in the microbial population between this and the other three composts during the early stages of the experiment. Statistical analysis of both the creosote degradation and the microbial respiration data indicates that the control was the least efficient in performance. Thus, the performance of all the treatments, including the control, in decreasing order, is as follows: sewage sludge > cow manure > vegetable waste > poultry manure > control.

Relative to the control, which showed a steady but slow degradation of the creosote, addition of the compost materials resulted in a markedly enhanced level of degradation of the pollutant (Fig.10.7). At the end of the treatment period only 26.5% of the total creosote hydrocarbons (Fig.10.2) had been degraded in the control, compared to 99% degradation in the compost-soil mixtures. A possible reason for the poor performance of the control could be the low concentration of microorganisms present, which is a result of the toxic concentration of creosote present in the soil, as was earlier stated. A high pollutant concentration is known to inhibit microbial growth (Herson and Baker,1994; Tate,1995;Alexander,1999). Although improved aeration was expected to boost the rate of microbial growth in the soil, it was observed that the increase in microbial population was very slow. The relatively low temperature regime that prevailed in the control experiment throughout most of the treatment period could also have contributed to the low level of creosote degradation. The temperature data from the manually recorded day temperatures showed that temperatures fluctuated along with the mean day temperatures in all the treatments. Data collected from the data logger for the period that it was functional showed that temperatures in the control decreased to very low levels

(6°C) at night, mirroring changes in ambient temperature. Although the pH of the control rose slightly in the first few months of treatment (Fig.10.1), it apparently did not increase sufficiently to allow for the prolific growth of microorganisms required to degrade the high levels of creosote present. Most of the creosote loss observed in the control experiment was believed to be due to volatilization and breakdown of the lower molecular mass components of creosote (Fig.10.5).

Because of the continued presence of some of the higher molecular mass components of creosote until the sixteenth month, the composting process was allowed to continue for a further four months to see if these substances would be completely removed from the soil matrix. Results shown in Fig.10.5 demonstrate that with the exception of chrysene and benzo(a)pyrene, the very recalcitrant higher molecular mass components were removed to below detectable limits in all the composted treatments. Where residual concentrations were detectable, they were found to be below 1mg $kg^{-1}$. Degradation of such high concentrations of creosote would normally have taken more than two years to complete (Overcash, 1979). However, the choice of compost material, improved aeration and closely monitored water content of the soil-compost matrix greatly accelerated the rate of creosote degradation.

## 10.4 CONCLUSIONS

From the experimental results obtained it can be concluded that:

- compost bioremediation can be used to treat soils heavily contaminated with creosote in a reasonable period of time.

- higher molecular mass (4-5 ring) PAHs can be effectively removed from contaminated soils in a short period of time.
- all the compost materials studied were useful for bioremediating creosote-contaminated soils without additional nutrient amendment.
- there was no significant difference in the overall degradation of creosote by the microbial populations present in the different compost materials at the end of the experimental period.
- the rate of creosote removal was very high in the first four months of the treatment period. This period constitutes the crucial period in the composting of hydrocarbon materials.
- ambient air temperatures influence the compost temperature to a lesser extent during the mesophilic stages of incubation.
- changes in pH in the selected compost materials are within the acceptable range for composting and no amendments are required to alter or control the pH

## 10.5 Recommendations

It is recommended that methods of improving aeration without incurring high costs should be developed, as it was observed that aeration was not very uniform throughout the soil-compost matrix. This will not only ensure even degradation of the pollutant in the entire system but will ensure faster breakdown of the organic matter.

# CHAPTER 11

# GENERAL DISCUSSION

## 11.1 INTRODUCTION

Arable land and groundwater are two scarce natural resources in South Africa. With the high rate of industrialization and population growth, these two resources have come under increasing pressure over the past one to two decades. Hydrocarbon pollutants have continued to feature prominently on the list of hazardous materials contaminating soil and water in the country. Co-disposal and land-filling are the most common practices used for treating contaminated soil in South Africa (Pearce *et al.*, 1995). Although bioremediation is not totally unknown in the country, it has seldomly been considered as an option for the treatment of contaminated land (Pearce and Ollerman, 1994; Lees, 1996; Snyman, 1996). The lack of legislation and standards defining acceptable levels of most contaminants in the environment has greatly hampered the exploitation of bioremediation potentials in the country.

It has been argued that the ever-tightening environmental legislation in the U.S.A. is the principal driving force behind clean-up operations, particularly with costs of site remediation being substantially lower than possible litigation costs. With legislation stipulating that a percentage of the profit of all U.S companies should be spent on environmental protection and pollution remediation, it was estimated that the annual U.S bioremediation market would reach about US $600 million by

the year 2000, and the global market about US $1.3 billion (Glass, 1995).

With the passing of the South African National Environmental Management Act in 1998, the government's attitude towards protection of the environment has become increasingly positive. Clauses entrenched in the Act, such as the right to a clean environment by every citizen of South Africa, the right of the environment to be protected from damage, the principle of "co-operative governance", which mandates all tiers of government, communities and industry to jointly address environmental management issues, and the "polluter pays" principle, which puts the responsibility of cleaning up any pollution on the defaulter, are efforts towards preventing environmental pollution (National Environmental Management Act 107 of 1998).

In spite of this new initiative on environmental protection, most industries pay very little attention to safe handling of hazardous materials. Many communities are unaware of the hazardous nature of substances used and spilled in their neighbourhoods. Certain manufacturing industries receive tax incentives and protection from the government to encourage them to create employment. Such industries tend to pay less attention to environmental pollution regulations because of the privileges they enjoy from the government (McGugan, 1995). The petrol pipeline leakage from a petroleum refinery in Durban, which was reported to have continued uncontrolled over a long period, is one such case (Reported by KwaZulu-Natal Department of Agriculture and Environmental Affairs).

The wood treatment industry in South Africa is a large and growing one. Most of the treatment plants treat their wood with creosote, a hazardous substance containing many known carcinogens (AAFP,

1993; Kästner *et al.*, 1998; Stapleton *et al.*, 1998). In spite of the potential danger of creosote in the environment, pollution caused by poor handling has continued unabated. There is hardly any regulatory legislation relating to creosote contamination of the environment. The apathy with which creosote contamination is treated in South Africa can be seen from the fact that there was no record of any remediation project on a creosote-contaminated site when the present study was begun. There are also no known funds dedicated to such projects; unlike the situation in the petroleum and mining industries, where the industry and government have funded projects directed at ameliorating pollution problems.

The unavailability of trained bioremediation personnel has often led to non-specialized civil engineering firms, with poor knowledge of the biological principles employed in bioremediation, being contracted to carry out bioremediation projects. This situation, the lack of a multi-disciplinary approach and the setting of unrealistic clean-up targets, have in the past meant poor results from bioremediation projects around the world (Baker and Herson, 1994; Alexander, 1999).

The main focus of this thesis, therefore, was to develop a cost-effective bioremediation technology for cleaning up sites contaminated with creosote hydrocarbons. Where necessary, bioremediation technologies developed for other applications were adapted and optimized. The project was also aimed at providing knowledge and information to the government, industry and communities in South Africa, and to the global community, on the potential of bioremediation for the treatment of creosote-contaminated soils. Wood was being treated with creosote on a daily basis at the site chosen for this project and different levels of creosote contamination were evident in the soil at different

locations on the premises. However, only areas with high concentrations of creosote (>250 000mg $kg^{-1}$ soil) were used in the study so as to provide useful information for the treatment of very highly polluted soils.

## 11.2 Bioremediation Technologies Used

Two main bioremediation technologies were employed: (i) a modified landfarming approach in which the soil was treated *in situ*, and (ii) compost bioremediation in which contaminated soil was co-composted with various compostable materials in an *ex situ* treatment system. A laboratory experiment was used to evaluate the potential of fungal bioremediation to remove the persistent, high molecular mass PAHs present in the creosote-contaminated soil. These technologies are discussed in Chapters 1, 7, 8, 9 and 10.

### 11.2.1 Landfarming

Landfarming was chosen as a possible treatment after considering the range of available options discussed in Chapter 1 and the treatability tests discussed in Chapters 3-6 of the present work. Standard landfarming techniques were modified, after assessing the available site information such as soil type, topography, water table, distance from the nearest body of surface water and porosity of the soil. The present work has shown that by making preliminary investigations, such as enrichment tests to determine the presence of microorganisms capable of catabolizing creosote, desorption of creosote molecules from soil particles using surfactants, growth rate of

microorganisms in cultures containing creosote components and optimization of soil physical and chemical conditions for microbial bioremediation of creosote contaminated soil, the problem of "trial and error" during the treatment programme can be largely eliminated and costs considerably reduced. For example, in the optimization of physical and chemical parameters of the soil, it was proved that the nutrient supplementation C:N 25:1 was most appropriate for the growth of the creosote-catabolizing microbial populations. This finding eliminated the use of unnecessarily large quantities of nutrient supplements during the pilot-scale and full-scale landfarming experiments, thereby saving considerable amounts of time and money. Although it was established that all the surfactants tested could enhance desorption of creosote from contaminated soil and consequently increase degradation rates, a further trial on the use of surfactants was terminated since it was estimated that an additional treatment would reduce the cost effectiveness of the project. The amount of water (60-70% field capacity) needed for sustaining microbial activity during the period of treatment was established experimentally. It was shown that indigenous soil microorganisms present in the contaminated soil were capable of catabolizing creosote.

The pilot-scale landfarming experiment was successful in that it was able to demonstrate that simple and inexpensive materials such as plastic troughs could be used to carry out reliable tests. In this experiment the use of sophisticated equipment was avoided and capital investment and treatment time reduced to a minimum. Experimental results showed that although the indigenous soil microorganisms present in the creosote-contaminated soil were capable of catabolizing creosote when amended with adequate amounts of nutrients, further amendment with organic supplements, such as manure, or application of an enriched indigenous microbial biosupplement, was necessary

to accelerate the rate of creosote breakdown in the soil. It was also established that there was no significant difference in the creosote-degrading capacity of the indigenous soil microbial biosupplement (88%) and the microbial populations present in sewage sludge (86%), cow manure (85%) and poultry manure (84%). It was therefore considered more cost-effective in the full-scale landfarming project to use sewage sludge rather than the adapted indigenous microbial biosupplement, since production of the latter involved: (i) labourious enrichment procedures requiring expensive, specialized facilities capable of handling large-scale production of inocula; (ii) devising suitable dispersion equipment and (iii) making repeated applications of the enriched culture. In the absence of such preliminary tests, capital would have been invested in large-scale enrichment experiments and on repeated applications of indigenous soil microbial biosupplements, as this was initially thought to be a viable option. Block *et al.* (1992) earlier claimed that bioremediation projects that failed were those that were not preceded by a treatability study. It is important to note that pressures exerted by industry, the community and the government to clean up a specific site within a short period may make a prolonged treatability test not feasible. In such a situation, basic assessments of the treatability of the contaminated soil, such as the presence of the requisite microorganisms in the soil and the hydrology of the site, should be carried out in one to three weeks to determine a suitable technology for application. This will depend on factors such as the extent of the contamination, the cleanup target level based on the intended future use of the land and on whether the contaminated soil would best be treated *in situ* or *ex situ*.

The full-scale landfarming project proved that a combination of landfarming, biostimulation and bioaugmentation enhances bioremediation of creosote-contaminated soil. The concentration of

creosote present in the soil was very high (310 000mg $kg^{-1}$) and the treatment strategy applied thus required careful manipulation to accelerate the breakdown rate. It was obvious that conventional biostimulation by inorganic nutrient addition, as practised in landfarming operations, would not be adequate to boost the population of the required microorganisms to the levels necessary to degrade such a high concentration of creosote. It was therefore necessary to bioaugment the autochthonous microbial population by the addition of a supplementary source of microorganisms. Sewage sludge was found to be useful in this regard because of its high microbial count, large carbon substrate reserve and large inorganic nutrient content to sustain the microbial population while it adapted to the creosote carbon.

The stepwise application of biostimulation techniques and bioaugmentation with intervals between each step to allow for establishment, and subsequent assessment, of each treatment phase proved to be useful, as periods of increases and decline for monitoring changes in the microbial population over time could be readily identified and correlated with creosote degradation, and appropriate actions taken promptly. This helped sustain microbial activity throughout the period of landfarming. The results from monitoring of pH and water content during the pilot-scale experiment were useful in maintaining appropriate physical conditions in the soil during the period of treatment.

Although some of the higher molecular mass components of creosote were recalcitrant to microbial attack and persisted in the soil in different amounts (pyrene 28.5mg kg-1, fluoranthene 17.5, chrysene 47.5, benzo(a)pyrene 17.5), the concentration of the parent substrate was reduced at the end of the treatment period from 310 000mg $kg^{-1}$ to 1763mg $kg^{-1}$, which was below the target value of

2000mg $kg^{-1}$. Achievement of this goal was possibly due to the period of treatment being extended beyond the originally set 8 months. Despite this extension of the treatment period the higher molecular mass components tested for were not completely removed. However, none of the phenolic components were detectable after four months and the lower molecular mass polycyclic aromatic hydrocarbons (PAHs) tested were reduced to undetectable levels within 8 months.

Measurement of respiratory activity over the treatment period proved to be an effective way of determining soil microbial activity because it increased in parallel with increase in microbial population. Although the culturable microorganisms present at different stages of the landfarming experiment were not specifically identified, micrographs revealed the presence of a consortium of microorganisms which included various bacteria, actinomycetes and fungi.

### 11.2.2 Fungal bioremediation

Two experiments were set up in an attempt to achieve total removal of selected higher molecular mass components of the creosote tested. The first attempt involved the use of fungi in a laboratory experiment. Fungi are known to produce enzymes that break down cellulose and lignin, two complex carbonaceous substances present in wood. This capability of fungi has been harnessed to degrade a wide range of hydrocarbons (McGugan, 1997; Kotterman *et al.*, 1999; Clemente *et al.*, 1999; Pointing, 2001). In the present experiment the fungi demonstrated the capacity to remove selected higher molecular mass components of creosote faster than was observed in the pilot-scale landfarming experiment. However, growth of the organisms could not be sustained long enough to

ensure complete removal of all the compounds tested. The release of large numbers of spores during the experiment was a problem that would need to be addressed if fungi were to be used on a larger scale for the treatment of hydrocarbon-contaminated soil. Although large numbers of spores would lead to the proliferation of fungal biomass, in an unprotected environment it would constitute a health hazard, as the spores would be carried away in the air to cause the usual health problems associated with fungal spores. This experiment showed that both lignolitic, non-lignolitic and cellulolytic fungi are capable of breaking down components of creosote in soil.

### 11.2.3 Co-composting of creosote-contaminated soil with different compostable materials

The second attempt to achieve complete removal of the tested higher molecular mass PAHs from the creosote-contaminated soil entailed co-composting of the contaminated soil with different compostable materials. This experiment attempted to remediate contaminated soil from the location with the highest concentration (380 000mg $kg^{-1}$) of creosote in the soil. Addition of all four compost materials, either separately or together, resulted in the removal of the higher molecular mass components of creosote tested, to levels below 1.0mg $kg^{-1}$,within 19 months. Fluoranthene and pyrene were completely removed within 16 months. This was faster than the removal rate achieved in the landfarming project. However, benzo(a)pyrene and chrysene persisted until the seventeenth and eighteenth months of treatment, respectively. This experiment demonstrated that these substances can be completely removed from contaminated soils by co-composting with appropriate compostable materials. Although many of the creosote components tested (phenolics and lower molecular mass PAHs) were rapidly degraded, the higher molecular mass components continued to be detected for much of the experimental period. This could be due to insufficient aeration. A system of aeration which would allow even distribution of air

throughout the system might possibly accelerate the decomposition of the more recalcitrant components. Such an air supply might prevent the temperature within the compost heaps from rising above the optimal temperature for activity of the creosote-degrading microorganisms. Again, the cost effectiveness of composting large amounts of contaminated soil would be questionable. Moving (possibly transporting) the soil and construction of the required structures would increase the cost of treatment. The co-composting process was, however, found to be an effective technology for removing the very recalcitrant creosote components from contaminated soil.

## 11.3 RECOMMENDATIONS

This thesis reports the results of the experiments incorporating both *in situ* and *ex situ* bioremediation technologies applied to a Mispah (lithosol: FAO) soil in South Africa heavily contaminated with creosote. These technologies were shown to be suitable and viable options for use under South African conditions and should therefore be further investigated for possible application on large-scale bioremediation projects. More research projects relating to bioremediation of creosote-contaminated soils should be carried out, particularly on the topics of large-scale application of fungal remediation and cost reduction in co-composting contaminated soils.

It is recommended that the government produce unequivocal guidelines on permissible levels of creosote components in soil and water and make the user industries and the public aware of such information. This would provide an official basis for monitoring contamination levels on behalf of the government and would encourage the wood preservation industry to establish systems to

control their pollution levels on continuous basis.

The government should introduce monitoring and law enforcement facilities to regularly check on the state of contamination in the industry and prosecute offenders when necessary. It should also put in place environmental education strategies that address the issue of awareness of the danger of different types of hazardous materials released into the environment, so that individuals living in communities exposed to such pollutants could help the government monitor the use of such substances in their community.

It is suggested that research organisations such as the National Research Foundation, the Council for Scientific and Industrial Research and government departments responsible for protecting the environment, such as the Departments of Environmental Affairs and Tourism, Agriculture, Water Affairs and Forestry and Land Affairs, encourage research on an on-going basis for the development of remedial technologies for the treatment of creosote-contaminated sites in South Africa, by funding research projects on this problem, as they do for petroleum and mining waste contamination throughout the country. The results of such research should be made available to the public and other interested researchers to help foster a general awareness of environmental issues.

# REFERENCES

Abdul, A.S., Gibson, T.L., Ang, C.C., Smith, J.C. and Sobcznski, R.E. 1992. *In situ* washing of polychlorinated biphenyls and oils from a contaminated site. *Ground Water* 30: 219-231.

Aelion, C.M., C.M. Swindoll, and F.K. Pfaender.1987. Adaptation to and biodegradation of xenobiotic compounds by microbial communities from a pristine aquifer. *Appl. Environ. Microbiol.* 53: 2212 - 2217.

Alexander, M. 1981. Biodegradation of chemicals of environmental concern. *Science* 211:132-138.

Alexander, M. 1977. Introduction to Soil Microbiology. John Wiley and Sons, New York.

Alexander, M. 1999. Biodegradation and bioremediation. Academic Press, San Diego.

Alshawabkeh, A.N. 2001. Potential for enhancement of In Situ bioremediation of petroleum contaminated soils by electrochemical method. In Proceedings of the First International Congress on Petroleum Contaminated soils, Sediments and Water, London. 14-17 August, 2001.

American Academy of Family Physicians (AAFP).1993. Polycyclic aromatic hydrocarbon toxicity; Environmental medicine. *American Association of Family Physician.* 47 (3): 623-629.

Ananthaswamy, H.N., Eisenstark, A. 1977. Repair of hydrogen peroxide induced single strand breaks in *E. coli* DNA. *J. Bacteriol.* 130: 187-191.

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

Andersson, B.E. and Henrysson, T. 1996. Accumulation and degradation of dead-end metabolites

during treatment of soil contaminated with polycyclic aromatic hydrocarbons with five strains of white rot fungi. *Appl. Microbiol. Biotechnol.* 46: 647-652.

Andrew, J.F. 1968. A mathematical model for the continuous culture of microorganisms. *Biotech. Bioeng.* 10: 707-723.

Annweiler, E., Michealis, W. and Meckenstock, R.U. 2001. Anaerobic cometabolic conversion of benzothiophene by a sulphate reducing enrichment culture and in a tar-oil-contaminated aquifer. *Appl environ Microb* 67: 5077-5083.

Annweiler, E., Richnow, H.H., Antranikian, G. Hebenbrock, S., Garms, C., Franke, S., Francke,W. and Michealis, W. 2000. Naphthalene degradation and incorporation of naphthalene-derived carbon into biomass by the thermopile *Bacillus thermoleovorans. Appl. Environ. Microbiol.* 66: 518-523.

Anselmo, A.M., Mateus, M., Cabral, J.M.S. and Novais, M. 1985. Degradation of phenol by immobilized cells of *Fusarium flocciferum. Biotech. Lett.* 7: 889-894.

Anselmo, A.M. and Novais J.M. 1984. Isolation and selection of phenol-degrading microorganisms from an industrial effluent.*Biotech. Lett.* 9: 601-606.

Anselmo, A.M., Cabral, J.M.S. and Novais, J.M. 1989. The adsorption of *Fusarium flocciferum* spores on elite particles and their use in degradation of phenol. *Microbiol. Biotechnol.* 31: 200-203.

Arvin, E., Jensen, B., Godsy, E.M. and Grbic-Galic, D. 1988. Microbial degradation of oil and creosote related aromatic compounds under aerobic and anaerobic conditions. In: Wu, Y.C.(ed). International Conference on Physiochemical and Biological Detoxification of Hazardous Wastes, May 3-5, 1988. Atlantic City, N.J. Vol. II. Technomic, Lancaster, PA. pp. 828-847.

Arvin, E. and Flyvbjerg, J. 1992. Groundwater pollution arising from the disposal of creosote waste. *J. IWEM.* 6(6): 646-652.

Atagana, H.I., Haynes, R.J. and Wallis, F.M. 2000. Bioremediation of creosote contaminated soils by microbial intervention. In: G.B. Wickramanayake, A.R.Gavaska, J.T. Gibbs, J.L. Means (eds), Case studies in the remediation of chlorinated and recalcitrant compounds. Battelle Press, Columbus.

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

Atlas, R.M. and R. Bartha. 1987. Microbial Ecology: Fundamentals and Applications. Benjamin/Cummings Publishing Company, Menlo Park , California.

Atlas, R.M. and R. Bartha. 1972. Degradation and mineralization of petroleum in seawater: Limitation by nitrogen and phosphorus. *Biotechnol. Bioeng.* 14:308-318.

Atlas. R.M. 1991. Bioremediation of Fossil Fuel Contaminated Soils. In R.E. Hinchee and R.F. Olfenbuttel (eds), *In situ* Bioreclamation. Butterworth-Heinemann, Boston. pp. 14-32.

Auret, B.J., Boyd, D.R. Robison, P.M., Watson C.G., Daly, J.W. and Jerina, D.M. 1971. The NIH shift during the hydroxylation of aromatic substrates by fungi. *Chem. Commun.* 1585-1587.

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

Autry, A.R. and Ellis, G.M. 1992. Bioremediation: An effective remedial alternative for petroleum hydrocarbon contaminated soil. Paper presented at the American Institute of Chemical Engineers, Spring National Meeting, New Orleans, U.S.A.

Bachmann, A. and Zehnder, A.J.B. 1988. Engineering significance in fundamental concepts in xenobiotic biodegradation in soil. In Contaminated soil '88. Kluwer academic publishers Netherlands.

Baeseman, J.L. and Novak, P.J. 2001. Effect of various environmental conditions on the transformation of chlorinated solvents by *Methanosarcina thermophila* cell exudates. *Biotechnol Bioeng.* 75: 634-631.

Baker, K.H. and D.S. Herson. 1994. Bioremediation. McGraw-Hill, Toronto.

Barbeau, D.S., Ellis, T.G. and Grady Jr, C.P.L. 1995. Oxygen leakage during respirometric measurements: Acaution on the use of PTFE tape. *Water Research* 29: 1211-1212.

Bardos, R.P., Forsythe, S., Westlake, K. 1996. The co-treatment of municipal and industrial waste. In M. de Bertoldi, P. Sequi, B. Lemmes and T Papi (eds), The Science of Composting. Blackie Academic and Professional, London. pp. 767-783.

Barkay, T. and Pritchard. 1988. Adaptation of aquatic microbial communities to pollutants. *Microbiol. Sci.* 5:165-169

Barles, R.W., C.G. Daughton and D.P.H. Hsieh. 1979. Accelerated parathion degradation in soil inoculated with acclimated bacteria under field conditions. *Arch. Environ. Contam. Toxicol.* 8: 647-660.

Barnes, P.W., Heaston, M.S. and Wharry, S. 2000. Composting treatment of explosives-contaminated soil at Pueblo Chemical Depot. In: G.B. Wickramanayake, A.R.Gavaska, J.T. Gibbs, J.L. Means (eds), Case studies in the remediation of chlorinated and recalcitrant compounds. Battelle Press, Columbus.

Baroon, Z., Mataqui, K., Yateem, A., Al-Othman, A. and Balba, M. 2001. Enhanced microbial degradation of hydrocarbons in oil-contaminated soil: Microcosm study. In Proceedings of the First International Congress on Petroleum Contaminated soils, Sediments and Water, London. 14-17 August, 2001.

Barr, D.P. and Aust, S.D. 1994. Mechanisms white rot fungi use to degrade pollutants. *Environ.Sci. Technol.* 28 (2): 79-87.

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

Bartha, R and Bossert, I. 1984. The treatment and disposal of petroleum refinery wastes. In Atlas, R.M (ed). Petroleum Microbiology. Macmillan, N.Y. 553 - 577.

Bartha, R. and Bossert, I. 1984. The treatment and disposal of refinery wastes. In: Atlas R.M. (Ed). Petroleum Microbiology. MacMillan, New York, U.S.A. pp. 435-473.

Bartha, R. and Atlas, R.M. 1977. The microbiology of aquatic oil spills. *Adv. Appl. Micrbiol.* 22: 225-266.

Bazelel, L., Hadar, Y. And Cerniglia, C.E. 1996. Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus. Appl. Environ. Microbiol.* 62: 292-295.

Benger, M. 1966. The disposal of liquid and solid effluents from oil refineries, Proceedings of the 21st Industrial Waste Conference, Purdue University, West Lafayette, Indiana. pp. 759-768.

Beppu, I. and K. Arima. 1969. Induction by mercuric ion of extensive degradation of cellular ribonucleic acid in *E. coli. J. Bacteriol.* 98: 888-897.

Bewley, R.J.F. 1992. Bioremediation of contaminated ground. p 270-281. In J.F. Rees. (ed),

Contaminated Land Treatment Technologies. Elsevier Applied science. London.

Black, W.V., Ahlert, R.C., Kosson, D.S. and Brugger, J.E. 1991. Slurry-Based biotreatment of contaminants sorbed onto soil constituents. In. R.E. Hinchee and R.F Olfenbttel (Eds), *On site Bioremediation: Processes for Xenobiotic and hydrocarbon Treatment.* Butterworth-Heineman, Stoneham, Massachusetts. pp. 408-421.

Block, R., Kabrick, R., Stroo, H. and Swett, G. 1992. Bioremediation of petroleum contaminated soils - Why doesn't it work sometimes? Paper presented at the American institute of chemical engineers, Spring National Meeting, New Orleans, U.S.A.

Bogan, B.W. and Lamar, R.T. 1999. Surfactant enhancement of white rot fungal PAH soil remediation. In: Bioremediation technologies for polycyclic aromatic hydrocarbon compounds. (Eds) Leeson,A. and Allemen, B.C. Battelle Press, Columbus. pp 81-86.

Bogan, B.W. and Lamar, R.T. 1996. Polycyclic aromatic hydrocarbon-degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular lignolitic enzymes. *Appl. Environ. Microbiol.* 62: 1597-1603.

Bogan, B.W., Szajkowics, A.M. and Paterek, J.R. 2000. Recent GTI advances in phytoremediation of polycyclic aromatic hydrocarbons. In proceedings of the GTI's Site Remediation Technologies and Environmental Management Practices in the Utility Industry, Orlando, Florida. 4-7 December, 2000.

Bogan, B.W. and Lamar, R.T. 1995. One-electron oxidation in the degradation of creosote polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 61: (7) 2631-2635.

Bollag, J.-M. and Bollag, W.B. 1995. Soil contamination and the feasibility of biological

remediation. In: Bioremediation science and application. (Eds) Skipper, H.D. and Turco, R.F. Soil scince special publication number 43. pp 1-12.

Boonchan, S., Britz, M.L. and Stanley, G.A. 2000. Degradation and mineralization of high-molecular weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. *Appl. Environ. Microbiol.* 60: (3) 1007-1019.

Boronin, A.M. 2001. Microorganisms for bioremediation of oil-contaminated sites. In Proceedings of the First International Congress on Petroleum Contaminated soils, Sediments and Water, London. 14-17 August, 2001.

Bossert, I. and Bartha, R. 1984. The fate of petroleum hydrocarbons in soil ecosystems. In: Petroleum Microbiology. (Ed) Atlas, R.M. MacMillan Publishing company, London. pp 399-434.

Bossert, I.D. and Bartha, R. 1986. Structure-bioderadability relationships of polycylic aromatic hydrocarbons in soil. *Bull. Environ. Contam. Toxicol.* 37: 490-495.

Bouchard, M., Pinnsonneault, L. and Tremblay, C. 2001. Biological monitoring of environmental exposure to polycyclic aromatic hydrocarbons in subjects living in the vicinity of a creosote impregnation plant. *Int Arch Occ Env Hea* 74: 505-513.

Bouchez, M., Blanchet, D. and Vandecasteele, J-P. 1995. Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain association: inhibition phenomena and cometabolism. *Appl. Microbiol. Biotechnol.* 43. pp. 156-164.

Bourquin, A.W., Accashian, J.V., DeLaet, J.D. 2001. Assessment of biodegradation processes at a complex site: Decision strategy and implementations for enhanced bioremediation. In proccedings

of the Sixth Annual In Situ and On site Bioremediation Conference, San Diego, California, June 3-7, 2001. Battelle Press, Columbus Ohio.

Boyle, C.D. 1995. Development of a practical method for inducing white rot fungi to grow into and degrade organopollutants in soil. *Can. J. Microbiol.* 41: 345-353.

Bradford, M.L. and Krishnamoorthy, R.A.J. 1991. Consider bioremediation for waste site cleanup. *Chemical Engineering Progress.* 87: 80-85.

Breedveld, G.D. and Sparrevik, M 2000. Nutrient-limited biodegradation of PAH in various soil strata at a creosote contaminated site. *Biodegradation* 6: 391-399.

Brender, J., Suarez, L., Hendricks, K., Holt, N. and Pichette, J. 1994. Health risks among residents living at former site of a creosote wood treatment facility. American Jounal of Epidermiology. 139 (11): 550.

Briglia, M., Rainey, F.A., Stackebrandt, E., Schraa, G. and Salkinoja-Salonen, M.S. 1996. *Rhodococcus percolatus* sp. Nov., a Bacterium degrading 2,4,6-Trichlorophenol. *Int. J. Syst. Bacteriol.* 46: 23-30.

Brilkov, A.V., Pechurkin, N.S. and Litvinov, V.V. 1980. Substrate inhibition and limitation of *Candida tropicalis* growth by phenol during continuous chemostat and pH-stat cultivation. *Microbiologija* 99 (3) 466-472.

Britto, R., Sherrard, J.H. and Truax, D.D. 1992. Development of a kinetic model for continous flow bioreactor treatment of petroleum contaminated soils. *47th Purdue Industrial Waste Conference Proceedings.* Lewis Publishers, Michigan. 133-142.

Britton, L.N. 1984. Microbial degradation of aliphatic hydrocarbons. In Gibson, D.T. (ed), Microbial

degradation of organic compounds. Marcel Dekker, Inc., NY. pp. 89-129.

Brock, T.D., and M. T. Madigan. 1991. Biology of microorganisms. 6th ed. Prentice-Hall, Englewood Cliffs. New Jersey.

Broholm,K. And Arvin, E. (2001) Biodegradation of creosote compounds: Comparison of experiments at different scales. *Ground Water Monitor* 21: 101-108.

Brown, R.A., Hicks, R.J. and Hicks, P.M. 1993. Use of air sparging for *in situ* bioremediation. *Proceedings of the second On Site and In Situ Bioreclamation Symposium*, Lewis Publishers, San Diego, CA.

Brown , R.A., Dey, J.C., and McFarland, W.E. 1991. Integrated site remediation combining groundwater treatment, soil vapor extraction and bioremediation. In R.E. Hinchee and R.F. Olfenbuttel (eds) *In Situ* biormediation and investigation for hydrocarbons and contaminated site remediation. Butterworth-Heineman, Stoneham, MA

Bull, A.T. and Slater, J.H. 1976. The teaching of continuous culture. In : Dean, A.C.R., Evans, C.G.T. and Melling, J. (Eds). Continuous culture 6: Application and New Fields. Ellis Hoorwood, Chichester. pp. 49-68.

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

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

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

Bungay, H.R. and Bungay, M.L. 1968. Microbial interaction in continuous culture. *Advances in Applied Microbiology.* 10: 269-290.

Buzea, D.C. and DeStefanis, E.J. 1999. Accelerated bioremediation as an alternative to conventional remedial technologies. In Proceedings of The Fifth International In Situ and On Site Bioremediation Conference, San Diego, May 19-22, 1999.Battelle Press, Columbus Ohio.

Campanella, B.F., Bock, C. and Schroder, P. 2002. Phytoremediation to increase the degradation of PCBs and PCDD/Fs-Potential and limitations. *Environ Sci Pollut.* 9: 73-85.

Carberry, J.B. 1994. Bioremediation of hydrocarbon-Contaminated soils using indigenous microbes. In D.L. Wise and D.J. Trantolo (eds) Remediation of hazardous waste contaminated soils. Marcel Dekker Inc. New York.

Carriere, P.P.E. and Mesania, F.A. 1995. Enhanced biodegradation of creosote-contaminated soil. *Waste Management.* 15 (8): 579-583.

Casillas, R.P., Crow Jr, S.A., Heinze, T.M., Deck, J. and Cerniglia, C.E. 1996. Initial oxidative and subsequent conjugative metabolites produced during the metabolism of phenanthrene by fungi. *J. Ind. Microbiol.* 16: 205-215.

Catallo, W.J. and Portier, R.J. 1992. Use of indigenous and adapted microbial assemblages in the removal of organic chemicals from soils and sediments.

Cerniglia, C.E. and Heitkamp, M.A. 1989. Microbial degradation of PAHs in the aquatic environment. In U. Vuranasi (ed), Metabolism of PAHs in aquatic environment. CRC. Press. Boca

Raton, Florida.

Cerniglia, C.E. 1992. Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation.* 3: 351-368.

Cerniglia, C.E., Freeman, J.P. and Mitchum, R.K. 1982. Glucuronide and sulfate conjugation in the fungal metabolism of aromatic hydrocarbons. *Appl. Environ. Microbiol.* 43: 1070-1075.

Cerniglia, C.E., Herbert, R .L. Dodge, R. H. Szaniszlo, P.J. and Gibson, D.T. 1979. Some Approaches to Studies on the Degradation of Aromatic Hydrocarbons by Fungi, In A.L Bourquin and H. Pritchard (eds), Microbial Degradation of Pollutants in Marine Environments. EPA Report No. EPA-600/9-79-012.EPA, Washington, DC.

Cerniglia, C.E. 1997. Fungal metabolism of polycyclic aromatic hydrocarbons: past , present and future applications in bioremediation. *J. Ind. Microbiol. Biotechnol.* 19: 324-333.

Cerniglia, C.E., Hebert, R.L., Szaniszlo, P.J. and Gibson, D.T. 1978. Fungal transformation of naphthalene. *Arch. Microbiol.* 117: 135-143.

Cerniglia, C.E. and Crow, S.A. 1981. Metabolism of aromatic hydrocarbons by yeasts. *Arch. Microbiol.* 129: 9-13.

Cerniglia C.E. 1993. Biodegradation of polycyclic aromatic hydrocarbons. *Curr. Opin. Biotechnol.* 4: 331-338.

Cerniglia, C.E. and Gibson, D.T. 1977. Metabolism of Naphthalene by *Cunninghamella elegans. Appl. Environ. Microbiol.* 34: 363 -370.

Cho, J.S., Kampbell, D.H., Wilson, J.T. and Digiulio, D.C. 1990. Soil bioventing demonstration

project. *National Technical Information Services Doc no* 91-162628/XAB. Newark. U.S.A.

Civilini, M., Domenis, C., de Bertoldi, M., and Sebastianutto, N. 1996. Composting and selected microorganisms for bioremediation of contaminated materials. In M. de Bertoldi, P. Sequi, B. Lemmes and T Papi (eds), The Science of Composting. Blackie Academic and Professional, London. pp. 884-891.

Clemente, A.R., Falconi, F.A., Anasawa, T.A, and Durrant, L.R. 1999. Degradation of aromatic pollutants by a non-basidiomycete ligninolytic fungus. In: Bioremediation technologies for polycyclic aromatic hydrocarbon compounds. (Eds) Leeson, A. and Alleman, B.C. Battelle Press, Columbus. pp 105-110.

Coe, R.H. 1952. Bench scale biological oxidation of refinery waste with activated sludge. *Sewage and Indust. Wastes.* 24: 731-749.

Cole, M.A., Liu, X. and Zhang, L. 1995. Effect of compost addition on pesticide degradation in planted soils. In Hinchee, R.E, Anderson, D.B. and Hoeppel, R.E. (eds), Bioremediation of recalcitrant organic. Battelle Press, Columbus, Ohio.

Connolly, M., Howe, F. and Mazur, M. 1999. Full-Scale bioremediation PAH. In Leeson, A. Alleman, B.C. (Eds), Bioremediation technologies for polycyclic aromatic hydrocarbon compounds. Battelle Press, Columbus.

Connor, J.R. 1988. Case study of soil venting. *Poll. Eng.* 20: 74-78.

Cook, S.V., Chu, A. Goodman, R.H. 2002. Leachability and toxicity of hydrocarbons, metals and salt contamination from flare pit soil. *Water Air Soil Poll.* 133: 297-314.

Cooney, J. 1984. The fate of petroleum pollutants in fresh-water ecosystems. In. Atlas, R.M. (Ed). Petroleum Microbiology. MacMillan, New York. pp. 399-433.

Cort, T. and Bielefeldt, A. 2000. Mechanism of nonionic surfactant inhibition of pentachlorophenol biodegradation. In proceedings of the 2000 conference on hazardous waste research, Denver, Colorado, USA. May 23-25, 2000.

Cort. T. and Bielefeldt, A. 2000. Effects of surfactants and temperature on PCP biodegradation. *J. Environ. Eng.* 126: 635-643.

Coutts, D.A.P., Senior, E. and Balba, M.T.M. 1987. Multistage hemostat investigation of interspecies interactions in a hexanoate-catabolizing microbial association isolated from anoxic landfill. *J. Appl. Bact.* 62: 251-260.

Criddle, C. 1993. The kinetics of co-metabolism. *Biotech. Bioengineering.* 41: 1048-1056.

Dagley, S and Gibson, D.T. 1965. The bacterial degradation of catechol. *Biochem J.* 95: 466-474.

Dagley, S. 1984. Introduction. In D.J. Gibson (ed), Microbial Degradation of Organic compounds. Marcel Dekker, Inc., New York. pp. 1-11.

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

De Wildeman, S., Nollet, H., Van Langenhove, H. and Verstraete, W. 2001. Reductive bioremediation of 1,2-dichloroethane by methanogenic grannular sludge in lab-scale UASB reactors. *Advances in Environmental research* 6: 17-27.

Dean, B.S., Lopez, G. and Kreyelok, E.P. 1992. Environmentally induced methhemoglobinemia in

infants. *Journal toxicology: Chemical Toxicology*. 30:127-131.

Deever, W.R. and White, R.C. 1978. Cited by Wilson, G.B. Sikora, L.J. and Parr, J.F. 1983. Composting of chemical industrial wastes prior to land application. In Parr, J.F., Marsh, P.B. and Kla, J.M. (eds), Treatment of hazardous wastes, Noyes Data Corporation, New Jersey, pp. 263-273.

Demple, B., Johnson, A. and Fung, D. 1986. Exonuclease III and endonuclease IV remove 3' blocks from DNA synthesis primers in $H_2O_2$ damaged *Escherichia coli*. *Proc. Natl. Acad.Sci. USA* 83: 7731-7735.

Demple, B. and Linn, S. 1982. 5,6-saturated thymine leisions in DNA production by ultraviolet light or hydrogen peroxide. *Nucleic Acids Res.* 10: 3781-3789.

Deuel, L.E., Brown, K.W. and Thomas, J.C. 1978. Soil disposal of API pit waste. In the 85$^{th}$ National Meeting of American Institute of Chemical Engineers, Philadelphia, PA.

Dhawale, S.W., Dhawale, S.S. and Dean-Ross, D. 1992. Degradation of phenanthrene by *Phanerochaete chrysosporium* occurs under ligninolitic as well as nonligninolitic conditions. *Appl. Environ. Microbiol.* 58: 3000-3006.

Di Leo, C., Barbaro, A., Bittoni, A., Colapietro, P., Fabiani, F. and Robertiello, A. 1999. Case History: Reclamation of a former manufacturing gas plant. In Leeson, A. Alleman, B.C. (Eds), Bioremediation technologies for polycyclic aromatic hydrocarbon compounds. Battelle Press, Columbus.

Diaz, L.F., Savage, G.M. and Golueke, C.G. 1996. Stabilization of hazardous wastes through biotreatment. In de Bertoldi, M., Bert, P. and Tiziano, P. (eds), The Science of Composting. Blackie Academic and Professional, London, pp. 1152-1156.

Dibble, J.T. and Bartha, R. 1979. Effect of environmental parameters on the biodegradation of oil sludge. *Appl Environ Microbiol* 37: 729-739.

Dick, V.B., Case, N.L. and Boyle, S.L. 2001. Enhanced bioremediation of high contaminat concentrations in source residual area. In proccedings of the Sixth Annual In Situ and On site Bioremediation Conference, San Diego, California, June 3-7, 2001. Battelle Press, Columbus Ohio.

Diehl, S.V. and Borazjani, H. 1998. Enhanced biodegradation of organic wood-preservative contaminated wastewater by commercial surfactants. In R.A. Meyers (ed), Encyclopedia of environmental analysis and remediation. pp3436-3451.

Duffy, B.E., Oudijk, G. and Guy, J.H. 1999. Enhanced aerobic bioremediation of petroleum UST releases in Puerto Rico. In Proceedings of The Fifth International In Situ and On Site Bioremediation Conference, San Diego, May 19-22, 1999.Battelle Press, Columbus Ohio.

Duncan, M., Bohn, H.L. and Burr, M. 1982. Pollutant removal from wood and coal fuel gases by soil treatment. *J. Air, Poll. Cont. Assoc.* 32:1175-1179.

Dyreborg, S. and Arvin, E. 1995. Inhibition of nitrification by creosote-contaminated water. *Wat. Res.* 29 (6): 1603-1606.

Dyreborg, S., Arvin, E., Broholm, K. and Löfvall, M. 1995. Biodegradation of creosote compounds coupled with toxicity studies. In R.E. Hinchee, F.J. Brockman and C.M. Vogel (eds), Microbial processes for remediation. Battelle Press, Columbus Ohio. pp. 213-221.

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

Edgehill, R.U. 1994. Pentachlorophenol removal from slightly acidic mineral salts, commercial sand,

and clay soil by recovered *Arthrobacter* strain ATCC 33790. *Appl Microbiol. Biotechnol* 41: 142-148.

Edgehill, R.U. and Finn, R.K. 1983. Microbial treatment of soils to remove pentachlorophenol. *Appl Environ Microbiol* 45: 1122-1125.

Efroymson,R.A. and Alexander, M. 1995. Reduction of mineralization of low concentrations phenanthrene because of sequestering in non-aqueous-phase liquids. *Environ. Sci. Technol.* 29: 515-521.

Eggen, T., Araneda, E., Vethe, Ø. and Sveum, P. 1999. Degradation of aged creosote-contaminated soil by *Pleurotus ostreatus.* In: Bioremediation technologies for polycyclic aromatic hydrocarbon compounds. (Eds) Leeson, A. and Alleman, B.C. Battelle Press, Columbus. pp 99-104.

Ellis, B., Harold, P. and Rees, J.F. 1999. Recycling of concentrated refinery tar residues using bioremediation. In Leeson, A. and Alleman, B.C. (eds), Bioremediation Technologies for polycyclic aromatic hydrocarbon compounds. Battelle, Columbus. pp.25-30.

Ellis, B. 1994. Reclaiming contaminated land: *In situ / ex situ* remediation of creosote- and petroleum hydrocarbon-contaminated sites. In: Bioremediation: Field experience. (Eds) Flatham, P.E., Jerger, D.E. and Exner, J.H. Lewis Publishers, London. pp 107-128.

Eriksson, M., Dalhammar, G. and Borg-Karlson, A.-K. 1999. Aerobic degradation of a hydrocarbon mixture in natural uncontaminated potting soil by indigenous microorganisms at 20°C and 6°C. *Appl. Microbiol. Biotechnol.* 51: 532-535.

Eriksson, M., Dalhammar, G. and Borg-Karlson, A.-K. 2000. Biological degradation of selected hydrocarbons in an old PAH/creosote contaminated soil from a gas work site. *Appl Microbiol Biotechnol.* 53: 619-626.

Feinstein, M .S., Miller, F.C. and Strom, P.E. 1986. Waste treatment composting as a controlled system. In Rhelm, H.J.and Reed, G. (eds), Biotechnology Vol. 8 Microbial degradation. VCH Publishers, NY. 363-398.

Ferris, J.P., Fasco, M.J., Stylianopoulou, F.L, Jerina, D.M., Daly, J.W. and Jeffrey, A.M. 1973. Monooxygenase activity in *Cunninghamella bainieri:* evidence for a fungal system similar to liver microsomes. *Arch. Biochem.Biophys.* 156: 97-103.

Field, J.A., de Jong, E., Feijoo-Costa, G. and de Bont, J.A.M. 1993. Screening for lignolytic fungi applicable to the biodegradation of xenobiotics. *Trends Biotechnol.* 11: 44-49.

Field, J.A., De Jong, E., Costa, G.F. and de Bont, J.A.J. 1992. Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. *Appl. Environ. Microbiol.* 58: (7) 2219-2226.

Findley, M., Fogel, S., Conway, L. and Taddeo, A. 1995. Field treatment of coal tar-contaminated soil based on results of laboratory treatability studies. In Young, L.Y. and Cerniglia, C.E. (eds), Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss, A John Whiley and Sons ,Inc., Publication, New York, pp. 487-514.

Flyvbjerg, J., Arvin, E., Jensen, B.K. and Olsen, S.K. 1993. Microbial degradation of phenols and aromatic hydrocarbons in creosote-contaminated groundwater under nitrate reducing conditions. *J. Contam. Hydrol.* 12: 133-150.

Foght, J.M. and Westlake, D.W.S. 1988. Degradation of polycyclic aromatic hydrocarbons and aromatic heterocycles by a *Pseudomonas* sp. *Can. J. Microbiol.*

Forster, J.C. 1995. Determination of the gravimetric water content and soil dry mass. In Alef, K. and Nannipieri, P. (eds), Methods in applied soil microbiology and biochemistry. Academic Press,

London.

Forth, H.D. 1984. Fundamentals of soil science.7th Ed. John Wiley and Sons, Inc., NY.

Foster, T. J. 1983. Plasmid determined resistance to antimicrobial drugs and toxic ions in bacteria. *Microbiol. Rev.* 47: 361- 407.

Fowler, M.G., Brooks, P.W., Northcott, M., King, M.W.G. Baker, J.F. and Snowdon, L.R. 1994. Preliminary results from a field experiment investigating some creosote components in a natural aquifer. *Org. Geochem.* 22 (3-5): 641-649.

Fritsche, W. 1992. Degradation of xenobiotics by fungi. In: Preprints of international symposium on soil decontamination using biological processes, Karlsruhe, Germany. 6-9 December 1992. Dechema, Frankfurt am Main. pp 31-36.

Fu, M.H. and Alexander, M.1992. Biodegradation of styrene in samples of natural environments. *Environ. Sci. Technol.* 26: 1540-1544.

Gagne, F., Trottier, S., Blaise, C., Sproul., J. and Ernst, B. 1995. Genotoxicity of sediment extracts obtained in the vicinity of a creosote-treated wharf to rainbow trout hepatocytes. *Toxicology Letter.* 78 (3) 175-182.

Gaudy, A.F. and Gaudy, E.T. (1980). Microbiology for Environmental Scientists and Engineers. McGraw-Hill Series in Water Resources and environmental Engineering. NY.
Ghoshal, S., Ramaswami, A. and Luthy, R.G. 1996. Biodegradation of naphthalene from coal tar and heptamethylnonane in mixed batch systems. *Environ. Sc. Technol.* 30: 1282-1291.

Ghoshal, S. and Luthy, R.G. 1998. Biodegradation kinetics of naphthalene in nonaqueous phase liquid-water mixed batch system: Comparison of model predictions and experimental results. *Biotech. Bioengin.* 57: 356-366.

Gibson, D.T. (Ed) 1984. Microbial degradation of organic compounds. Marcel Dekker, New york.

Gjssing, E.T. and Berglind, L. 1981. Adsorption of PAH to aquatic humus.*Arch. Hydrobiol.* 92: 24-30.

Gibson, D.T., and V. Subramanian. 1984. Microbial Degradation of Aromatic Hydrocarbons. In D.T. Gibson (ed), Microbial Degradation of Organic Compounds. Marcel Dekker, inc., New York. p. 182-252.

Glaser, J.A. and Lamar, R.T. 1995. Lignin-degrading fungi as degraders of pentachlorophenol and creosote in soil. In: Bioremediation science and applications. (Eds) Skipper, H.D. and Turco, R.F. Soil Science Society of America special publication number 43. pp 117-134.

Glass, D.J., Rapheal, T., Valo, R. and van Eyk, J. 1995. International activities in bioremediation: Growing markets and opportunities. In Hinchee, R.E., Kittel, J.A. and Reisinger, H.J. (eds), Applied bioremediation of petroleum hydrocarbons. Battelle Press, Columbus, Ohio, U.S.A.

Godsy, E.M., Goerlitz, D.F., and Grbic-Galic, D. 1992. Methanogenic biodegradation of creosote contaminants in natural and simulated groundwater ecosystems. *Ground Water.* 30: 232-242.

Gold, M.H., Wariishi, H. Valli, K. (1989). Extracellular peroxidases involved in lignin degradation by the white rot basidiomycete *Phanerochaete chrysosporium. ACS Sym Ser.* 389: 127-140.

Goldstein, R.M., L.M. Mallory, and M. Alexander. 1985. Reasons for possible failure of inoculation to enhance biodegradation. *Appl. Environ. Microbiol.* 50: 977-983.

Goldfarb, A.S. and Vogel, G.A. 1994. Tecnical aspects of site remediation: Soil vapor vacuum extraction. *Waste Management.* 14: 153-159.

Golgstein, N. 2001. Advances in composting contaminated soil. *BioCycle* 42: 60-63.

Gramss, G., Voight, K.-D. And Kirsche, B. 1999. Oxidoreductase enzymes liberated by plant roots and their effects on soil humic materials. *Chemosphere.* 38: 1481-1494.

Gramss, G. and Rudeschko, O. 1998. Activities of oxidoreductase enzymes in tissue extracts and sterile root exudates of three crop plants, and some properties of the peroxidase component. New Phytologist. 138: 401-409.

Harmsen, J., van den Toorn, A., Heersche, J., Riedstra, D. and van der Kooij, A. 1999. Use of residual substrate from mushroom farms to stimulate biodegradation of poorly available PAH. In A. Leeson and B.C. Alleman (eds), Bioremediation Technologies for Polycyclic Aromatic Hydrocarbon Compounds. Battelle Press, Columbus, Ohio.

Gray, N.C.C., Cline, P.R., Moser, G.P. and Gannon, D.J. 2000. Bioremediation of toxaphene contaminated soil. In: G.B. Wickramanayake, A.R.Gavaska, J.T. Gibbs, J.L. Means (eds), Case studies in the remediation of chlorinated and recalcitrant compounds. Battelle Press, Columbus.

Griffin, D.M. 1972. The ecology of soil fungi. Chapman and Hall Publishers, London. pp. 29-43.

Grover, R. 1967.Studies on the degradation of 4-amino-3,5,6-trichloropicolinic acid in soil. *Weed Res.* 7: 61-67.

Guerin, W.F. and Boyd, S.A. 1992. Differential bioavailability of soil-sorbed naphthalene to two bacterial species. *Appl. Environ. Microbiol.* 58: 1142-1152.

Gurujeyalakshmi, G. and Oriel, P. 1989. Isolation of phenol-degrading bacillus stearothermophilus and partial characterization. *Appl. Environ. Microbiol.* 55: 500-502.

Gutnik, D.L. and Rosenberg, E. 1977. Oil tankers and pollution: a microbiological approach. *Annu. Rev. Microbiol.* 31: 379.

Hadas, A., Sofer, M., Molina, J.A.E., Barak, P. and Clapp, C.E. 1992. Assimilation of nitrogen by soil microbial populations: $NH_4$ versus organic N. *Soil Biol. Biochem.* 24: 137-143.

Haggblom, M.M. and Valo, R. J. 1995. Bioremediation of chlorophenol wastes. In L.Y. Young and C.E. Cerniglia (eds), Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss, New York. pp. 389-434.

Halliwell, B. and Gutteridge, J.M.C. 1985. Free radicals in biology and medicine. Clarendon Press, Oxford.

Hamaker, J.W. 1972. Decomposition: quantitative aspects. In C.A.I. Goring and J.W. Hamaker (eds),Organic chemicals in the soil environment" Marcel Dekker, Inc., NY. pp 253-340.

Hammel, K.K., Gai, W.Z., Green, B. and Moen, M.A. 1992. Oxidative degradation of phenanthrene by the ligninolytic fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 58: 1832-1838.

Hamoda, M.F. and Al-Haddad, A.A. 1989. Treatment of petroleum refinery effluents in a fixed film reactor. *Wat. Sci. Techn.* 20: 131-140.

Hansch, C. and Fujita, T. 1964. A method for the correlation of biological activity and chemical structure. *J. Am. Chem. Soc.* 36: 1616-1626.

Hansen, L.D., Nestler, C., Ringelberg, D., Pritchard, H. and Jones-Meehan, J. 2000. Bioremediation of PAH/PCP contaminated soils from Popile wood treatment facility. In G.B.Wickramanayake, A.R.Gavaskar, J.T.Gibbs and J.L. Means (eds), Case studies in the remediation of chlorinated and recalcitrant compounds. Battelle Press, Columbus, Ohio.

Harker, A.R., Kim, Y. and Matrubutham, U. 1994. Application of genetic engineering to the field of bioremediation. In Wise, D.L. and Trantolo, D.J. (eds), Remediation of hazardous waste contaminated soils. Marcel Dekker, Inc., New York. pp. 77-96.

Harris, J.C. 2000. Land treatment of contaminated soil from wood-treating sites. In: G.B. Wickramanayake, A.R.Gavaska, J.T. Gibbs, J.L. Means (eds), Case studies in the remediation of chlorinated and recalcitrant compounds. Battelle Press, Columbus.

Heikkila, P.R., Hameila, N. Pyy, L. and Paunu, P. 1987. Exposure to creosote in the impregnation and handling of impregnated wood. *Scandinavian Journal of Work and Environmental Health.* pp. 431-437.

Heitkamp, M.A., Franklin, W. And Cerniglia, C.E. 1988a.Microbial metabolism of polycyclic aromatic hydrocarbons: Isolation and characterization of a pyrene-degrading bacterium. *Appl. Environ. Microbiol.* 54:2549-2555.

Heitkamp, M.A., Freeman, J.P., Miller, D.W. and Cerniglia, C.E. 1988b. Pyrene degradation by a *Mycobacterium* sp.: identification of ring oxidation and ring fission products. *Appl. Environ. Microbiol.* 54. 2556-2565.

Hicks, P., Pahr, M.R., Messier, J.P. and Gillespie, R. 2001. Aerobic bioremediation of MTBE and BTEX at a USCG facility. In proccedings of the Sixth Annual In Situ and On site Bioremediation Conference, San Diego, California, June 3-7, 2001. Battelle Press, Columbus Ohio.

Higson, F.K. 1991. Degradation of xenobiotics by white rot fungi. *Rev. Environ. Contamin. Toxicology*. 122: 111-152.

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

Hill, D.L., Phelps, T.J., Palumbo, A.V., White, D.C., Strandberg, G.W. and Donaldson, T.L. 1989. Bioremediation of polychlorinated biphenyls: Degradation Capabilities in Field Lysimeters. *Appl. Biochem. Biotech.* 20/21: 233-243.

Hinchee, R.E. and Arthur, M. 1991. Bench-scale studies of the soil aeration process for bioremediation of petroleum hydrocarbons. *J. Appl. Biochem. Biotechnol.* 28/29: 901-906.

Hinchee, R.E., Downey, D.C., Du Pont, R.R., Aggerwal, P. and Miller, R.N. 1991. Enhancing biodegradation of petroleum hydrocarbons through soil venting. *J. Haz. Mat.* 27: 315-325.

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

Hofmann, K.H. 1986. Oxidation of naphthalene by *Saccharomyces cerevisiae* and *Candida utilis*. *J. Basic. Microbiol.* 26: 109-111.

Hofrichter, M., Günther, T. and Fritsche, W. 1993. Metabolism of phenol, chloro- and nitrophenols by the *Penicillium* strain *Bi 7/2* isolated from a contaminated soil. *Biodegradation* 3: 415-421.

Holladay, D.W., Hancher, C.W. and Scott, C.D. 1978. Biodegradation of phenolic waste liquors in stirred tank, packed-bed and fluidized-bed bioreactors. *J. Wat. Pollution Fed.* 49: 2573-2589.

Holusha, J. 1991. Using bacteria to control pollution. The New York Times. C6, March 13.

Hoover, D.G., Borgonovi, G.E., Jones, S.H. and Alexander, M. 1986.Anomalies in mineralization of low concentration of organic compounds in lake water and sewage. *Appl. Environ. Microbiol.* 51: 226-232.

Hughes, J.B., Beckles, D.M.., Chandra, S.D. and Ward, C.H. 1997. Utilization of bioremediation processes for the treatment of PAH-contaminated sediments. *J. Industrial Microbiology & Biotechnology.* 18:152-160.

Hughes,T.J., Claxton, L.D., Brooks, L., Warren, S., Brenner, R. and Kremer, F. 1998. Genotoxicity of bioremediated soils from the Reilly tar site, St. Louis Park, Minnesota. *Environmental Health Perspectives.* 106: 1427-1433.

Hunter, J.V., Finstein, M.S., Suler, D.J and Bopal, R.R. 1981. Cited by Feinstein, M .S., Miller, F.C. and Strom, P.E. 1986. Waste treatment composting as a controlled system. In Rhelm, H.J.and Reed, G. (eds), Biotechnology Vol. 8 Microbial degradation. VCH Publishers, NY. 363-398.

Iranzo, M., Sainz-Pardo, I. and Boluda, R. 2001. The use of microorganisms in environmental remediation. *Ann Microbiol.* 51: 135-145.

Jamison, V.M., Raymond, R.L. and Hudson J.O. 1975. Biodegradation of high-octane gasoline in ground water. *Dev. Ind. Microbiol.* 16: 305-312.

Jannasch, H.W. 1967. Enrichment of aquatic bacteria in continuous culture. *Arciv. Für Mikrobiologie.* 59: 165-173.

Jerina, D.M., Selander, H., Yagi, H., Wells, M.C., Davey, J.F., Mahadevan, V. and Gibson, D.T. 1976. Dihydrodiols from anthracene and phenanthrene. *J. Am. Chem. Soc.* 98: 5988-5996.

Jobson, A., McLaughlin, M., Cook, F.D. and Westlake, D.W.S. 1994. Effect on amendment on the microbial utilization of oil applied to soil. *Appl. Microbiol.* 27: 166-171.

Jøgenson, B.B. 1977. Bacterial sulfate-reduction within microniches of oxidized marine sediments. *Marine Biology.* 41: 7-17.

Jones, G.L., Jansen, F. and McKay, A.J. 1973. Substrate inhibition of the growth of bacterium NCIB 8250 by phenol. *J. Gen. Microbiol.* 74: 139-148.

Kaake, R.H., Roberts, D.J., Stevens, T.O. Crawford, R.L and Crawford, D.L. 1992. Bioremediation of soils contaminated with the herbicide 2-*sec*-Butyl-4,6-Dinitrophenol (Dinoseb). *Appl. Environ. Microbiol.* 58: 1683-1689.

Karickhoff, S.W. 1980. Sorption kinetics of hydrophobic pollutants in natural sediments. In Baker, R.A. (Ed). Contaminants and sediments. Vol. 2. Fate and transport studies, modeling, toxicity. Ann Arbor science, Ann Arbor. pp.193-205.

Karlehagen, S., Anderson, A. and Ohlson, C. 1992. Cancer incidence among creosote-exposed workers. *Scandinavian Journal of Work and Environmental Health.* pp. 26-29.

Kästner, M, Breuer-Jammali, M and Mahro, B. 1998 Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. *Appl. Environ. Microbiol.* 64 (1) 359-362.

Kennedy, R.S., Finnerty, W.R., Sudarsana, K. and Young, R.A. 1975. *Arch. Microbiol.* 102: 75.

Kennes, C. and Lema, J.M. 1994. Simultaneous biodegradation of *p*-cresol and phenol by the basidiomycete *Phanerochaete chrysosporium. J. Ind. Microbiol.* 13: p. 311-314.

Kerry E. 1990. Microorganisms colonizing plants and soil subjected to different degrees of human activity, including petroleum contamination, in the Vestfold hills and MacRobertson Land, Antarctica. *Polar Biol.* 10 : 423-430.

Kiehlmann, E.., Pinto, L. and Moore, M. 1996. The biotransformation of chrysene to *trans*-1,2-dihydroxy-1,2-dihydrochrysene by filamentous fungi. *Can. J. Microbiol.* 42: 604-608.

Kilbane, J. J., D. K. Chatterjee, and A.M. Chakrabarty. 1983. Detoxification of 2,4,5, Trichlorophenoxyacetic acid from contaminated soil by *Pseudomonas cepacia. Appl. Environ. Microbiol.* 45: 1697-1700.

Kimball, S.L. 1994. The use of surfactants to enhance pump-and-treat process for *in situ* soil remediation. In D.L. Wise and D.J. Trantolo (eds). Remediation of hazardous waste contaminated soils. Marcel Dekker Inc. New York.

Kincannon, D.L., Stover, E.L., Nicols, V. And Medley, D. 1983. Removal mechanisms for toxic priority pollutants. *J. Wat. Pollution Control Fed.* 55: 139-148.

King, R.B., Long, G.M. and Sheldon, J.K. 1992. Practical Environmental Bioremediation. Lewis Publishers, CRC Press Inc., Boca Raton, FL.

Kingsbury, G.C., Sims, R.C., White J.B. 1979. Multimedia goals for environmental assessment. EPA 600/7-79-176b, PB 81-166 548.

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

Kittel, J.A., Hinchee, R.E, Hoeppel, R. and Miller, R. 1995. Bioslurping-Vacuum-enhanced free-product recoverycoupled with bioventing: A case study. In *Proceedings of the In Situ and On Site Bioreclamation Third International Symposium,* San Diego, CA.

Knackmuss, H.-J. 1992. Potentials and limitations of microbes to degrade xenobiotics. In Soil decontamination using biological processes. Organized by Deutsche Gesellschaft für Chemisches Apparatewesen, Chemisce Technik und Biotechnologie e.V, Vereinigung für Allgemeine und Angewandte Mikrobiologie e.V and EFB Task Group on Soil Decontamination using Biological processes. pp3-9.

Koenigsberg, S.S. and Farone, W.A. 1999. The use of Hydrogen Release Compound (HRC™) for CAH bioremediation. In Proceedings of The Fifth International In Situ and On Site Bioremediation Conference, San Diego, May 19-22, 1999.Battelle Press, Columbus Ohio.

Koenigsberg, S.S. and Sandefur, C. 2001. Efficacy of Oxygen Release Compound: A six year review. In proceedings of The17th Annual International Conference on Contaminated Soils, Sediments and Water. University of Massachusetts, Amherst, Massachusetts, 22-24 October, 2001.

Kotterman, K., van Lieshout, J., Grotenhuis, T. and Field , J. 1999. Development of white rot fungal technology for PAH degradation. In Leeson, A. and Alleman, B.C. (eds), Bioremediation Technologies for polycyclic aromatic hydrocarbon compounds. Battelle, Columbus. pp.25-30.

Kubota, H. and Nakasaki, K. 1991. Accelerated thermophilic composting of garbage. *BioCycle* 32: 66-68.

Kuyukina, M.S., Ivshina, I.B., Ritchkova, M.I., Kostarev, S.M., Philp, J.C., Cunningham, C.J. and Christofi, N. 2001. Bioremediation of crude oil contaminated soil using slurry-phase biological treatment and landfarming techniques. In Proceedings of the First International Congress on Petroleum Contaminated soils, Sediments and Water, London. 14-17 August, 2001.

Lajoie, C.A. and Strom, P.F. 1994. Biodegradation of polynuclear aromatic hydrocarbons in coal tar oil contaminated soil. In D.L. Wise and D.J. Trantolo (eds) Remediation of hazardous waste contaminated soils. Marcel Dekker Inc. New York.

Lamar, R.T., Larsen, M.J., Kirk, T.K. and Glaser, J.A. 1987. Growth of the white rot fungus *Phanerochaete chrysosporium* in soil. In Land disposal, remedial action, incineration and treatment ofhazardous waste: Proceedings of the 13th Annual Research Symposium. US EPA. pp. 419-424.

Lambert, M., Kremer, S. and Anke, H. 1992. Degradation of PAHs by fungi: comparison of deuteromycetes isolated from polluted and non-polluted soil with saprophytic basidiomycetes. In: Preprints of international symposium on soil decontamination using biological processes, Karlsruhe, Germany. 6-9 December 1992. Dechema, Frankfurt am Main. pp 333-341.

Larson, J.R., Voegeli, V.J. 2001. Aerobic and anaerobic bioremediation of 1,1-DCE and vinyl chloride in groundwater. In proccedings of the Sixth Annual In Situ and On site Bioremediation Conference, San Diego, California, June 3-7, 2001. Battelle Press, Columbus Ohio.

Launen, L., Pinto, L., Wiebe, C., Kiehlmann, E. and Moore, M. 1995. The oxidation of pyrene and benzo(a)pyrene by nonbasidiomycete soil fungi. *Can. J. Microbiol.* 41. p 477-488.

Leavitt, M.E., Graves, D.A. and Lang, C.A. 1991. Evaluation of bioremediation in a coal-coking waste lagoon. In Sayler, G.S., Fox, R. and Blackburn, J.W. (eds), Environmental Biotechnology for Waste Treatment, Plenum Press, NY, pp. 71-84.

Lee, M.D., Thomas, J.M., Borden, R.C., Bedient, P.B., Ward, C.H. and Wilson, J.T. 1988. Biorestoration of aquifers contaminated with organic compounds. CRC *Crit. Rev. Environ. Control.* 18: 29-89.

Lees, Z.M. 1996. Bioremediation of oil-contaminated soil: A South African case study. A PhD thesis

submitted to the University of Natal, Pietermaritzburg, South Africa.

Leland, J.E., Mullins, D.E. and Berry, D.F. 2001. Evaluating environmental hazards of land applying composted diazinon using earthworm bioassays. *J. Environ Sci Heal. A.* 36: 821-834.

Lewandowski, G., Salerno, S., McMullen, N., Gneiding, L. and Abramowitz, D. 1986. Biodegradation of toxic chemicals using commercial preparations. *Environmental Progress* 5(3): 212 - 217.

Lewandowski, G., Baltzix, B. and Varuntanya, C.P. 1988. The use of pure cultures as a means of understanding the performance of mixed cultures in the biodegradation of phenolics, in biotechnology for degradation of toxic chemicals in hazardous wastes. Noyes Publication, Park Ridge, New Jersey.

Li, K.Y. and Zhang, Y.B. 1996. Oxygen transfer limitation in a respirometer. *Water Environment Research* 68: 36-41.

Limbert, E.S.B. and Betts, W.B. (1995). Kinetics of bio-oxidation of a medium comprising phenol and a mixture of organic contaminants. *Appl. Microbiol. Biotechnol.* 43. pp 165-170.

Linkenheil, R. 1988. On-site biological treatment of creosote-contaminated soils. In Omen, G.S. (ed), Environmental Biotechnology: Reducing Risks form Environmental chemicals through biotechnology. Plenum Press, New York, p. 455.

Liu, Z., Laha, S. and Luthy, R.G. 1991. Surfactant solubilization of polycyclic aromatic hydrocarbon compounds in soil water suspensions. *Water Sci. Technol.* 23: 475-485.

Londry, K.I., Fedorak, P.M. and Suflita, J.M. 1997. *Appl. Environ Micrbiol.* 63: 3170-3173.

Loske, D., Hütterman, A., Majcherczyk, A., Zadra□il, F., Lorsen, H. and Waldinger, P. 1989. Use of white rot fungi for the clean-up of contaminated sites. In Coughlan, M.P. and Amaral Collaço, M.T. (eds), Advances in Biological Treatments of Lignocellulosic materials. Elsevier, London, pp. 311-322.

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

Lynch, J.M. and Poole, N.J. (Eds). 1979. Microbial ecology: A conceptual approach. Blackwell Scientific Publications, Oxford, 1979.

MacGillivray, A.R. and Shiaris, M.P. 1993. Biotransformation of polycyclic aromatic hydrocarbons by yeasts isolated from coastal sediments. *Appl. Environ. Microbiol.* 59: 1613-1618.

Madhosnigh, C. 1958. The metabolic detoxification of 2,4-dinitrophenol by *Fusarium oxysporium Can. J. Microbiol.* 7: 553-567.

Malins, D.C., Krohn, M.M., Myers, M.S., Rhodes., L.D., Brown, D.W., Krone, C.A., McCain, B.B. and Chan, S.-L. 1985. Toxic chemicals in sediments and biota from a creosote polluted habour: relationship with hepatic neoplasms and other hepatic lesions in English sole (*Parophrys vetulus*) *Carcinogenesis* 6: 1463-1469.

Manohar, S. and Karegoudar, T.B. 1998. Degradation of naphthalene by cells of *Pseudomonas* sp. strain NGK1 immobilized in alginate, agar and polyacrylamide. *Appl. Microbiol. Biotechnol.* 49: 785-792.

Martens, R. And Zadrazil, F. 1992. Screening of white rot fungi for their ability to mineralize polycyclic aromatic hydrocarbons in soil. In: Preprints of international symposium on soil decontamination using biological processes, Karlsruhe, Germany. 6-9 December 1992. Dechema,

Frankfurt am Main. pp 31-36.

Mason, C.A., Sticher, P. and Hamer, G. 1992. Microbes to the rescue? Factors affecting the survival and maintenance of activity of introduced bacteria for environmental clean-up programmes. *EAWAG-News* 32/33: 4-9.

McAllister, K.A., Lee, H. and Trevors, J.T. 1996. Microbial degradation of pentachlorophenol. *Biodegradation.* 7: 1-40.

McDermott, J.B., Unterman, R., Brennan, M.J., Brooks, R.E. Mobley, D.P., Scwartz, C.C. and Dietrich, D.K. 1989. Two strategies for PCB soil remediation: biodegradation and surfactant extraction. *Environ. Progress* 8: 46-51.

McGugan, B.R. 1997. Exploitation of indigenous fungi in low-cost ex-situ attenuation of oil-contaminated soil. An M.Sc. Thesis submitted to the Department of Microbiology and Plant Pathology, University of Natal, Pitermaritzburg, South Africa.

Merkel, G.J., S.S. Stapleton, and J.J. Perry. 1978. Isolation of peptidoglycan of gram-negative hydrocarbon-utilizing thermophilic bacteria. *J. Gen. Microbiol.* 109: 141-148.

Meyer, S. and Steinhart, H. 2001. Fate of PAHs and hetero-PAHs during biodegradation in a model soil/compost-system: Formation of extractable metabolites. *Water Air Soil Poll.* 132: 215-231.

Middaugh, D.P., Hemmer, M.J. and Lores, E.M. 1988. Teratological effects of 2,4-dinitrophenol, 'produced water' and naphthalene on embryos of the inland silverside *Menidiabeyllina. Dis. Aquat. Org.* 4: 53-65.

Pinsky, C. and Bose, R. 1988. Pyridine and other coal tar constituents as free radical generating environmental neurotoxicants. *Mol. Cell. Biochem.* 84:217-222.

Mihelcic, J.R and R.C. Luthy. 1991. Sorption and microbial degradation of naphthalene in soil-water suspensions under denitrification conditions. *Environ. Sci. Technol.* 25: 169-177.

Mihelcic, J.R and R.C. Luthy. 1988. Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. *Appl. Environ. Microbiol.* 54: 1182-1187.

Miller, M.E. and Alexander, M. 1991. Kinetics of bacterial degradation of benzylamine in a montmorillonite suspension. *Environ. Sci. Tech.* 25:240-245.

Miller, R.M. 1995. Surfactant-Enhanced bioavailability of slightly soluble organic compounds. In Skipper, H.D. and Turco R.F. (eds). Bioremediation science and application. Soil science special publication number 43.

Molmann, F.W. 1929. The biochemical oxidation of phenol wastes. *Amer. J. Public Health.* 19: 145-151.

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

Mueller, J.G., S.E. Lantz, B.O. Blattmann, and P.J. Chapman. 1991. Bench-scale evaluation of alternative biological treatment processes for the remediation of pentachlorophenol- and creosote-contaminated materials: solid-phase bioremediation. *Environ. Sci. Technol.* 25: 1045-1055.

Mueller, R.H. and Babel, W. 1994. Phenol and its derivatives as heterotrophic substrates for microbial growth - an energetic comparison. *Appl. Microbiol. Biotechnol.* 42: 446-451.

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

Mulder, H. Breure, A.M. and Rulkens W.H. 2001. Prediction of complete bioremediation periods for PAH soil pollutants in different physical states by mechanistic models. *Chemosphere.* 43: 1085-1094.

Mulkins-Phillip, G.J. and Stewart, J.E. 1974. Effect of 4 dispersants on biodegradation and growth of bacteria on crude oil. *Appl. Microbiol.* 28: 547-552.

Muniz, H., Koenigsberg, S.S. and Lapus, K. 2001. Accelerating aerobic biodegradation of GRO/DRO: A five year study. In proccedings of the Sixth Annual In Situ and On site Bioremediation Conference, San Diego, California, June 3-7, 2001. Battelle Press, Columbus Ohio.

Murray, W., Dooley, M. and Koenigsberg, S.S. 2001. Enhance bioremediation of chlorinated solvents. In proccedings of the Sixth Annual In Situ and On site Bioremediation Conference, San Diego, California, June 3-7, 2001. Battelle Press, Columbus Ohio.

Nakahara, T., Hisasuka, K. and Minoda, Y. 1981. *J. Ferment. Technol.* 59: 415-418.

Nelson, A.S. 1994. Passive remediation delivers results. *Pollution Engineering.* Sept: 40-43.

Nicholas, D. 1973. Wood deterioration and its prevention by preservative treatments. Vol.II. Preservatives and Preservative Systems. Syracus, University Press. Syracus. pp. 2-17.

Nishino, S.F. and Spain, J.C. 2001. Technology status review: Bioremediation of dinitrotoluene (DNT). WWW.estcp.org/documents/techdocs/ONT_Report.

Nohynek, L., Suhonen, E., Nurmiaho-Lassila, E.-L., Hantula, J. and Salkinoja-Salonen. 1995. Description of four pentachlorophenol-degrading bacterial strains as *Sphingomonas chlorophenolica* sp. nov. *Syst. Appl. Microbiol.* 18:527-538.

Novotný, C., Erbanová, P., Šašek, V., Kubátová, A., Cajthaml, T., Lang, E., Krahl, J. and Zadra□il, F. 1999. Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi. *Biodegradtion.* 10: 159-168.

Nyholm, N., Lindgaard-Jorgensen, P. and Hansen, N. 1984. *Ecotoxicol. Environ. Saf.* 8: 451-470.

O'Melia, B.C., Siegel, R., Dupuy, G., Compeau, G.C., Thomas, R. and Syverson, T. 1999. *In Situ* bioremediation of degradable contaminants from a former wood treatment facility. In Leeson, A. Alleman, B.C. (Eds), Bioremediation technologies for polycyclic aromatic hydrocarbon compounds. Battelle Press, Columbus.

Oberbremer, A., Müller-Hertig, R.and Wagna, F. 1990. Effect of the addition of microbial surfactant on hydrocarbon degradation in a soil population in a stirred reactor. *Appl. Microbiol. Biotechnol.* 32: 485-489.

Oren, A. 1988. The Microbial Ecology of the Dead Sea. p 193-229. In K.C. Marshal (ed), Advances in microbial ecology, vol. 10. Plenum Press, New York .

Otte, M., Gagnon, J., Comeau, Y., Matte, N., Greer, C.W. and Samson, R. (1994) Activation of an indigenous microbial consortium for bioaugmentation of pentachlorophenol/creosote contaminated soils. *Appl. Microbiol. Biotechnol.* 40 : 926-932.

Pachon, C.S. 2001. Update on the treatment technologies use in EPA's superfund program. In proceedings of The 17th Annual International Conference on Contaminated Soils, Sediments and Water. University of Massachusetts, Amherst, Massachusetts, 22-24 October, 2001.

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

Parkes, R.J. 1982. Methods for enriching, isolating and analyzing microbial communities in laboratory systems. In A.T. Bull and J.H. Slater (ed), Microbial Interactions and Communities Vol. 1. Academic Press, Jovanovich London. pp. 45-99.

Parkinson, D.T., Gray, T.R.G. and Williams, S.T. 1971. Methods for studying the ecology of soil microorganisms. IBP Handbook No.19. Blackwell Scientific Publications Ltd., Oxford.

Parr, J.F., L.J. Sikora, and W.D. Burge. 1983. Factors affecting the degradation and inactivation of waste constituents in soil. p 20-49. In J.F. Parr, P.B. Marsh and J.M. Kla (ed), Land Treatment of Hazardous wastes. Noyes publishing, Park Ridge New Jersey.

Paszczynski, A., Huynh, V.-B. And Crawford, R. 1985. Enzymatic activities of an extracellular manganese-dependent peroxidase from *Phanerochaete chrysosporium. FEMS Microbiol. Lett.* 29: p. 37-41.

Paul, E.A. and Clark, F.E. 1989. Soil microbiology and biochemistry. Academic Press, Inc., San Diego.

Pearce, K., Snyman, H., van Heerden, H., Greben, H. and Oellermann, R.A. 1995. Bioremediation technology for the treatment of contaminated soils in South Africa. Water Research Commission, Division of Water Technology, CSIR. WRC Report No. 543/1/95. Pretoria, South Africa.

Pearce,K. and Oellermann, R.A. 1994. Cited by Pearce, K., Snyman, H., van Heerden, H., Greben, H. and Oellermann, R.A. 1995. Bioremediation technology for the treatment of contaminated soils in South Africa. Water Research Commission, Division of Water Technology, CSIR. WRC Report No. 543/1/95. Pretoria, South Africa.

Pfaender, F.K., R.J. Shimp and R.J. Larson. 1985. Adaptation of estuarine ecosystems to the biodegradation of nitrilotriacetic acid: Effects of preexposure. *Environ. Toxicol. Chem.* 4:587-593.

Phillips, T.M., Liu, D., Seech, A.G., Lee, H. and Trevors, J.T. 2000. Bioremediation in field box plots of a soil conterminated with wood preservatives: A comparison of treatment conditions using toxicity testing as a monitoring technique. *Water, Air and Soil Pollution* 121: 173-187.

Piotrowski, M.R. 1991. Biodegradation of hydrocarbon contaminated surface water, groundwater and soils: The Microbial Ecology Approach. In P.T. Kostecki and E.J. Calabrese (eds), Hydrocarbon contaminated soils and groundwater; Analysis, Fate, Environmental and Public Health Effects Remediation Vol. 1. p.203-238.

Piccinini, S., Rossi, L., Bonazzi, G. and Dall' Orso, G. 1996. The Emilia-Romaga Experiment in animal manure composting. In M. de Bertoldi, P. Sequi, B. Lemmes and T Papi (eds), The Science of Composting. Blackie Academic and Professional, London. pp. 1275-1280.

Piotrowski, M.R. 1991. Bioremediation of hydrocarbon contaminated surface water, groundwater, and soils: The microbial ecology approach. In Kostecki, P.T and Calabrese, E.J. (Eds). Hydrocarbon contaminated soils and groundwater: Analysis Fate Environmental and public Health Effects Remediation. Vol. 1. Lewis Publishers, Chelsea, Michigan.

Pirt, S.J. 1975. Principles of microbes and cell cultivation. Blackwell, Oxford.

Platt, J.J., Backhus, D.A., Capel, P.D. and Eisenreich, S.J. 1996. Temperature-dependent sorption of naphthalene, phenanthrene and pyrene to low organic carbon sediments. *Environ. Sci. Technol.* 30: 751-760.

Poeton,.T.S., Stensel, H.D. and Strand S.E. 1999. Biodegradation of polyaromatic hydrocarbons by marine bacteria: effect of solid phase on degradation kinetics. *Water Research.* 33: 868-880.

Pointing, S.B. 2001. Feasibility of bioremediation by white-rot fungi. *Appl Microbiol Biot.* 57: 20-33.

Portier, R.J. 1989. Examination of site data and discussion of microbial physiology with regards to site remediation. In proceedings of the 10$^{th}$ National Congress: Superfund '89. Washington, D.C. Haz. Mat. Control.Res. Inst.

Portier, R.J., Hoover, D.G. and Miles, M.S. 1996. Microbial-assisted remediation of creosote- and pentachlorophenol-treated wood products. *J. Ind. Microbiol.* 17: 1-5.

Pothuluri, J.V., Heflich, R.H., Fu, P.P. and Cerniglia, C.E. 1992. Fungal metabolism and detoxification of fluoranthene. *Appl. Environ. Microbiol.* 58: 937-941.

Pothuluri, J.V., Freeman, J.P., Evans, F.E. and Cerniglia, C.E. 1990. Fungal transformation of fluoranthene. *Appl. Environ. Microbiol.* 56: 2974-2983.

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

Pothuluri, J.V., Freeman, J.P., Evans, F.E. and Cerniglia, C.E. 1992. Fungal metabolism of acenaphthene by *Cunninghamella elegans. Appl. Environ. Microbiol.* 58: 3654-3659.

Pothuluri, J.V., Selby, A., Evans, F.E., Freeman, J.P. and Cerniglia, C.E. 1995. Transformation of chrysene and other polycyclic aromatic hydrocarbon mixtures by the fungus *Cunninghamella elegans. Can. J. Bot.* 73: 1025-1033.

Potter, C.L., Glaser, J.A., Hermann, R. and Dosani, M.A. 1999. Remediation of contaminated East River sediment by composting technology. In A. Leeson and B.C. Alleman (eds), Bioremediation technologies for polyclic aromatic hydrocarbon compounds. The fifth international *in situ* and on-site bioremediation symposium. San Diego, California, April 19-22, 1999. Battelle Press, Columbus. pp. 31-36.

Potter, T.M. 1989. Analysis of petroleum contaminated soil and water: An overview. In: Calabrese, E.J. and Kostecki, P.T. (Eds). Petroleum contaminated soils (Vol. 2). Lewis Publishers, N.Y.pp. 97-109.

Pozdnyakova,N., Turkovskaya, O., Ignatov, V. 2001. Degradation of oil hydrocarbons by White-Rot fungi. In Proceedings of the First International Congress on Petroleum Contaminated soils, Sediments and Water, London. 14-17 August, 2001.

Pramer, D. And Bartha, R. 1972. Preparation and processing of soil samples for bioremediation studies. *Env. Lett.* 2: 217-224.

Prescot, L.M., Harley, J.P. and Klein, D.A. 1999. Microbiology. Wm.C. Brown and Company, Inc., Dubuque, Iowa.

Prokop, W.H. and Bohn, H.L. 1985. Soil bed system for control or rendering or rendering plant odors. *JAPCA*. 35: 1332.

Radwan, S.S., Sorkhoh, N.A. Fardoun, F and Al-Hassan R.H. (1995). Soil management enhancing hydrocarbon biodegradation in the polluted Kuwaiti desert. *Appl. Microbiol. Biotechnol.* 44 : 265-270.

Ramanand, K., Balba, M.T.M. and Duffy, J. 1993. Anaerobic metabolism of chlorinated benzene in soil under different redox potentials. *Proceedings of the Second On Site and In Situ Bioreclamation Symposium,* Lewis Publishers, San Diego, CA.

Raper, K.B. and Thom, C. 1968. A manual of the Penicillia. Hafner Pub. Co., New York. p.126-132.

Raymond, R.L., Hudson, J.O. and Jamieson, V.W. 1976. Oil degradation in soil. *Appl. Environ. Microbiol.* 31: 522-535.

Reid, B.J., Jones, K.C., Semple, K.T. and Fermor, T.R. 1999. Bioremediation potential of PAHs in compost. In Leeson, A. and Alleman, B.C. (eds), Bioremediation Technologies for polycyclic aromatic hydrocarbon compounds. Battelle, Columbus. pp.25-30.

Renoux, A.Y., Millette, D., Tyagi, R.D. and Samson, R.1999. Detoxification of fluorene, phenanthrene, carbazole and p-cresol in columns of aquifer sand as studied by the Microtox assay. *Water Research* 33 (9) 2045-2052.

Republic of South Africa. Government Gazette No. 19519, Act No. 107. National Environmental Management Act, 1998.

Richmond, M.H. 1968. *Essays Biochem* 4: 105-154.

Ringelberg, D. and Reynolds, M. 2001. Integrated analytical approach for determining bioremediation effectiveness. In proceedings of The17th Annual International Conference on Contaminated Soils, Sediments and Water. University of Massachusetts, Amherst, Massachusetts, 22-24 October, 2001.

Riser-Roberts, E.1992. Bioremediation of petroleum contaminated sites. C.K. Smoley, Boca Raton, Florida. pp.82-96.

Robert, D.J. and Fedorak, P. M. and Hrudey, S.E.1987. Comparison of the fate of methyl carbons of m-cresol and p-cresol in methanogenic consortia. *Can. J. Microbiol.* 33:335-338.

Rodriguez, R., Montalvo, C.P., Dendooven, L., Esparza, F.G. and Fernandez, L.L. 1999. Degradation of benzo(a)pyrene by white rot fungi. In: Bioremediation technologies for polycyclic aromatic hydrocarbon compounds. (Eds) Leeson, A. and Alleman, B.C. Battelle Press, Columbus. pp 93-98.

Rosenberg, E., Gottlieb, A. and Rosenber, M. 1983. Inhibition of bacterial adherence to hydrocarbon and epithelial cells by emulsan. *Infect. Immun.* 39: 1024-1028.

Rosenfeld, J.K. and R.H. Plumb, Jr.1991. Groundwater contamination at wood treatment facilities. *Ground Water Monit. Rev.* Winter: 133-140.

Ross, D. 1991. Slurry-phase bioremediation: case studies and cost comparison. *Remediation.* 1: 469-474.

Ross, D. 1987. Pilot-scale land treatment study. In Abstracts of the 8th Annual SETAC meeting. Environmental Risks: recognition, Assessment, and Management, Pensacola, Florida, Nov. 9-12, 1987.

Sack, U. and Fritsche, W. 1997. Enhancement of pyrene mineralization in soil by wood-decaying fungi. *FEMS Microbiol. Ecol.* 22: 77-83.

Sack, U., Heinze, T.M., Deck, J., Cerniglia, C.E., Martens, R., Zadrazil, F. and Fritsche, W. 1997. Comparison of phenanthrene and pyrene degradation by different wood-decaying fungi. *Appl. Environ. Microbiol.* 63: 3919-3925.

Sahasrabudhe, S.R., Amin, A.R. and Modi, V.V. 1985. Transformation of chlorinated benzoate and other benzene derivatives by *Aspergillus niger* and *Aspergillus japonicus. Appl. Microbiol. Biotechnol.* 21: 365-367.

Sahasrabudhe, S.R., Amin, A.R. and Modi, V.V. 1987. Dehalogenation of chlorinated derivatives of phenoxyacetic acid by *Aspergillus niger. Microbios Letters.* 34: 19-22.

Sanglard, D., M.S.A. Leisola and A. Fiechter. 1986. Role of extracellular ligninase in biodegradation of benzo(a)pyrene by *Phanerochaete chrysosporium. Enzyme Microb. Technol.* 8:209-212.

Sanseverino, J., Graves, D.A., Leavitt, M.E., Gupta, S. K. and Luthy, R.G. 1994. Surfactant-Enhanced bioremediation of polynuclear aromatic hydrocarbons in coke waste. In D.L. Wise and D. J. Trantolo (eds) Remwdiation of hazardous waste contaminated soils. Marcel Dekker, Inc. New york.

Šašek, V., Volfová, O., Erbanová, P., Vyas, B.R.M. and Matucha, M. 1993. Degradation of PCBs by white rot fungi, methylotrophic and hydrocarbon utilizing yeasts and bacteria. *Biotechnol. Lett.* 15: 521-526.

Savage, G.M., Diaz, L.F. and Golueke, C.G. 1985. Biological treatment of organic toxic wastes. *BioCycle* 26: 30-33.

Sayles, G.D., Brenner, R.C., Hinchee, R.E., Leeson, A., Vogel, C.M. and Miller, R.N. 1994. Bioventing of jet fuel spills1: Bioventing in a cold climate with soil warming at Eielson AFB, Alaska. In Symposium on Bioremediation of Hazardous Wastes. San Francisco, U.S.A.

Schaefer, A. 2000. Environmental concerns linked to railroad creosote. *Environ Sci Technol* 34: 502A-502A.

Schirmer, M., Molson, J.W. and Frind, E.O. 2000. Bioremediation modelling of a dissoleved gasoline plume applying independent laboratory and field parameters. *J Cont Hydrol.* 46: 339-374.

Schmidt, S.K., K.M. Scow and M.Alexander. 1987. Kinetics of *p*-nitrophenol mineralization by a *Pseudomonas* sp.: Effects of second substrates. *Appl. Environ. Microbiol.* 53: 2617-2623.

Scow, K.M., Li, D., Manilal, V.B. and Alexander, M. 1990. Mineralization of organic compounds at low concentrations by filamentous fungi. *Mycol. Res.* 94: (6) p 793-798.

Sellers, K. 1999. Fundamentals of hazardous waste site remediation. Louis Publishers, Boca Raton. pp. 326.

Semprini, L., Roberts, P.V., Hopkins, G.D. and McCarthy, P.L. 1991. *In Situ* bioremediation of carbon tetrachloride, freon-133, freon-11 and1,1,1-TCA under anoxic conditions. In: Hinchee, R.E., Offenbuttel, B. (Eds). *In Situ* bioreclamation : Application and Investigations for Hydrocarbon and Contaminated Site Remediation. Butterworth-Heineman, Stoneham, Massachussetts. pp.41-58.

Shailubhai, K. and Sahasrabudhe, S.R. 1983. Degradation of chlorinated phenoxyacetic acid and benzoic acid by *Aspergillus niger*. *FEMS Microbiology Letters*. 18: 279-282.

Shailubhai, K., Sahasrabudhe, S.R., Vora, K.A. and Modi V.V. 1984. Degradation of chlorobenzoates by *Aspergillus niger*. *Experimentia* 40: 406-407.

Sharpley, A.N. 1991. Effect of soil pH on cation and anion solubility. *Commun. Soil sci. Plant Anal.* 22: 827-841.

Shiohara, K., Diehl, S.V. and Borazjani, H. 2001. Use of commercial surfactants for enhanced biodegradation of organic wood-preservative contaminated processwater. In proceedings of the 2001 Mississipi Water Resources Conference. Raymond, Mississipi, USA. 10-11 April, 2001.

Shrout, J.D. and Parkin, G.F. 2000. Inhibition of anaerobic perchlorate biotransformation by Fe(0). In G.B.Wickramanayake, A.R.Gavaskar, J.T.Gibbs and J.L. Means (eds), Case studies in the remediation of chlorinated and recalcitrant compounds. Battelle Press, Columbus, Ohio.

Sims, J.L., Sims, R.C. and Matthews, J.E. 1990. Approach to bioremediation of contaminated soil. *Haz. Waste Haz. Mater.* 7: 117-147.

Sims, R.C. and Overcash, M.R. 1981. Land treatment of coal conversion wastewaters. In Environmental Aspects of Coal Conversion Technology VI: A symposium on coal-based synfuels. EPA Report No. EPA-600-9-82-017. Environmental Protection Agency, Washington, D.C., pp. 218-230.

Sims, R.C., Sims, J.L. 1986. Cleanup of contaminated soils. In: Brown, K.W., Carlile, B.L., Miller, R.H., Rutledge, E.M. and Runge, E.C.A (eds). Utilization, Treatment and Disposal of Waste on Land. Soil Society of America, Inc.

Sims, R.C., Sims, J.L., Sorenson, D.L., Doucette, W.J. and Hastings, L.L. (1986). Waste/Soil treatability studies for four complex industrial wastes: Methodologies and Results. Volume 2. Waste lodging impacts on soil degradation, transformation and Immobilization. EPA-600/6-86-003a,b, U.S. EPA, Ada., Oklahoma.

Sims, J.L., Sims, R.C. and Matthew, J.E. 1989. Bioremediation of contaminated surface soils. Robert S. Kerr Environmental Research Laboratory, U.S. EPA office of research and development, Ada, Okla., EPA-600/9-89/073.

Singer, M.E. and Finnerty, W.R. 1984. Microbial metabolism of straight-chain and branched alkanes. In Atlas, R.M. (ed) Petroleum Microbiology. Macmillan, New York. pp. 1-60.

Slater, J.H. 1978. In The oil industry and microbial ecosystems. Chater, K.W.A. and Somerville, H.J. (Eds), Heyden and Sons Ltd, London. p. 137.

Slater, J.H. and Bull, A.T. 1982. Environmental microbiology: biodegradation. Philosophical transactions of the Royal Society, London.297: 515-597.

Slater, J.H. and Lovatt, D. 1984. Biodegradation and the significance of microbial communities. In Gibson, D.T. (Ed). Microbial degradation of organic compounds. Marcel Dekker Inc., N.Y. p 439-

481.

Smith, S and Mason, J.R.(1999). Microbial bioremediation *in situ* of land contaminated with organic chemicals. *Progress in Environmental Science.* 1 (1) 71 - 87.

Snyman, H.G. 1996. The microbiology of *ex situ* bioremediation of petroleum hydrocarbon-contaminated soil. A PhD thesis submitted to the Department of Microbiology and Plant Pathology, University of Natal, Pietermaritzburg, South Africa.

Song, H., Wang, X. and Bartha, R. 1990. Bioremediation potentials of terrestrial fuel spill. *Appl. Environ. Microbiol.* 56: 652-656.

Spain, J.C., P.H. Pritchard and A.W. Bourquin. 1980. Effects of adaptation on biodegradation rates in sediments/water cores from estuarine and freshwater environments. *Appl. Environ. Microbiol.* 40: 726-734.

Spain, J.C. and van Veld, P.A. (1983). Adaptation of natural microbial communities to degradation of xenobiotic compounds: Effects of concentration, exposure time, inoculums, and chemical structure. *Appl. Environ. Microbiol.* 45: 428-435.

Spain, J.C., Pettigrew, C.A. and Haigler, B.E. 1991. Biodegradation of mixed solvents by a strain of *Pseudomonas*. In Sayler, G.S., Fox, R. and Blackburn, J.W. (eds), Environmental Biotechnology for Waste Treatment, Plenum Press, NY, pp. 175-184.

Standefer, J.I. and Van Lith, C. 1993. Biofilters minimize emissions. *Environ Prot.* 4: 48-58.

Stanier, R.Y., J.L. Ingraham, M.L. Wheelis, and P.R. Painter. 1986. The Microbial world. 5th ed. Prentice-Hall, Englewood Cliffs, New Jersey.

Stapleton, R.D., Dwayne, D.C., Savage, G.S., Sayler, G.S. 1998. Biodegradation of aromatic hydrocarbons in an extremely acidic environment. *J. Appl. Environ. Microbiol.* 64: 4180-4184.

Stegmann, P., Goede, J.F. and Ginster, M. 1994. Implementing bioremediation-ongoingimprovements in an effective solution to coping with hydrocarbon wastes. *Chemical Technology.* (March/April): 22-27.

Stegmann, R., Lotter, S. and Heerenklage, J. 1991. Biological treatment of oil contaminated soils in a bioreactor. In R.E. Hinchee and R. Olfenbuttel (eds), On-site bioreclamation processes for xenobiotic and hydrocarbon treatment. Butterworth-Heinemann. pp. 188-208.

Straube, G., Hensel, J., Niedan, C. and Straube, E. 1990. Kinetic studies of phenol degradation by *Rhodococcus sp.* P1 I. Batch cultivation. *Antonie van Leeuwenhoek.* 57: 29-32.

Stroo, H.F., Mahaffey, W. and Bourquin, A.W. 1989. Development of an *in situ* bioremediation system for a creosote contaminated site, in international conference on physicochemical and biological detoxification of hazardous wastes. Technomics publishing co. Inc., Lancaster. pp. 919-936.

Stryer, L. 1988. Biochemistry. Third Edition. W.H. Freeman and Co. New York. p. 412-413.

Subramanian, C., Sekaran, G., Padmavathi, S. and Chanrakasan G. 1995. Removal of phenolic compounds from waste water using mutant strain of *Pseudomonas pictorum*. *J. Gen. Appl. Microbiol.* 41: 229-237.

Suflita, J.M., Gibson, S.A. and Beeman, R.E. 1988. Aerobic biotransformation of pollutant chemicals in aquifers. *J. Ind. Microb.* 3: 179-194.

Sullivan E.R., Zang, X.M. and Phelps, C. 2001. Anaerobic mineralization of stable-isotope-labelled

2-methylnaphthalene. *Appl Environ Microb.* 67: 4356-4357.

Sutherland, J.B., Selby, A.L., Freeman, J.P., Evans, F.E. and Cemiglia, C.E. 1991. Metabolism of phenanthrene by *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 57: 3310-3316.

Sutherland, J.B., Rafti, F., Khan, A.A. and Cerniglia, C.E.1995. Mechanisms of polycyclic aromatic hydrocarbon degradation. In: Microbial transformation and degradation of toxic organic chemicals (Eds) Young, L.Y. and Cerniglia, C.E. Wiley Liss, New York. pp 269-306.

Swartz, R.C., Schults, D.W., Dewitt, T.H., Ditsworth, G.R. and Lamberson, J.O. 1990. Toxicity of fluoranthene in sediments to marine amphipods: a test of the equilibrium partitioning approach to sediment quality criteria. *Environ. Toxicol. Chem.* 9: 1071-1080.

Swindell, C.M., Aelion, C.M., Pfaender, F.K. 1988. Influence of mineral and organic nutrients onaerobic biodegradation and the adaptation responses of surface microbial communities. *Appl. Environ. Microbiol.* 54: 212-217.

Tagatz, M.E., Plaia, G.R., Deans, C.H. and Lores E.M. 1983. Toxicity of creosote-contaminated sediment to field- and laboratory colonized estuarine benthic communities. *Environ. Toxicol. Chem.* 2: 441-450.

Talaro, T. and Talaro, A. 1993. Foundations in microbiology. Wm. C. Brown Publishers, Dubuque. p. 806.

Talley, J.W., Hatzinger, P.B., Waisner, S.A., Goldstein, K., Navon, D., Heckelman, C.A. and Senick, M. 2000. Bioremediation of polycyclic aromatic hydrocarbons (PAHs) at the Watervliet Arsenal.In G.B.Wickramanayake, A.R.Gavaskar, J.T.Gibbs and J.L. Means (eds), Case studies in the remediation of chlorinated and recalcitrant compounds. Battelle Press, Columbus, Ohio.

Tan, K.H. 1998. Principles of soil chemistry. Third edition. Marcel Dekker Inc. New York.

Tatarko, M and Bumpus, J.A. 1993. Biodegradation of phenanthrene by *Phanerochaete chrysosporium:* on the role of role of lignin peroxidase. *Lett. Appl. Microbiol.* 17: 20-24.

Tate, R.L. 1995. Soil Microbiology. John Wiley and Sons, Inc., New York. pp. 398.

Testa, S.M. and Winegardner, D.L. 1991. Restoration of petroleum contaminated aquifers. Lewis publishers, Chelsea.

Thakker, M.E., Yagi, H., Levin, W., Wood, A.W., Cooney, A.H. and Jerina, D.M. 1985. Polycyclic aromatic hydrocarbon: metabolic activation to ultimate carcinogens. In Anderson, M.W. (Ed), Bioactivation of foreign compounds. Academic Press Inc., New York. p.177-192.

The Government of the Republic of South Africa. 1998. Government Gazette No. 19031, National Environmental Management Act 107 of 1998.

Thibault, G.T. and Elliot, N.W. 1980. Biological detoxification of hazardous organic chemical spills. In Proceedings of USEPA conference on control of hazardous material spills, Vanderbilt University, Nashville, TN. pp 398-402.

Thomas, J.M. and Ward, C.H. 1989. *In situ* biorestoration of organic contaminants in the subsurface. *Environ. Sci. Technol.* 23: 760-766.

Thompson, R. 1991. The chemistry of wood preservation. Royal Society of Chemistry, Cambridge.

Thornton-Manning, J.R., Jones, D.D. and Federle, T.W. 1987. Effects of experimental manipulation of environment factors on phenol mineralization in soil. *Environ. Toxicol. Chem.* 6: 615-621.

Tien, M., and Kirk, T.K. 1983. Lignin-degrading enzyme from the hymenomycetes *Phanerochaete chrysosporium. Burds. Science.* 221: 661-663.

Tien, M. and Kirk, T.K. 1988. Lignin peroxidase of *Phanerochaete chrysosporium. Methods in Enzymol.* 161: 238-249.

Torma, A.E. 1994. The basics of bioremediation. *Pollution Engineering.* June: 46-47.

Troy, M.A., Allen, L.B. and Douglas, E.J. 1994. Bioremediation of petroleum-contaminated soils at railroad facilities. In Wise, D.L. and Trantolo, D.J. (Eds), Remediation of hazardous waste contaminated soils. Marcel Dekker, Inc. New York. p.929.

Trzesicka-Mlynarz, D and Ward, O.P. (1995). Degradation of polycyclic aromatic hydrocarbons (PAHs) by a mixed culture and its component pure cultures, obtained from PAH-contaminated soil. *Can. J. Microbiol.* 41: 470-476.

USEPA 1998. Federal Remediation Technologies Roundtable, 1998. Remediation Case Studies: Ex Situ soil Treatment technologies (Bioremediation and Vitrification), EPA/542/R-98/011.

USEPA 1997. EPA's contaminated sediment management strategy. Office of Water and Solid Waste, US EPA Washington, D.C.

USEPA 1997. Federal Remediation Technologies Roundtable, 1997. Remediation Case Studies: Bioremedtion and vitrification, EPA/542/R-97/008.

USEPA 1990. International evaluation of *In situ* biorestoration of contaminated soil and groundwater. EPA/540/2-90/012.

USEPA. 1986. Test methods for evaluating solid waste, SW-846, Vol. I and II. Nov. 1986.

USEPA. 1995. How to evaluate alternative cleanup technologies for underground storage tank sites: A guide for corrective action plan reviewers (EPA 510-B-95-007).

USEPA. 1983. Methods for chemical analysis of water and wastes. Washington D.C.

Usinowicz, P.J. and Rozich, A.F. 1993. Thermophilic processes cuts biomass wastes. *Environ. Protect.* 4: 26-34.

Valli, K. and Gold, M.H. 1991. Degradation of 2,4-dichlorophenol by the lignin-degrading fungus *Phanerochaete chrysosporium*. *J. Bacteriol.* 173: 345-352.

Valo, R. and Salkinoja-Salonen, M. 1986. Bioremediation of chlorophenol-contaminated land. *Appl. Microbiol. Biotechnol.* 25: 68-75.

Van Rooj, J.G.M., Lieshout, E.M.A., Bodelier-Bade, N. and Jongeneelen, F.J. 1993. Effect of the reduction of skin contamination on the internal dose of creosote workers exposed to polycyclic aromatic hydrocarbons. *Scandinavian Journal of Work and Environmental Health.* pp. 200.

Veldkamp, H. and Jannasch, H.W. 1972. Mixed culture studies with the hemostat. *J. Appl. Chem. Biochem.* 22:105-123.

Verstrate, W., Vanloocke, R., Deborger, R. and Verlinde, A. 1976. Modeling of the breakdown and the mobilization of hydrocarbons in unsaturated soil layers. In Sharpley, J.M. and Kaplan, A.M. (eds), Proceedings of the 3rd International Biodegradation Symposium. Applied Science Publishers, Ltd., London. pp. 99-112.

Vipulanandan, C. and Krishnan, S. 1993. Leachability and biodegradability of high concentrations of phenol and o-chlorophenol. *Hazardous wastes and hazardous materials.* 10 : 1. pp.27-47.

Vipulanandan, C., Wang, S. and Krishnan, S. 1994. Bioremediation of phenol. In D.L. Wise and D.J. Trantolo (eds). Remediation of hazardous waste contaminated soils. Marcel Dekker Inc. New York.

Vogelbein, W.K., Fournie, J.W., Van Veld, P.A. and Huggett, R.J. 1990. Hepatic neoplasms in the mummichog, *Fundulus heteroclitus*, from a creosote contaminated site. *Cancer Res.* 50: 5978-5996.

von Rumker, R., Lawless, E.W. and Meiners, A.F. 1975. Production, distribution, use and environmental impact potential of selected pesticides. U.S. Environmental Protection Agency. EPA 540/-74-001.

Walter, U., Beyer, M., Klein, J. and Rehm, H-J. 1991. Degradation of pyrene by *Rhodococcus sp.* UW1. *Appl. Microbiol. Biotechnol.* 34: 671-676.

Wang, X., Yu, X. and Bartha, R. 1990. Effects of bioremediation on polynuclear aromatic hydrocarbon residues in soil. *Environ. Sci. Technol.* 24: 1086-1089.

Weber, O. Scholz, R.W. and Buhlman, R. 2001. Risk perception of heavy metal soil contamination and attitudes towards decontamination strategies. *Risk Anal.* 21: 967-977.

Weissenfels, W.D., Klewer, H.-J. and Langhoff, J. 1992. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: influence on biodegradability and biotoxicity. *Appl. Microbiol. Biotechnol.* 36. 689-696.

Weissenfels, W.D., Beyer, M. And Klein, J. 1990. Degradation of phenethrene, fluorene and fluoranthene by pure bacterial cultures. *Appl. Microbiol. Biotechnol.* 32: 479-484.

Wiggins, B.A., S. H. Jones, and M. Alexander. 1987. Explanation for the acclimation period preceding the mineralization of organic chemicals in aquatic environments. *Appl. Environ. Microbiol.* 53: 791-796.

William, R.T., P.S. Ziegenfuss, and P.J. Marks. 1988. Field Demonstration: Composting of Explosive-Contaminated Sediments at Louisiana Army Ammunition Plant (LAAP). Report No. AMXTH-IR-TE-88242. Final Report. U.S. Army toxic and Hazardous Materials Agency, Aberdeen Proving Ground, Maryland.

Williams, B.D. and Packer, P.E. 1979. Sewage sludge and other organic materials as amendments for revegetation of spent oil shale. In Utilization of municipal sewage effluent and sludge on forest and disturbed land. University Press, University Park, PA. 353-358.

Wilson, G.B. Sikora, L.J. and Parr, J.F. 1983. Composting of chemical industrial wastes prior to land application. In Parr, J.F., Marsh, P.B. and Kla, J.M. (eds), Treatment of hazardous wastes, Noyes Data Corporation, New Jersey, pp. 263-273.

Wilson, J.T., McNabb, J.F., Cochran, J.W., Wang, T.H., Tomson, M.B and Bedient. P.B. 1985. Influence of microbial adaptation on the fate of organic pollutants in ground water. *Environ. Toxicol. Chem.* 4721-726.

Wilson, S.C. and Jones, K.C. 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): A review. *Environmental Pollution.* 81: 229-249.

Winningham, J. Britto, R. Patel, M. and McInturff, F. 1999. A landfarming field study of creosote-contaminated soil. In Leeson, A. Alleman, B.C. (Eds), Bioremediation technologies for polycyclic aromatic hydrocarbon compounds. Battelle Press, Columbus.

Wiseman, A. and Woods, L.F.J. 1979. Benzo(a)pyrene metabolites formed by the action of yeast cytochrome P-450/P-448. *J. Chem. Technol. Biotechnol.* 29: 320-329.

Wlliams, R.T., Zeigenfuss, P.S. and Sisk, W.E. 1992. Composting of explosives and propellant contaminated soils under thermophilic and mesophilic conditions. *J. Indust. Microbiol.* 9: 137-144.

Wodzinski, R.S. and Bertolini, D. 1979. Physical state in which naphthalene and bibenzyl are utilized by bacteria. Appl. Microbiol. 23: 1077-1081.

Wodzinski, R.S. and Johnson, 1968. Yields of bacterial cells from hydrocarbons. *Appl. Microbiol.* 16: 1886-1891.

Wodzinski, R.S. and Coyle, J.E. 1974. Physical state of phenanthrene for utilization by bacteria. *Appl. Environ. Microbiol.* 27: 1081-1084.

Woodhull, P., Jerger, D., Barnes, P., Staponski, R. and Wharry, S. 1999. Composting of explosives-contaminated soils: A pilot- and Full-scale case history. In B.C. Alleman and A. Leeson (eds), Bioremediation of Nitroamatic and Haloaromatic compounds, Vol. 5(7) pp. 63-68. Battelle Press, Columbus, Ohio.

Woods, L.F.J. and Wiseman, A. 1979. Metabolism of benzo(a)pyrene by the cytochrome P-450/P-448 of *Saccharomyces cerevisiae. Biochem. Soc. Trans.* 7: 124-127.

Woodward, R. 1990. Evaluation of composting implementation: A Literature Review. Report No.AMXTH-IR-TE-88242. U.S. Army Toxic and Hazardous Materials Agency. Aberdeen Proving Ground, Maryland.

Wunder, T., Kremer, S., Sterner, O. and Anke, H. 1994. Metabolism of the polycyclic aromatic hydrocarbon pyrene by *Aspergillus niger* SK9317. *Appl. Microbiol. Biotechnol.* 42: 636-641.

Wyndham, R. C. 1986. Evolved aniline catabolism in *Acinetobacter calcoaceticus* during continuos culture in river water. *Appl. Environ. Microbiol.* 51: 781-789.

Yalkowski, S.H. and Valvani, S.C. 1979. Solubility and partitioning 2. Relationships between aqueous solubilities, partition coefficients and molecular surface areas of rigid aromatic

hydrocarbons. *J. Chem. Engin. Data.* 24:127-129.

Yamamoto, S., Katagiri, M., Maeno, H. and Hayaishi, O. 1965. Salicilate hydroxylase, a monooxigenase requiring flavin adenine dinuclotide. I. Purification and general properties. *J. Biol Chem.* 240: 3408-3413.

Yang, R.D. and Humphrey, A.E. 1975. Dynamic and steady state studies of phenol biodegradation in pure and mixed cultures. *Biotechnol. Bioeng.* 17: 1211-1235.

Yateem, A., Balba, M.T. and Al-Shayji, Y. 2002. Isolation and characterisation of biosurfactant-producing bacteria from oil-contaminated soil. *Soil Sediment Contam.* 11: 41-55.

Yeom, I.T., Ghosh, M.M. and Cox, C.D. 1996. Kinetic aspects of surfactant solubilization of soil-bound polycyclic aromatic hydrocarbons. *Environ. Sci. Technol.* 30:1589-1595.

Yerushalmi, L. and Guiot, S.R. (1998). Kinetics of biodegradation of gasoline and its hydrocarbon constituents. *Appl. Microbiol. Biotechnol.* 49: 475-481.

Zahiraleslamzadeh, Z.M. and Bensch, J.C. 2001. Enhanced bioremediation in clay soil. In proccedings of the Sixth Annual In Situ and On site Bioremediation Conference, San Diego, California, June 3-7, 2001. Battelle Press, Columbus Ohio.

Ziegenfuss, P.S., Williams, R.T. and Myler, C.A. 1991. Hazardous material composting. *J. Haz. Mater.* 28: 91-99.

Zitrides, T. G. 1978. "Mutant bacteria overcome growth inhibition in industrial waste facility." *Industrial Wastes.* 24: 42-44

Zobell, C.E. and J. Agosti. 1972. Bacterial oxidation of mineral oil at sub-zero Celsius. In Abstracts 72nd Annual meeting of the American Society for Microbiology. Abstract E11.

APPENDIX 1 Changes in total creosote concentration (mg $kg^{-1}$) during pilot-scale landfarming.

| WEEKS | TREATMENTS | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1a | 1b | Mean | 2a | 2b | Mean | 3a | 3b | Mean | 4a | 4b | Mean | 5a | 5b | Mean |
| 0 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 |
| 1 | 243300 | 245100 | 244200 | 195040 | 216000 | 205520 | 201055 | 217020 | 209037.5 | 185101 | 183350 | 184225.5 | 185351 | 236220 | 210785.5 |
| 2 | 240000 | 242100 | 241050 | 173070 | 178000 | 175535 | 156800 | 165362 | 161081 | 168711 | 170235 | 169473 | 183400 | 218000 | 200700 |
| 3 | 236000 | 240000 | 238000 | 180005 | 176000 | 178002.5 | 140533 | 139003 | 139768 | 165003 | 165123 | 165063 | 179100 | 181260 | 180180 |
| 4 | 233700 | 232000 | 232850 | 180500 | 182000 | 181250 | 140007 | 135336 | 137671.5 | 151365 | 156011 | 153688 | 175568 | 176333 | 175950.5 |
| 5 | 223800 | 223000 | 223400 | 183023 | 180008 | 181515.5 | 123655 | 133350 | 128502.5 | 125166 | 133552 | 129359 | 156400 | 175220 | 165810 |
| 6 | 222100 | 222000 | 222050 | 175340 | 175115 | 175227.5 | 121325 | 124002 | 122663.5 | 107656 | 124359 | 116007.5 | 137521 | 157385 | 147453 |
| 7 | 221850 | 221900 | 221875 | 161000 | 165235 | 163117.5 | 116906 | 116000 | 116453 | 105302 | 102568 | 103935 | 119000 | 105125 | 112062.5 |
| 8 | 221680 | 220800 | 221240 | 160850 | 163054 | 161952 | 111211 | 112211 | 111711 | 97501 | 97874 | 97687.5 | 115505 | 104551 | 110028 |
| 9 | 221600 | 220750 | 221175 | 160360 | 162552 | 161456 | 111100 | 111522 | 111311 | 97203 | 97580 | 97391.5 | 110566 | 100836 | 105701 |
| 10 | 221570 | 220500 | 221035 | 160090 | 162003 | 161046.5 | 111002 | 111222 | 111112 | 96537 | 97500 | 97018.5 | 107576 | 100522 | 104049 |
| 11 | 221500 | 220300 | 220900 | 159095 | 161820 | 160457.5 | 110852 | 111166 | 111009 | 96156 | 96850 | 96503 | 105005 | 100452 | 102728.5 |
| 12 | 221300 | 220000 | 220650 | 158001 | 161500 | 159750.5 | 110485 | 111035 | 110760 | 95922 | 96735 | 96328.5 | 102522 | 100252 | 101387 |
| 13 | 220600 | 219000 | 219800 | 155800 | 160755 | 158277.5 | 110200 | 110985 | 110592.5 | 95700 | 96570 | 96135 | 100844 | 100168 | 100506 |
| 14 | 220000 | 218700 | 219350 | 155500 | 160400 | 157950 | 110042 | 110366 | 110204 | 95530 | 96556 | 96043 | 1 00600 | 100052 | 100326 |
| 15 | 218500 | 215000 | 216750 | 153100 | 160114 | 156607 | 107805 | 109000 | 108402.5 | 95431 | 96358 | 95894.5 | 100320 | 98802 | 99561 |
| 16 | 218300 | 212000 | 215150 | 153000 | 159603 | 156301.5 | 107550 | 107510 | 107530 | 95205 | 96288 | 95746.5 | 100005 | 98560 | 99282.5 |

| WEEKS | TREATMENTS | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6a | 6b | Mean | 7a | 7b | Mean | 8a | 8b | Mean | 9a | 9b | Mean |
| 0 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 | 258257 |
| 1 | 237832 | 232500 | 235166 | 235510 | 212012 | 223761 | 238280 | 226822 | 232551 | 235367 | 216256 | 225811.5 |
| 2 | 219580 | 205350 | 212465 | 231158 | 231005 | 231081.5 | 227500 | 204750 | 216125 | 231147 | 225758 | 228452.5 |
| 3 | 183427 | 186410 | 184918.5 | 211465 | 208691 | 210078 | 215645 | 209745 | 212695 | 223854 | 206265 | 215059.5 |
| 4 | 115632 | 127930 | 121781 | 186265 | 192500 | 189382.5 | 200345 | 195699 | 198022 | 197301 | 192230 | 194765.5 |
| 5 | 80450 | 85365 | 82907.5 | 123035 | 165055 | 144045 | 162108 | 172547 | 167327.5 | 163569 | 156580 | 160074.5 |
| 6 | 65520 | 61750 | 63635 | 96856 | 136578 | 116717 | 134500 | 146033 | 140266.5 | 141174 | 151005 | 146089.5 |
| 7 | 36452 | 41475 | 38963.5 | 81258 | 95751 | 88504.5 | 102256 | 125258 | 113757 | 118100 | 135147 | 126623.5 |
| 8 | 45750 | 40420 | 43085 | 65542 | 74085 | 69813.5 | 86544 | 100104 | 93324 | 88612 | 109524 | 99068 |
| 9 | 43520 | 40168 | 41844 | 53358 | 55007 | 54182.5 | 55192 | 65554 | 60373 | 62701 | 75263 | 68982 |
| 10 | 33315 | 35051 | 34183 | 45525 | 51755 | 48640 | 49432 | 60980 | 55206 | 51495 | 60550 | 56022.5 |
| 11 | 32536 | 31022 | 31779 | 43802 | 48356 | 46079 | 45502 | 48258 | 46880 | 47752 | 52032 | 49892 |
| 12 | 35480 | 33475 | 34477.5 | 42911 | 42411 | 42661 | 44245 | 45496 | 44870.5 | 46523 | 45258 | 45890.5 |
| 13 | 26456 | 28006 | 27231 | 41790 | 43535 | 42662.5 | 43325 | 42617 | 42971 | 42135 | 41543 | 41839 |
| 14 | 28145 | 25540 | 26842.5 | 41025 | 40156 | 40590.5 | 41512 | 40112 | 40812 | 41600 | 40515 | 41057.5 |
| 15 | 31605 | 24596 | 28100.5 | 38558 | 38361 | 38459.5 | 40312 | 39698 | 40005 | 41101 | 40112 | 40606.5 |
| 16 | 31920 | 26355 | 29137.5 | 36456 | 35456 | 35956 | 38501 | 37825 | 38163 | 40074 | 40060 | 40067 |

APPENDIX 2 Reduction in total creosote concentration (mg/kg) during co-composting

| Treatments | Control | | | | Vegetable waste | | | | Sewage sludge | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Month | 1 | 2 | 3 | Mean | 1 | 2 | 3 | Mean | 1 | 2 | 3 | Mean |
| 0 | 381592 | 381592 | 381592 | 381592 | 381592 | 381592 | 381592 | 381592 | 381592 | 381592 | 381592 | 381592 |
| 1 | 365986 | 365983 | 366331 | 366100 | 353360 | 354521 | 349632 | 352504.33 | 238900 | 246500 | 258000 | 247800 |
| 2 | 342566 | 342561 | 342541 | 342556 | 317480 | 316794 | 308325 | 314199.67 | 115250 | 118500 | 135000 | 122916.67 |
| 3 | 341000 | 341002 | 338116 | 340039.33 | 223000 | 207693 | 239450 | 223381 | 99750 | 99750 | 102760 | 100753.33 |
| 4 | 340007 | 340005 | 337000 | 339004 | 119500 | 128330 | 128456 | 125428.67 | 91560 | 86750 | 91560 | 89956.667 |
| 5 | 337000 | 338011 | 335000 | 336670.33 | 109950 | 119895 | 119880 | 116575 | 84620 | 79550 | 84620 | 82930 |
| 6 | 335500 | 334505 | 332000 | 334001.67 | 101860 | 101750 | 101855 | 101821.67 | 73500 | 63500 | 73500 | 70166.667 |
| 7 | 334900 | 332510 | 331550 | 332986.67 | 85460 | 85465 | 85655 | 85526.667 | 49850 | 45330 | 55450 | 50210 |
| 8 | 333750 | 332255 | 331371 | 332458.67 | 68743 | 68854 | 68864 | 68820.333 | 40680 | 40450 | 40300 | 40476.667 |
| 9 | 332000 | 331008 | 331175 | 331394.33 | 55599 | 55599 | 55598 | 55598.667 | 30456 | 30550 | 30880 | 30628.667 |
| 10 | 331650 | 327652 | 327655 | 328985.67 | 42175 | 41675 | 43277 | 42375.667 | 20500 | 20845 | 20450 | 20598.333 |
| 11 | 323660 | 323665 | 324130 | 323818.33 | 29960 | 28581.3 | 29866 | 29469.1 | 12675 | 13500 | 13620 | 13265 |
| 12 | 323200 | 323485 | 323100 | 323261.67 | 24520 | 24550 | 24530.1 | 24533.367 | 8500 | 8500 | 9850 | 8950 |
| 13 | 322500 | 323152 | 322851 | 322834.33 | 21820 | 22825 | 21828 | 22157.667 | 3550 | 4350 | 4350 | 4083.3333 |
| 14 | 322200 | 322730 | 322560 | 322496.67 | 18350 | 16060 | 20350 | 18253.333 | 1800 | 2200 | 2255 | 2085 |
| 15 | 321921 | 321929 | 322123 | 321991 | 15000 | 15853 | 17855 | 16236 | 1500 | 1590 | 1600 | 1563.3333 |
| 16 | 321287 | 321188 | 321500 | 321325 | 14500 | 14735 | 15330 | 14855 | 1200 | 1400 | 1530 | 1376.6667 |
| 17 | 320500 | 320525 | 321260 | 320761.67 | 9179 | 7890 | 8900 | 8656.3333 | 1200 | 1300 | 1450 | 1316.6667 |
| 18 | 320190 | 320398 | 318720 | 319769.33 | 3630 | 2100 | 3100 | 2943.3333 | 800 | 850 | 980 | 876.66667 |
| 19 | 318940 | 316660 | 315410 | 317003.33 | 900 | 1035 | 1008 | 981 350 | 550 | 575 | 491.66 | 667 |

| Treatments | Cow manure | | | | Poultry manure | | | |
|---|---|---|---|---|---|---|---|---|
| Month | 1 | 2 | 3 | Mean | 1 | 2 | 3 | Mean |
| 0 | 381592 | 381592 | 381592 | 381592 | 381592 | 381592 | 381592 | 381592 |
| 1 | 282300 | 282300 | 302450 | 289016.67 | 256700 | 257500 | 258500 | 257566.67 |
| 2 | 187420 | 187550 | 190500 | 188490 | 205600 | 195650 | 195800 | 199016.67 |
| 3 | 128300 | 125500 | 125850 | 126550 | 178550 | 158680 | 168780 | 168670 |
| 4 | 106870 | 118500 | 115500 | 113623.33 | 151000 | 143000 | 155000 | 149666.67 |
| 5 | 99500 | 109250 | 105785 | 104845 | 130500 | 133420 | 139650 | 134523.33 |
| 6 | 92490 | 95550 | 95750 | 94596.667 | 128560 | 128550 | 135675 | 130928.33 |
| 7 | 76250 | 78590 | 70560 | 75133.333 | 123500 | 123650 | 125810 | 124320 |
| 8 | 53640 | 56250 | 55550 | 55146.667 | 113100 | 113350 | 113560 | 113336.67 |
| 9 | 48500 | 45640 | 45125 | 46421.667 | 98650 | 100000 | 100230 | 99626.667 |
| 10 | 42600 | 35260 | 34550 | 37470 | 85622 | 87650 | 89520 | 87597.333 |
| 11 | 30500 | 30670 | 30500 | 30556.667 | 81640 | 81550 | 83600 | 82263.333 |
| 12 | 23360 | 25500 | 25600 | 24820 | 65550 | 65850 | 66800 | 66066.667 |
| 13 | 20935 | 20955 | 20956 | 20948.667 | 43360 | 43980 | 45460 | 44266.667 |
| 14 | 18450 | 19600 | 17665 | 18571.667 | 40830 | 41500 | 42845 | 41725 |
| 15 | 15420 | 17450 | 15600 | 16156.667 | 36590 | 36730 | 36770 | 36696.667 |
| 16 | 11867 | 12895 | 13598 | 12786.667 | 3745 | 4152 | 4550 | 4149 |
| 17 | 5835 | 6189 | 4550 | 5524.6667 | 3250 | 3800 | 4150 | 3733.3333 |
| 18 | 2880 | 1025 | 1500 | 1801.6667 | 2356 | 2650 | 3000 | 2668.6667 |
| 19 | 820 | 648 | 685 | 717.66667 | 770 | 915 | 980 | 888.33333 |

APPENDIX 3

STATISTICAL RANKING OF MEANS OF RESIDUAL CONCENTRATIONS OF CREOSOTE DURING PILOT-SCALE LANDFARMING.

Standard Error of a Treatment Mean = 10280
Standard Error of a Difference = 14538

Least Significant Difference (0.05) = 28610

| Group | Ranked Means | Association letters | Treatment description |
|---|---|---|---|
| 6 | 87927.9 | A | Indigenous soil microbial biosupplement |
| 7 | 110639 | AB | Sewage sludge |
| 8 | 117742 | BC | Cow manure |
| 9 | 119915 | BC | Poultry manure |
| 4 | 126397 | BC | Water + aeration + MAP |
| 3 | 133301 | BC | Water + aeration |
| 5 | 139692 | C | Hydrogen peroxide |
| 2 | 173660 | D | Natural control |
| 1 | 226925 | E | Sterile control |

Means followed by the same letter are not significantly different

Duncan's New Multiple Range Test at alpha 0.05

| Number of means | Tabular Value | Critical Range |
|---|---|---|
| 2 | 2.789 | 28666.9 |
| 3 | 2.933 | 30145.7 |
| 4 | 3.022 | 31069.9 |
| 5 | 3.094 | 31807.1 |
| 6 | 3.155 | 32432.6 |
| 7 | 3.205 | 32946.9 |
| 8 | 3.246 | 33370.1 |
| 9 | 3.28 | 33721.3 |

| Group | Ranked Means | Association letters |
|---|---|---|
| 6 | 87927.9 | A |
| 7 | 110639 | AB |
| 8 | 117742 | AB |
| 9 | 119915 | B |
| 4 | 126397 | B |
| 3 | 133301 | B |
| 5 | 139692 | B |
| 2 | 173660 | C |
| 1 | 226925 | D |

Means followed by the same letter are not significantly different

Tukey's w-procedure (HSD) at alpha 0.05

Value used to judge significance of differences = 46098

| Group | Ranked Means | Association letters |
|---|---|---|
| 6 | 87927.9 | A |
| 7 | 110639 | AB |
| 8 | 117742 | AB |
| 9 | 119915 | AB |
| 4 | 126397 | AB |
| 3 | 133301 | ABC |
| 5 | 139692 | BC |
| 2 | 173660 | CD |
| 1 | 226925 | E |

Means followed by the same letter are not significantly different

Student-Newman-Keuls' Range Test at alpha 0.05

| Number of means | Tabular Value | Critical Range |
|---|---|---|
| 2 | 2.772 | 28493 |
| 3 | 3.348 | 34414.5 |
| 4 | 3.685 | 37876.8 |
| 5 | 3.924 | 40336.4 |
| 6 | 4.11 | 42247.3 |
| 7 | 4.247 | 43662.3 |
| 8 | 4.374 | 44967.4 |
| 9 | 4.484 | 46098 |

| Group | Ranked Means | Association letters |
|---|---|---|
| 6 | 87927.9 | A |
| 7 | 110639 | AB |
| 8 | 117742 | AB |
| 9 | 119915 | AB |
| 4 | 126397 | AB |
| 3 | 133301 | B |
| 5 | 139692 | B |
| 2 | 173660 | C |
| 1 | 226925 | D |

Means followed by the same letter are not significantly different

APPENDIX 4

STATISTICAL RANKING OF MEANS OF RESIDUAL CONCENTRATIONS OF CREOSOTE DURING CO-COMPOSTING EXPERIMENT.

Standard Error of a Treatment Mean = 24972
Standard Error of a Difference = 35316

Least Significant Difference (0.05) = 69503

| Group | Ranked Means | Association letters | Treatment description |
|---|---|---|---|
| 3 | 63601.9 | A | Sewage sludge |
| 4 | 82289 | A | Cow manure |
| 2 | 98496.9 | A | Vegetable waste |
| 5 | 116285 | AB | Poultry manure |
| 1 | 381400 | C | Control |

Means followed by the same letter are not significantly different

Duncan's New Multiple Range Test at alpha 0.05

| Number of means | Tabular Value | Critical Range |
|---|---|---|
| 2 | 2.789 | 69641.7 |
| 3 | 2.933 | 73234.3 |
| 4 | 3.023 | 75479.6 |
| 5 | 3.094 | 77270.5 |

| Group | Ranked Means | Association letters |
|---|---|---|
| 3 | 63601.9 | A |
| 4 | 82289 | A |
| 2 | 98496.9 | A |
| 5 | 116285 | AB |
| 1 | 381400 | C |

Means followed by the same letter are not significantly different

Tukey's w-procedure (HSD) at alpha 0.05

Value used to judge significance of differences = 97988

| Group | Ranked Means | Association letters |
|---|---|---|
| 3 | 63601.9 | A |
| 4 | 82289 | A |
| 2 | 98496.9 | A |
| 5 | 116285 | AB |
| 1 | 381400 | C |

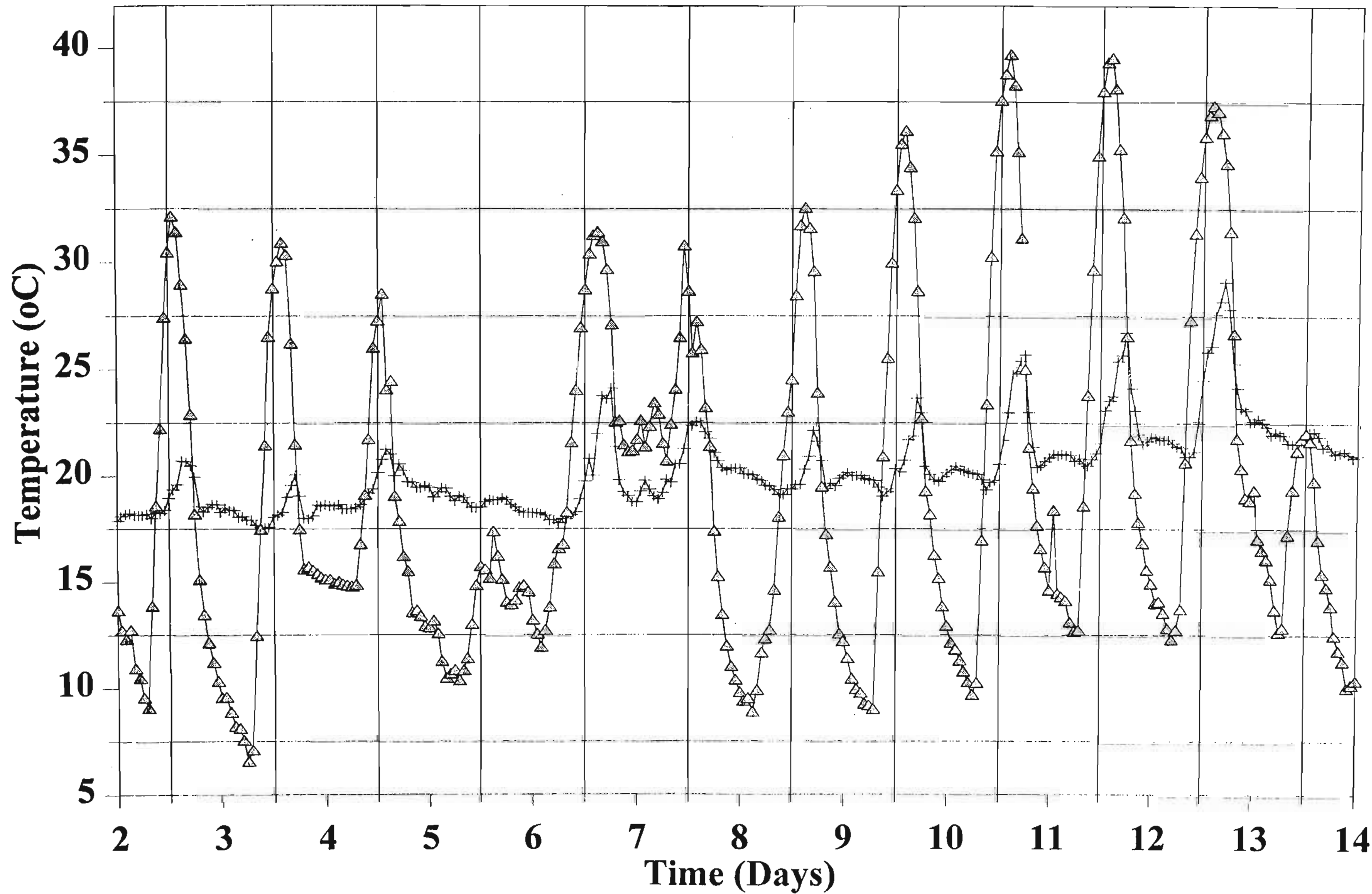
Means followed by the same letter are not significantly different

Student-Newman-Keuls' Range Test at alpha 0.05

| Number of means | Tabular Value | Critical Range |
| --- | --- | --- |
| 2 | 2.772 | 69217.6 |
| 3 | 3.348 | 83602.4 |
| 4 | 3.685 | 92013.4 |
| 5 | 3.924 | 97988.4 |

| Group | Ranked Means | Association letters |
| --- | --- | --- |
| 3 | 63601.9 | A |
| 4 | 82289 | A |
| 2 | 98496.9 | A |
| 5 | 16285 | AB |
| 1 | 381400 | C |

Means followed by the same letter are not significantly different



Temperature (oC)
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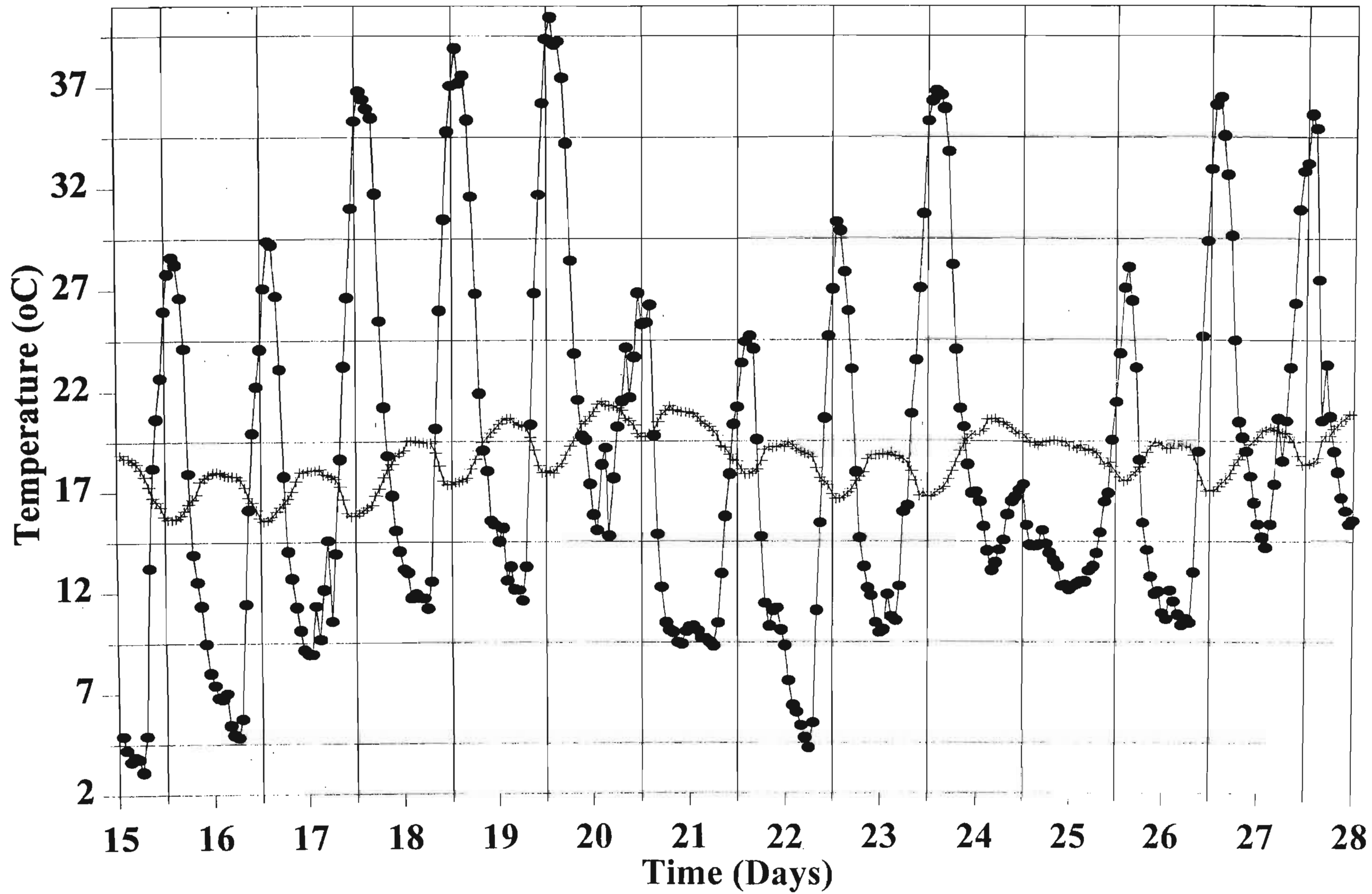
8
9
10
11
12
13
14
Time (Days)
TC-2
TC-4



Temperature (oC)
37
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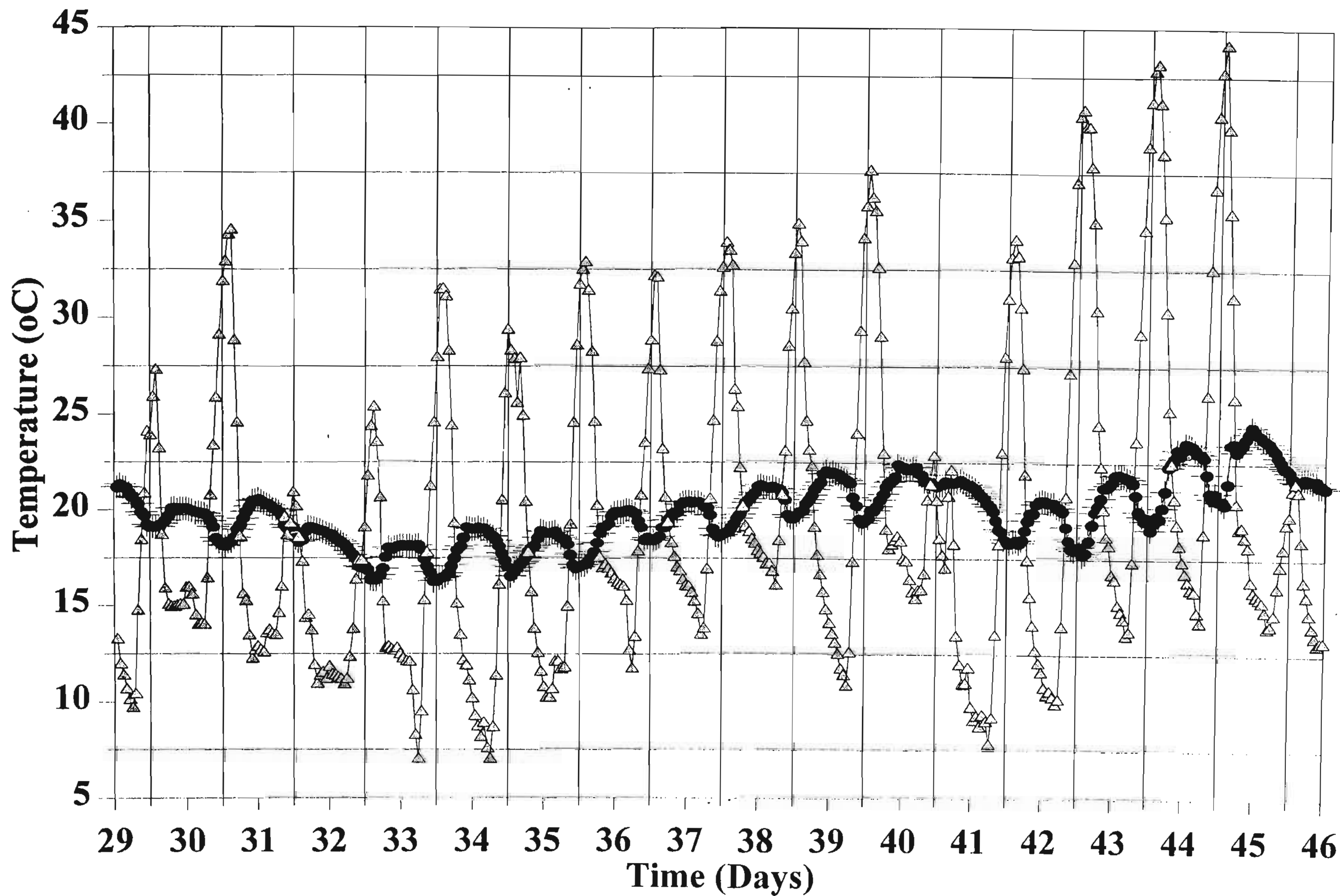
Time (Days)
TC-2
TC-4



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Temperature (oC)
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Time (Days)
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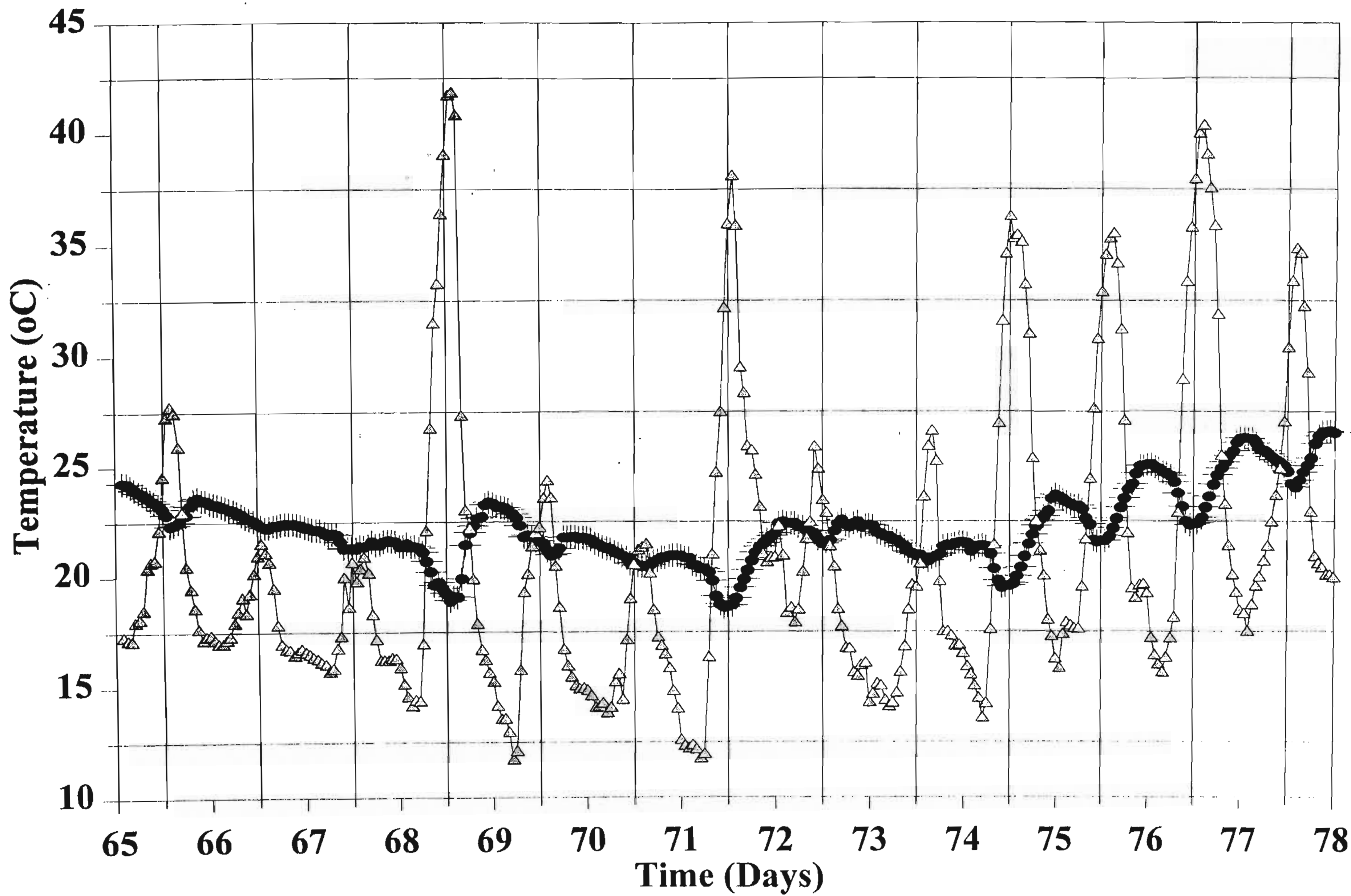
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Temperature (oC)
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Time (Days)
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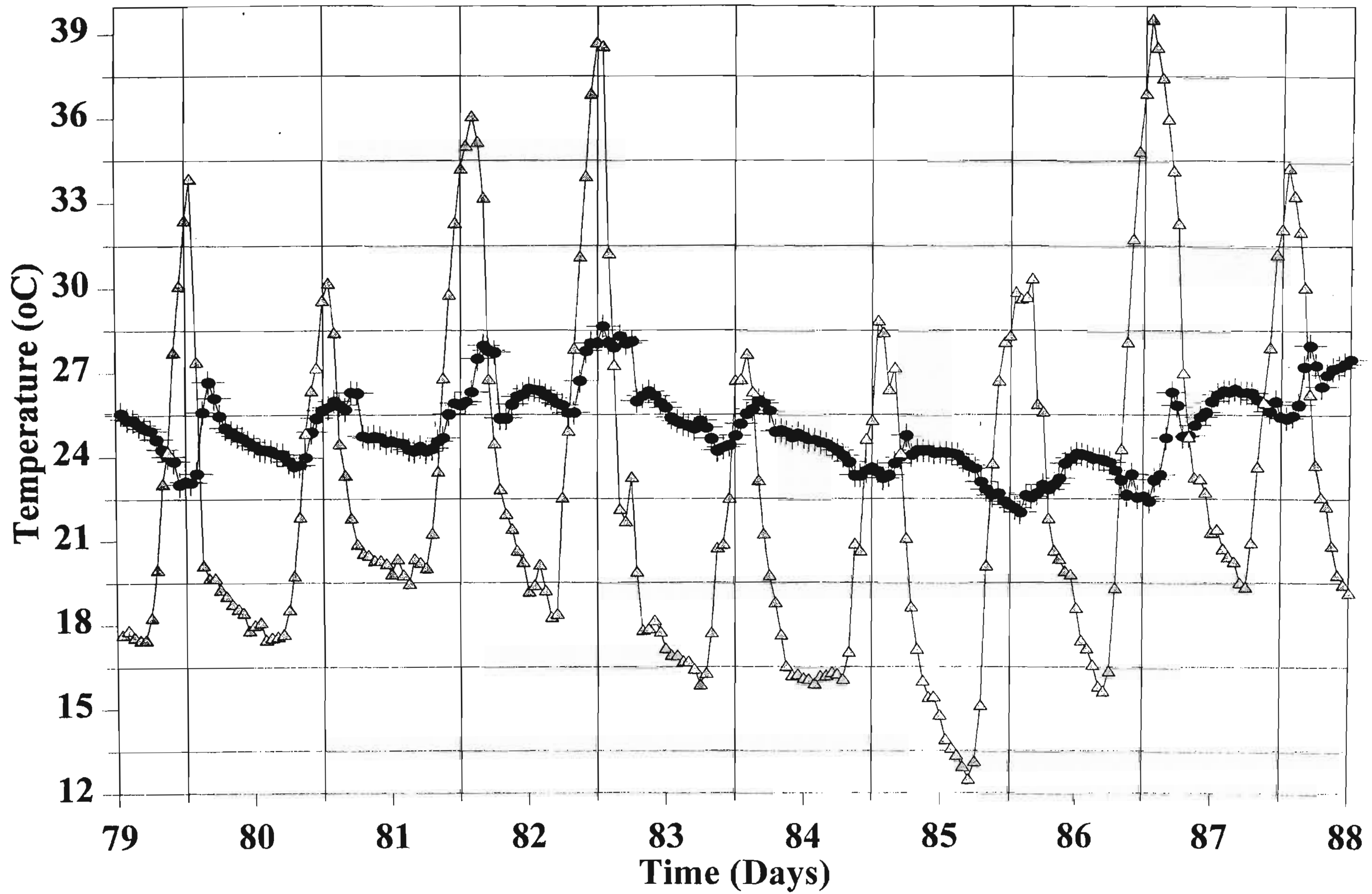
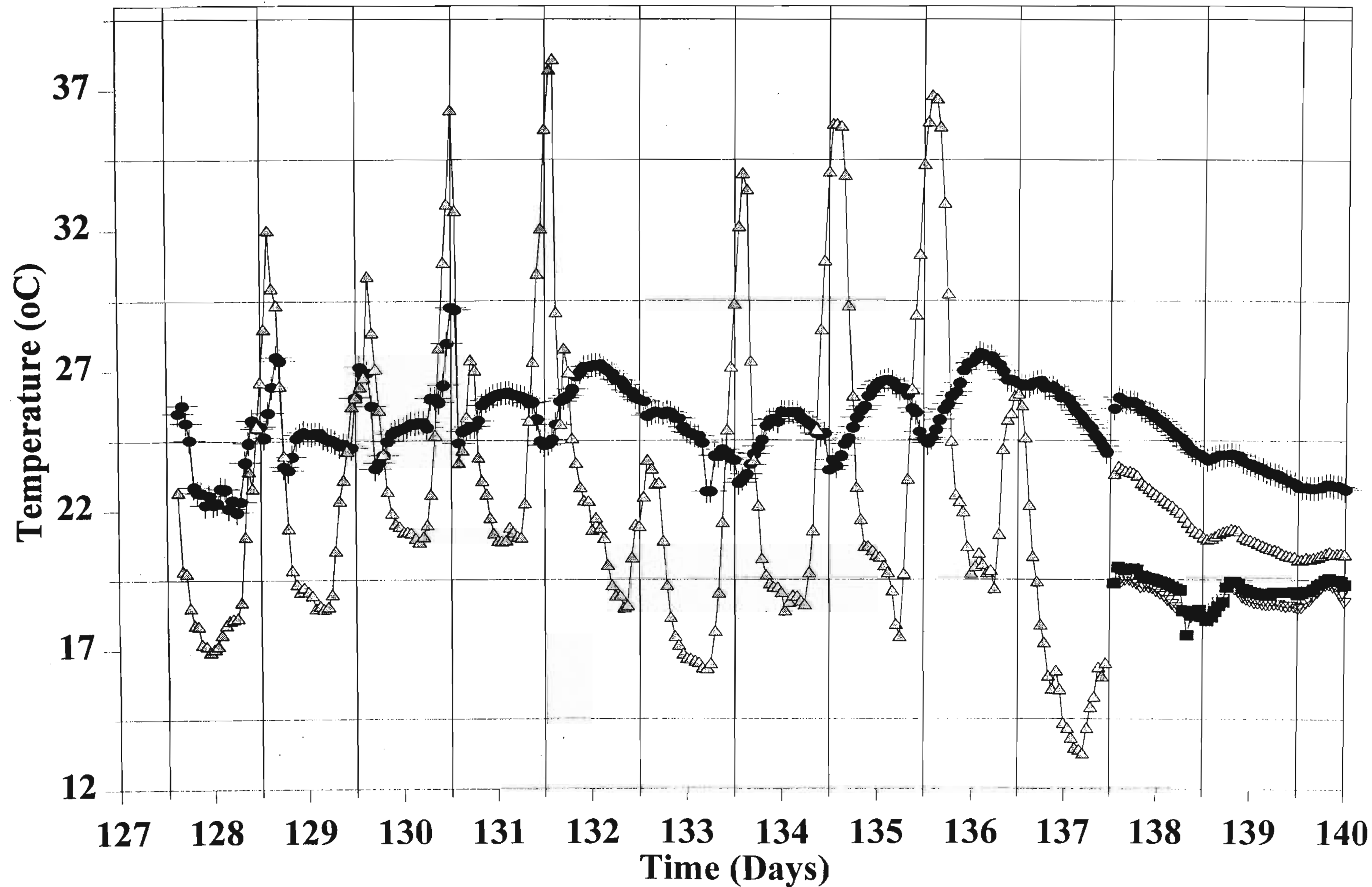
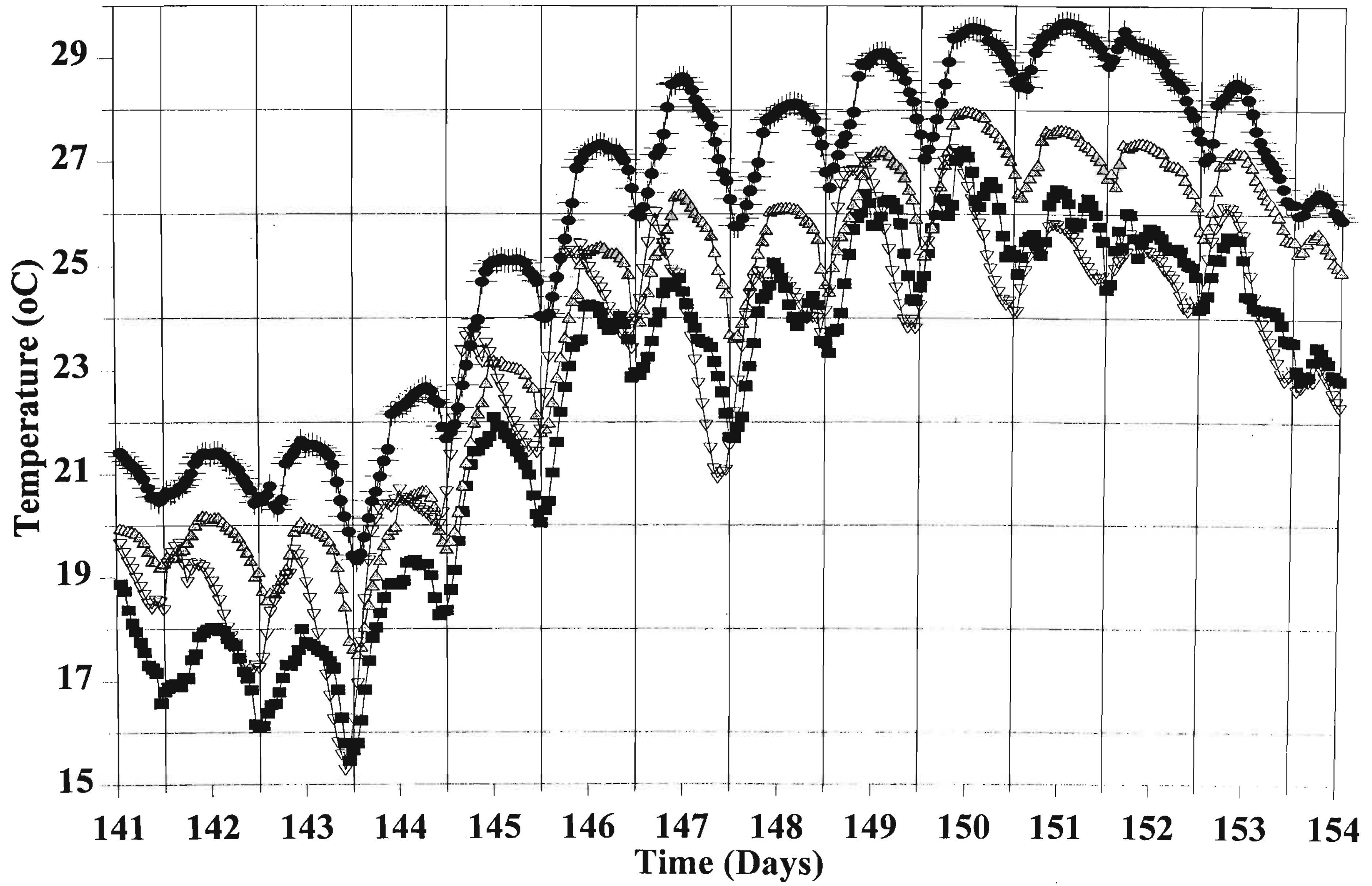
TC-3
TC-4

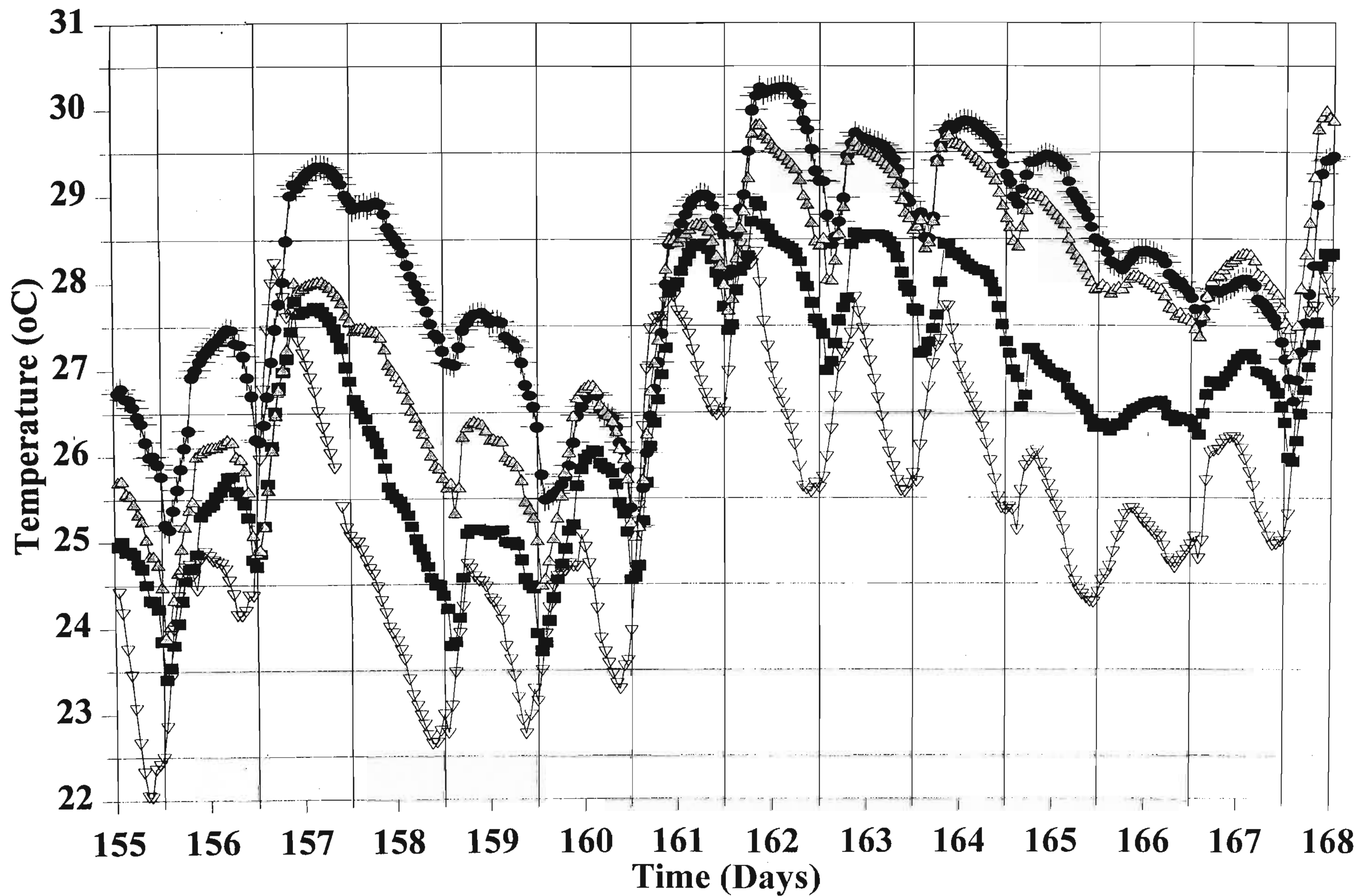

39
36
33
30
27
24
21
18
15
12
Temperature (oC)
79
80
81
82
83
84
85
86
87
88
Time (Days)
TC-2
TC-3
TC-4



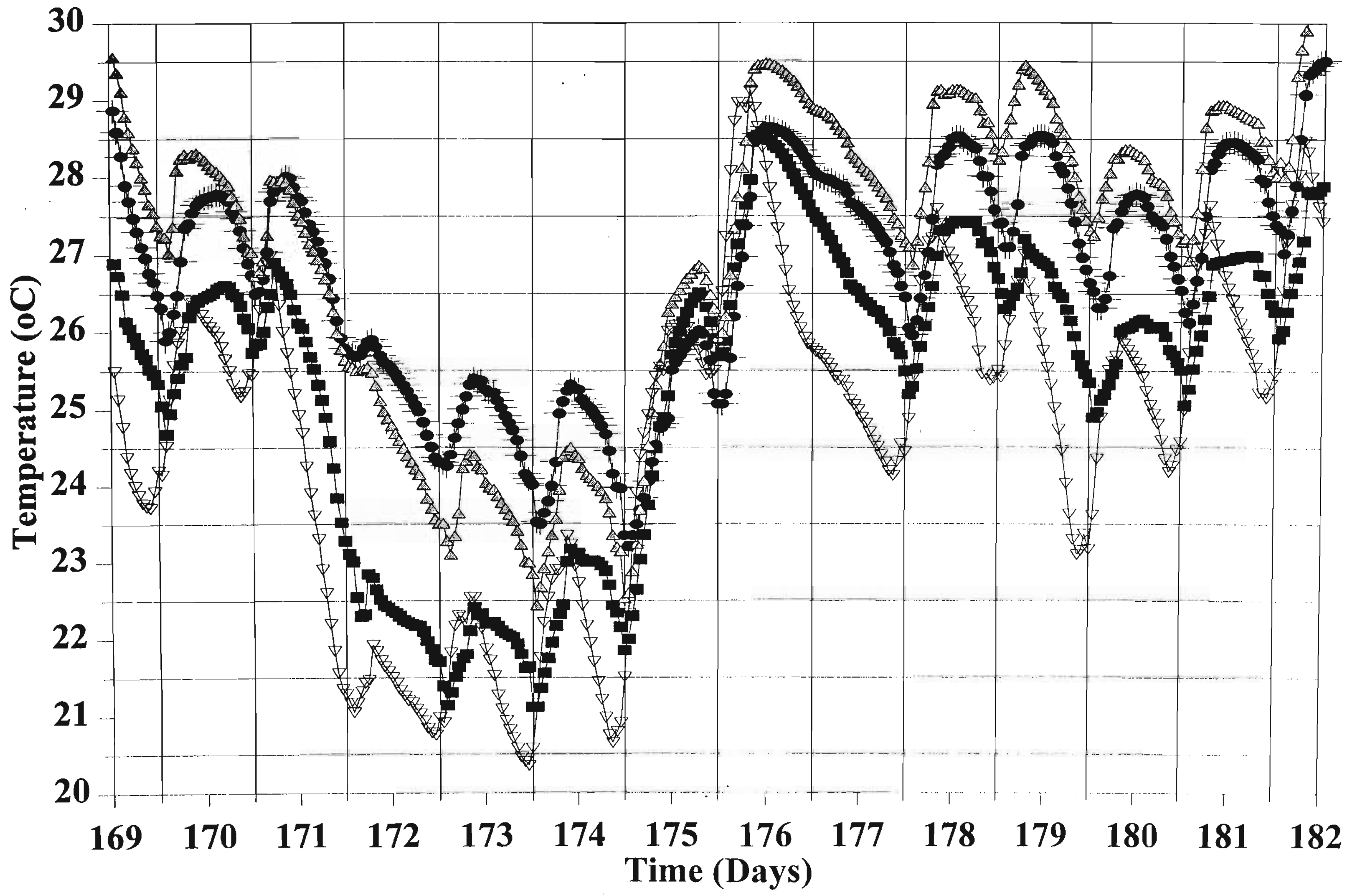
Temperature (oC)
37
32
27
22
17
12
127
128
129
130
131
132
133
134
135
136
137
138
139
140
Time (Days)
TC-1
TC-2
TC-3
TC-4
TC-5



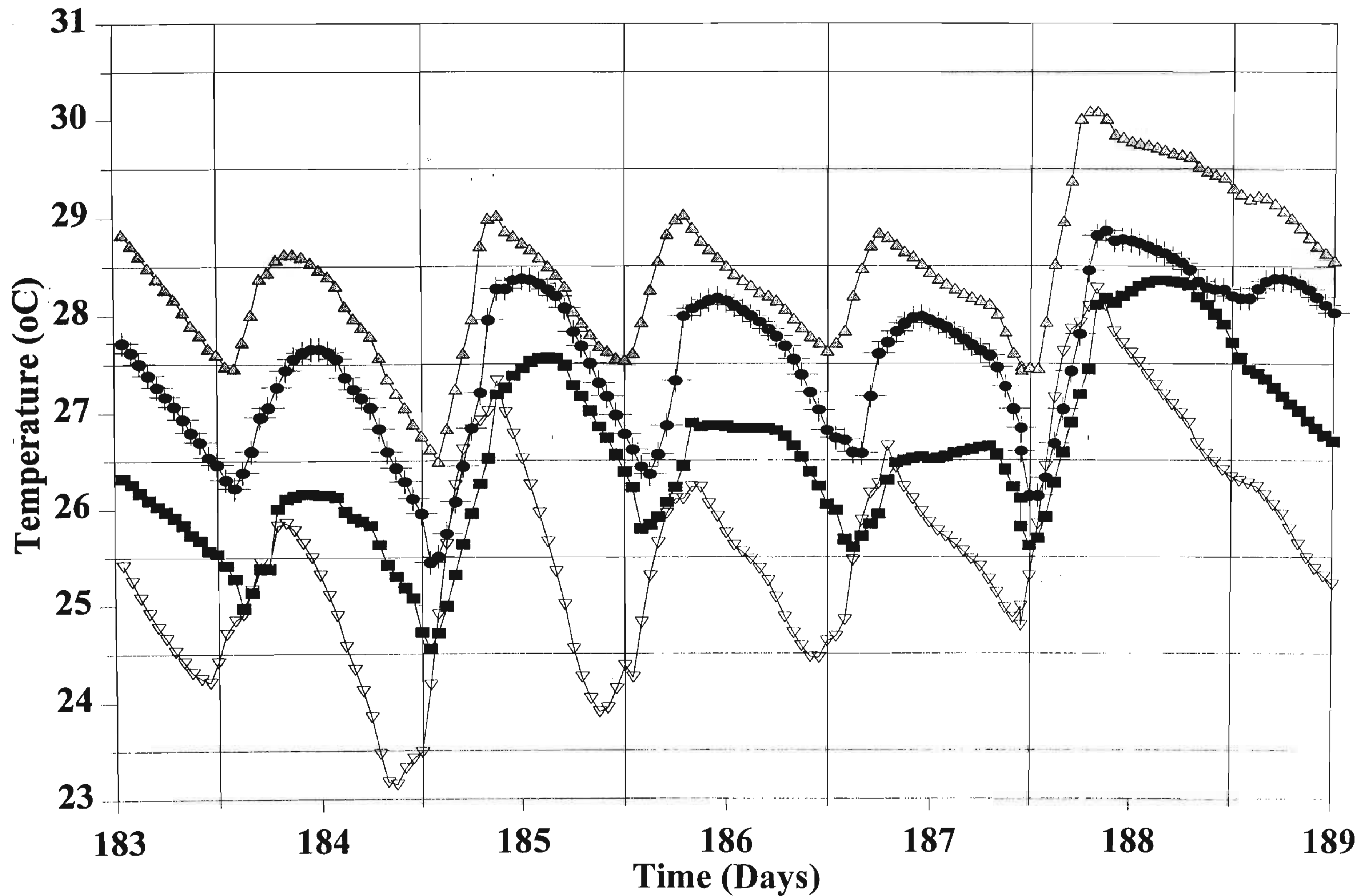
Temperature (oC)
29
27
25
23
21
19
17
15
141
142
143
144
145
146
147
148
149
150
151
152
153
154
Time (Days)
TC-1
TC-2
TC-3
TC-4
TC-5



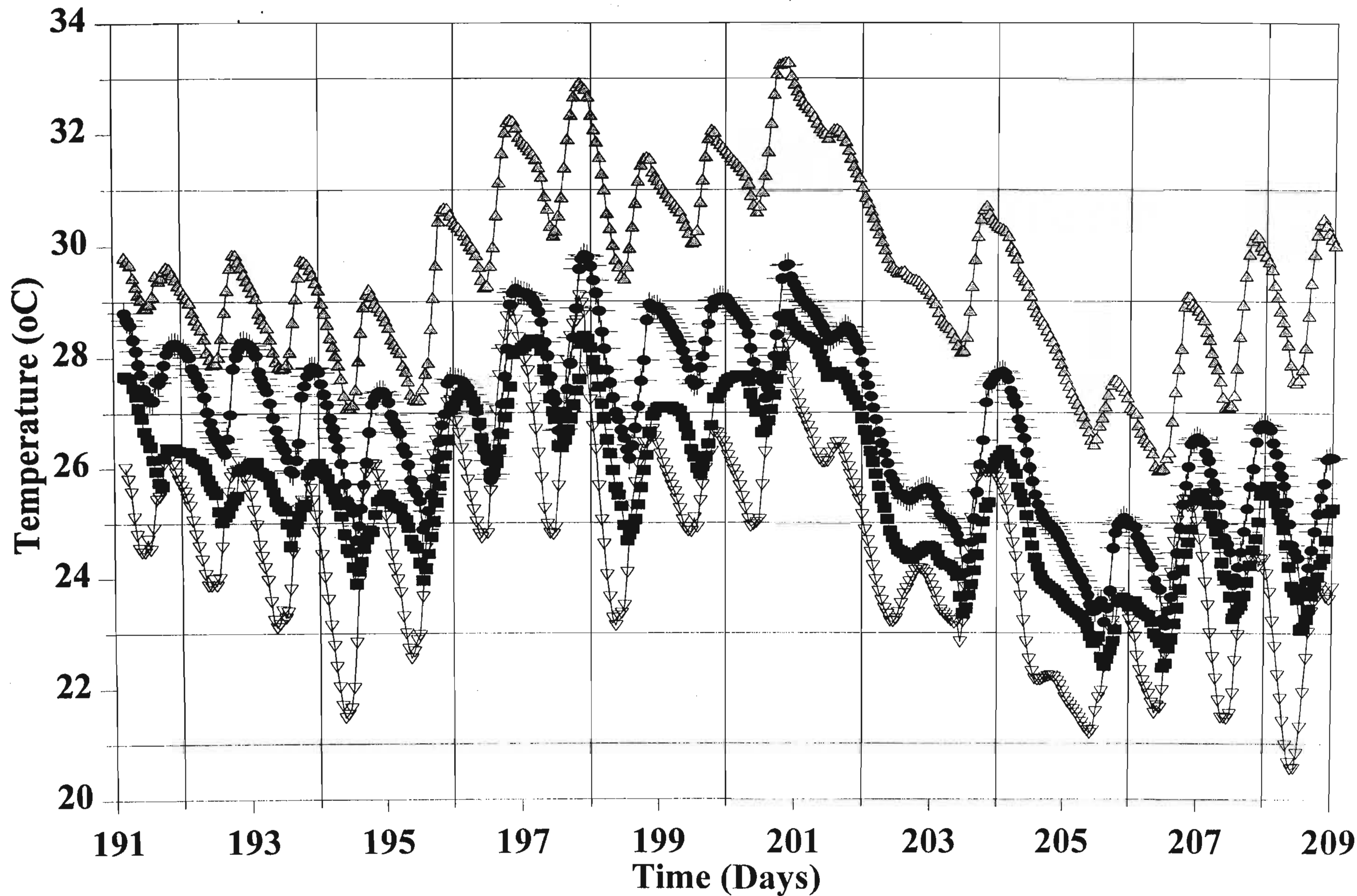
31
30
29
28
27
26
25
24
23
22
Temperature (oC)
155
156
157
158
159
160
161
162
163
164
165
166
167
168
Time (Days)
TC-1
TC-2
TC-3
TC-4
TC-5



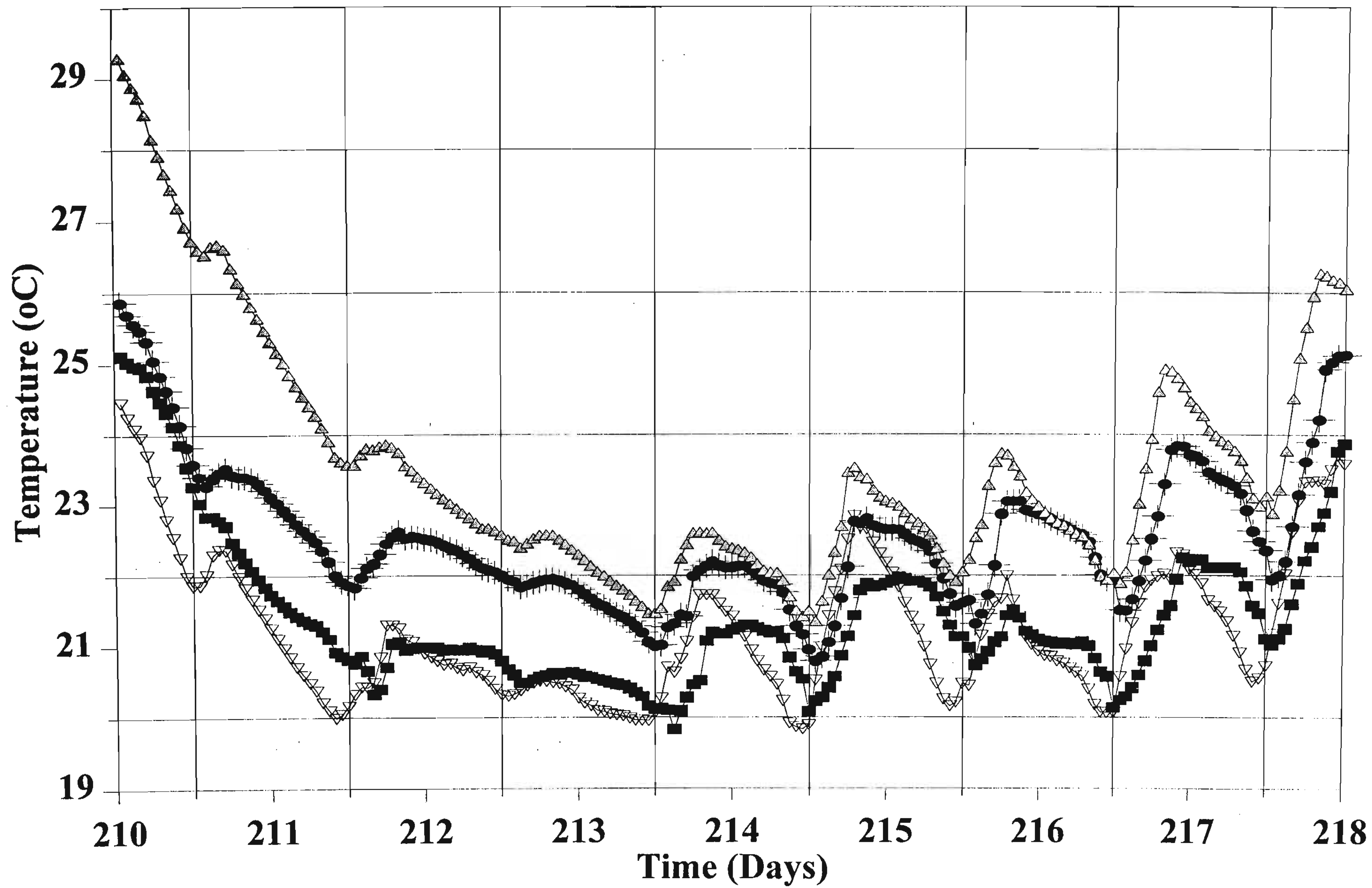
Temperature (oC)
30
29
28
27
26
25
24
23
22
21
20
169
170
171
172
173
174
175
176
177
178
179
180
181
182
Time (Days)
TC-1
TC-2
TC-3
TC-4
TC-5

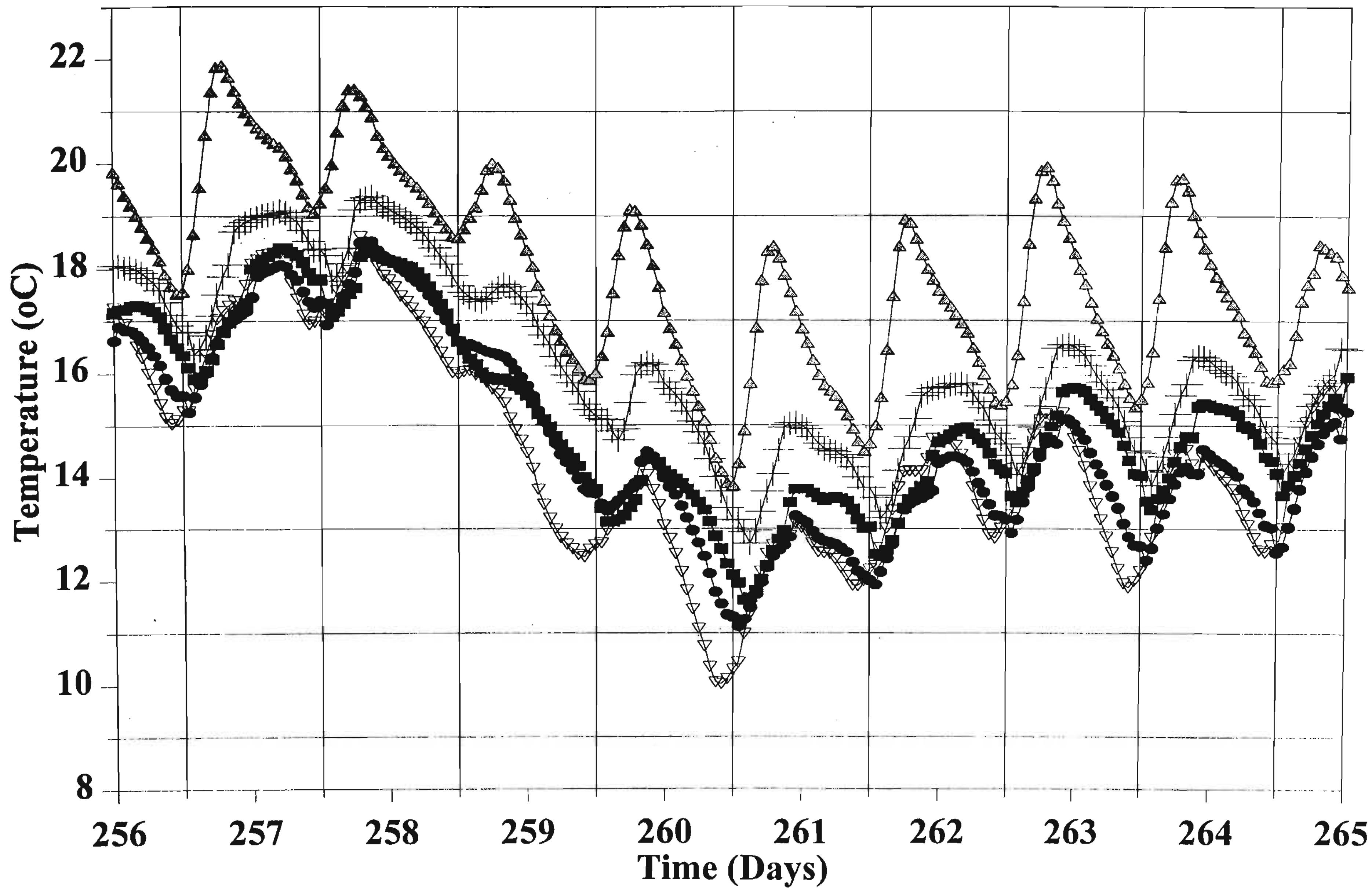


31
30
29
28
27
26
25
24
23
Temperature (oC)
183
184
185
186
187
188
189
Time (Days)
TC-1
TC-2
TC-3
TC-4
TC-5

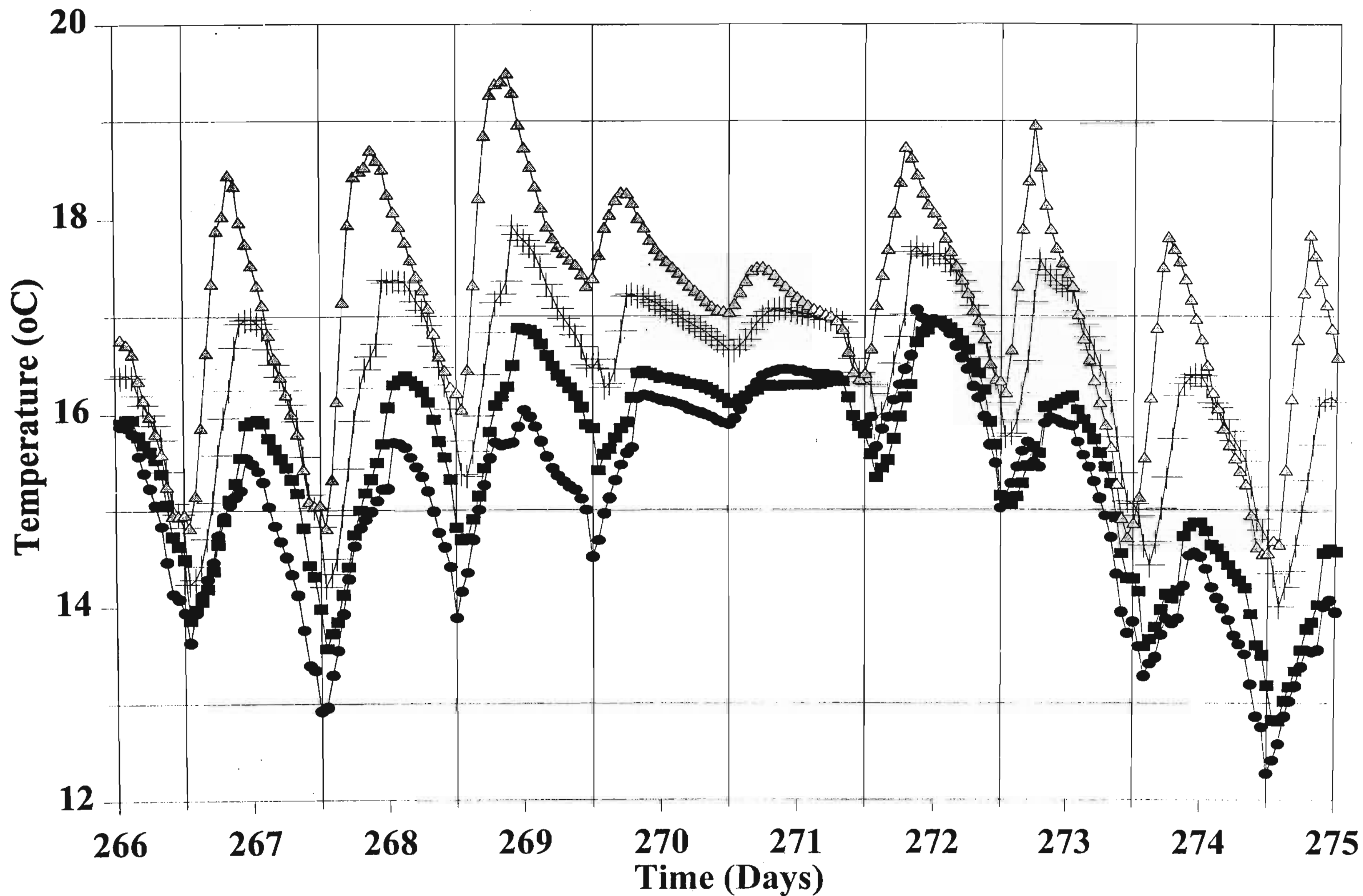


Temperature (oC)
34
32
30
28
26
24
22
20
191
193
195
197
199
201
203
205
207
209
Time (Days)
TC-1
TC-2
TC-3
TC-4
TC-5

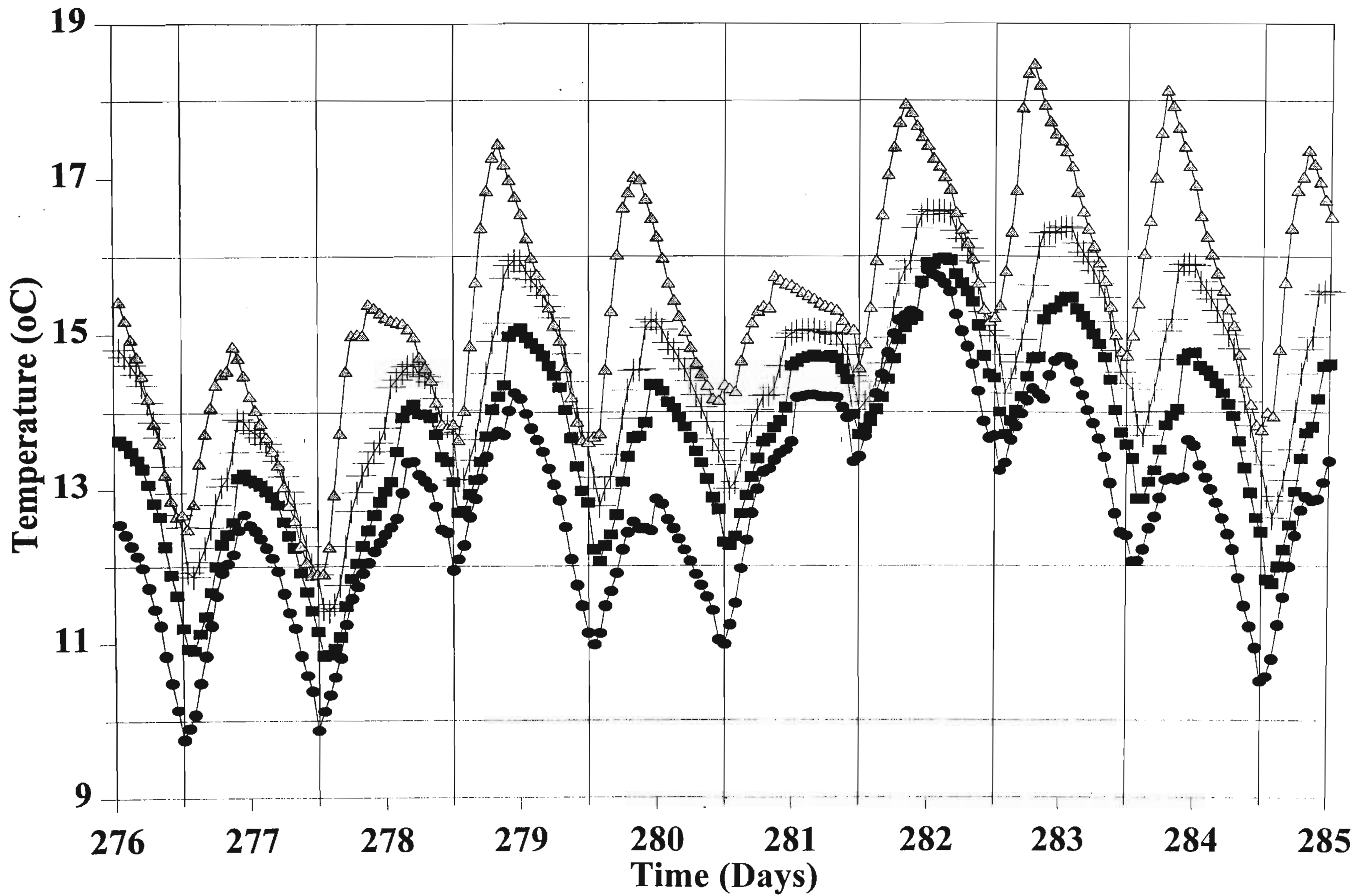

29
27
25
23
21
19
Temperature (oC)
210
211
212
213
214
215
216
217
218
Time (Days)
TC-1
TC-2
TC-3
TC-4
TC-5



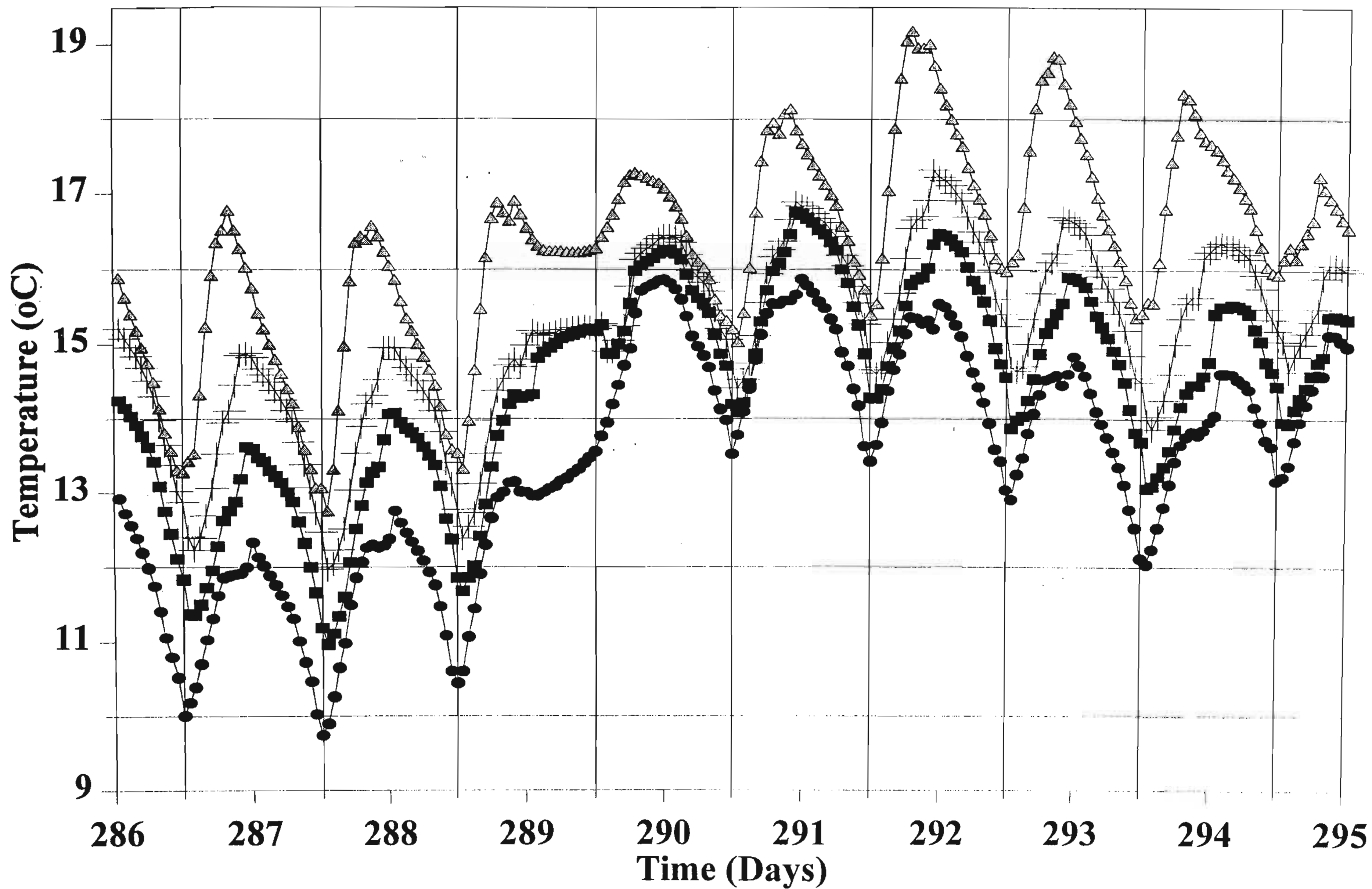
Temperature (oC)
22
20
18
16
14
12
10
8
256
257
258
259
260
261
262
263
264
265
Time (Days)
TC-1
TC-2
TC-3
TC-4
TC-5

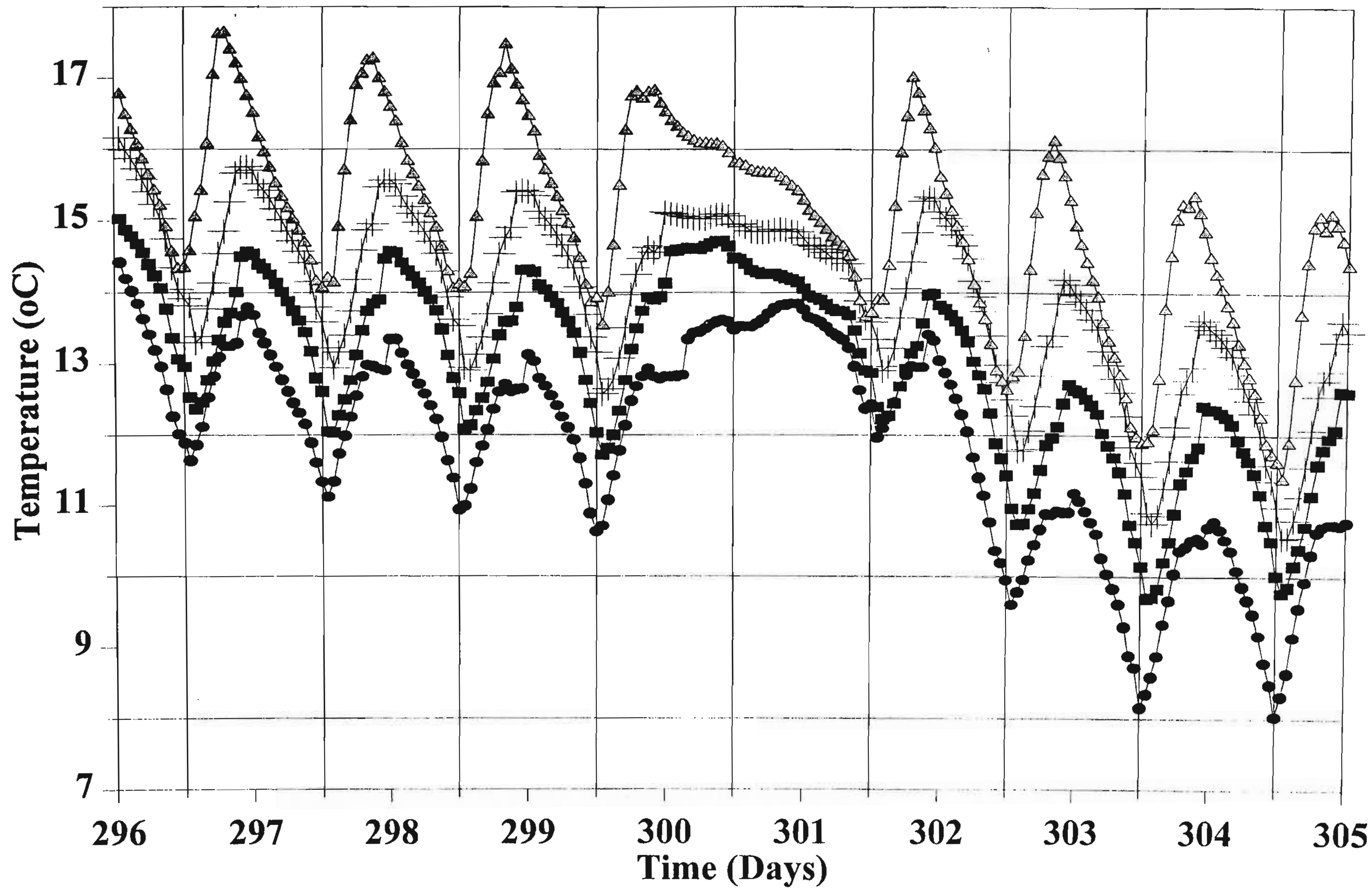


20
18
16
14
12
Temperature (oC)
266
267
268
269
270
271
272
273
274
275
Time (Days)
TC-1
TC-2
TC-3
TC-4

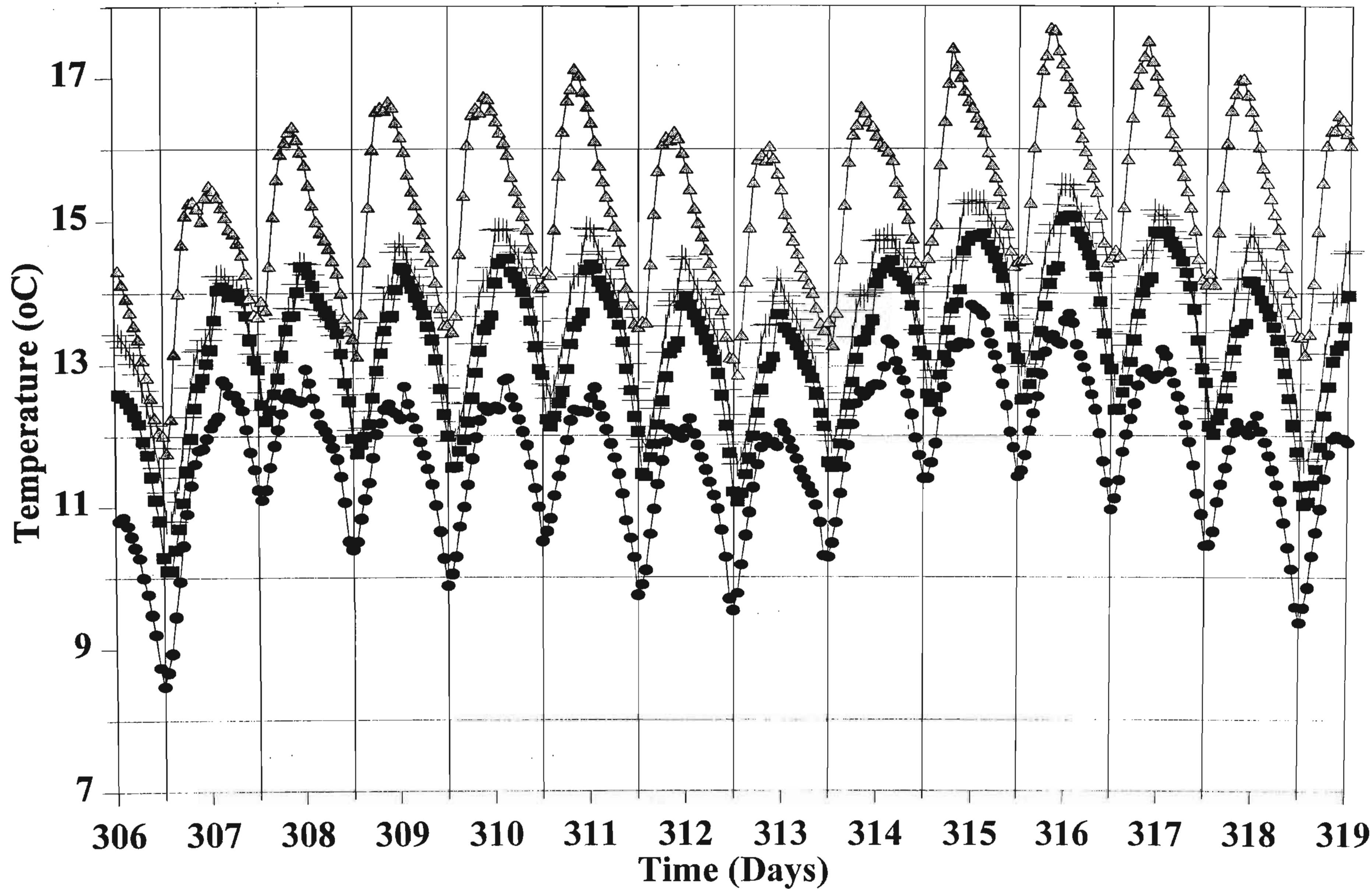


19
17
15
13
11
9
Temperature (oC)
276
277
278
279
280
281
282
283
284
285
Time (Days)
TC-1
TC-2
TC-3
TC-4

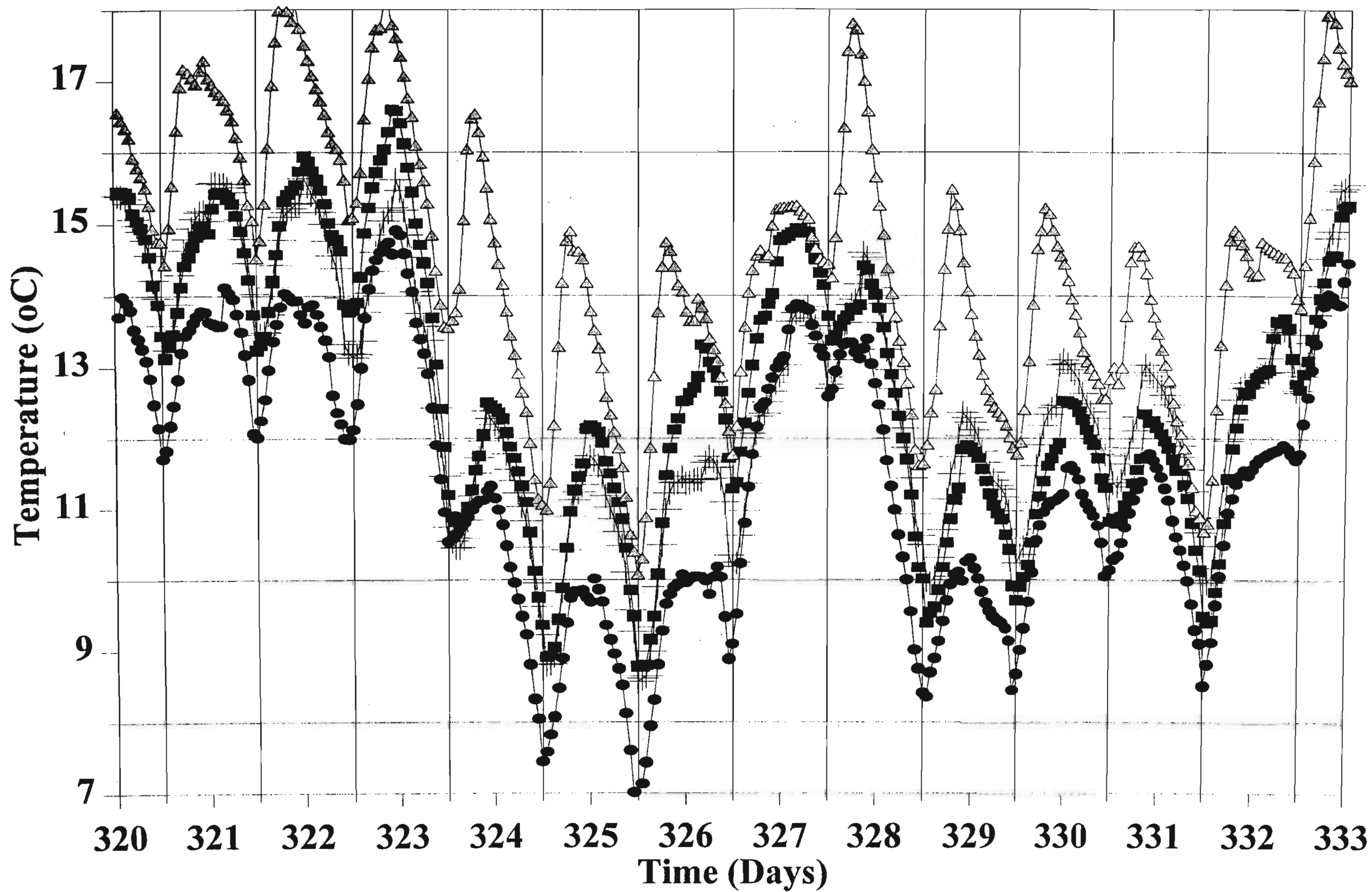

Temperature (oC)
19
17
15
13
11
9
286
287
288
289
290
291
292
293
294
295
Time (Days)
TC-1
TC-2
TC-3
TC-4



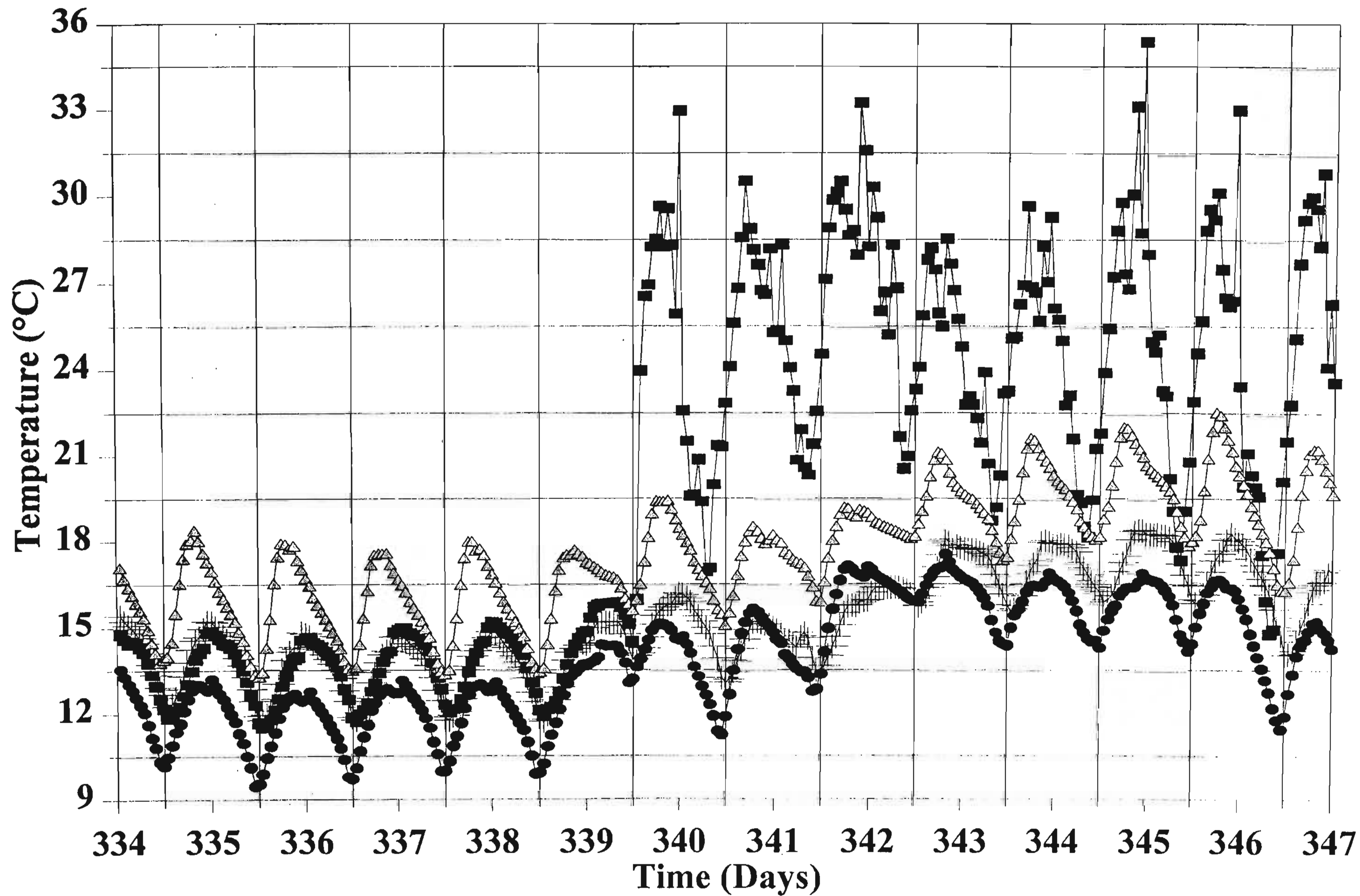
Temperature (oC)
17
15
13
11
9
7
296
297
298
299
300
301
302
303
304
305
Time (Days)
TC-1
TC-2
TC-3
TC-4

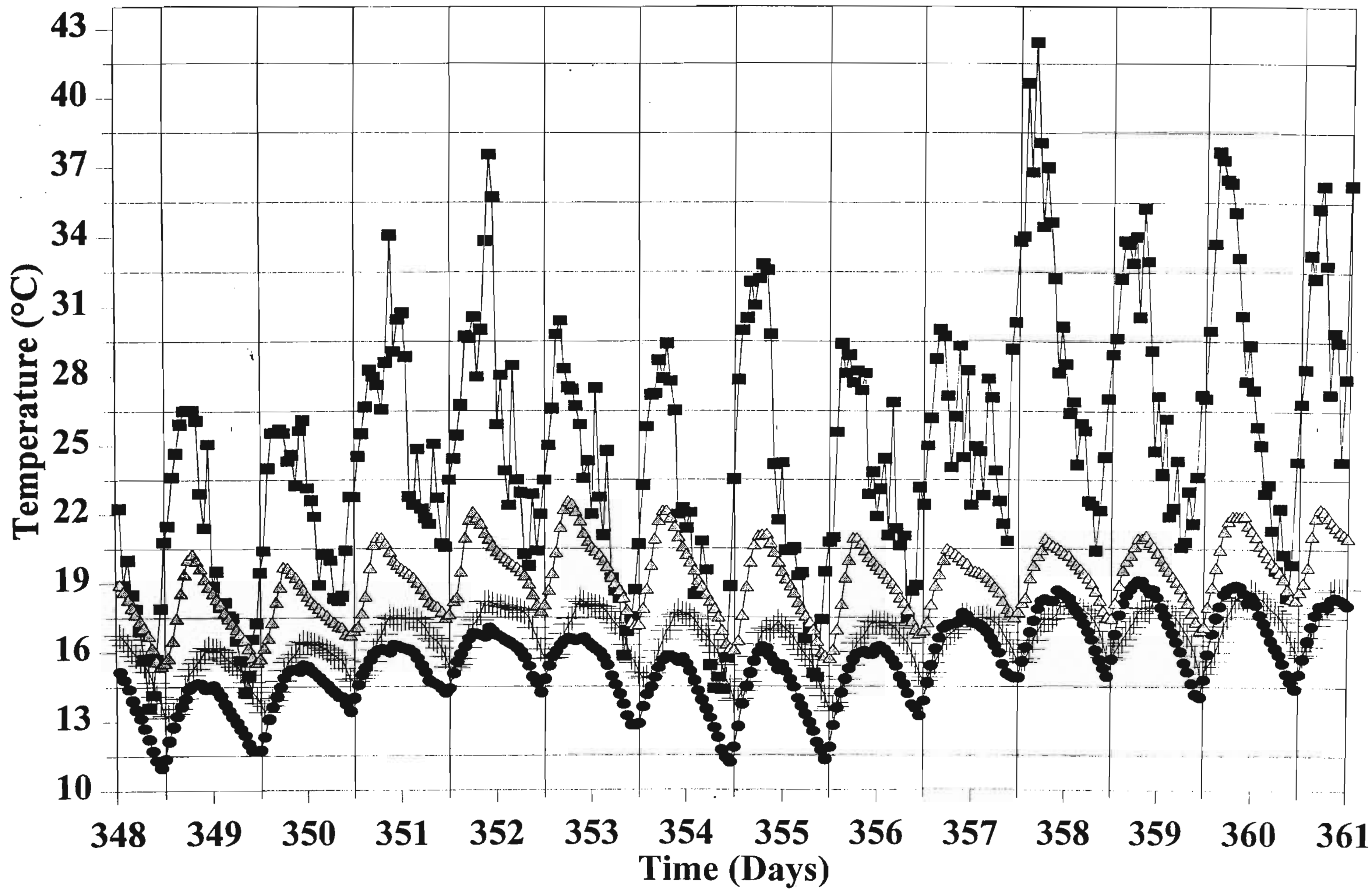


Temperature (oC)
17
15
13
11
9
7
306
307
308
309
310
311
312
313
314
315
316
317
318
319
Time (Days)
TC-1
TC-2
TC-3
TC-4

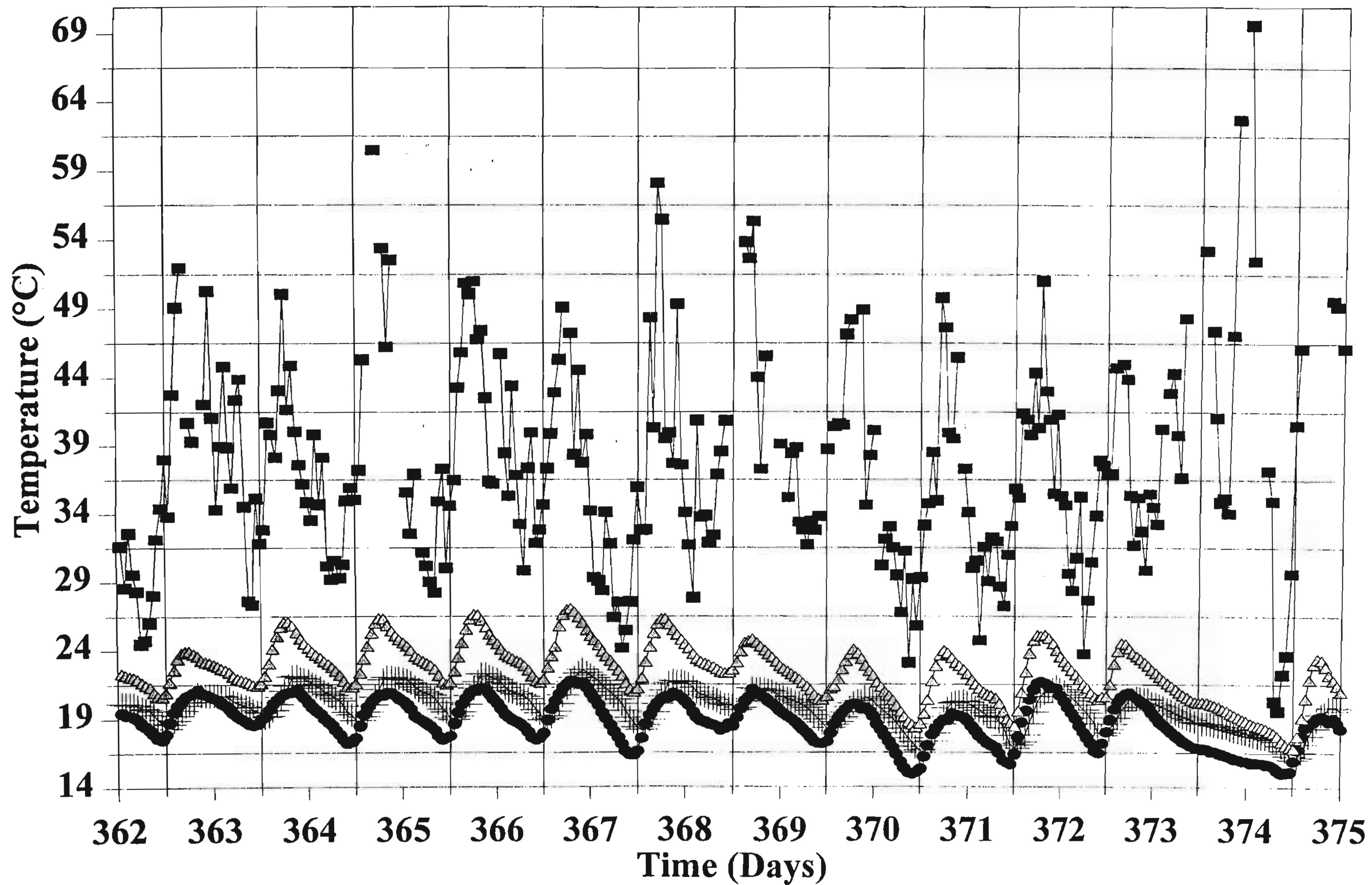


Temperature (oC)
17
15
13
11
9
7
320
321
322
323
324
325
326
327
328
329
330
331
332
333
Time (Days)
TC-1
TC-2
TC-3
TC-4

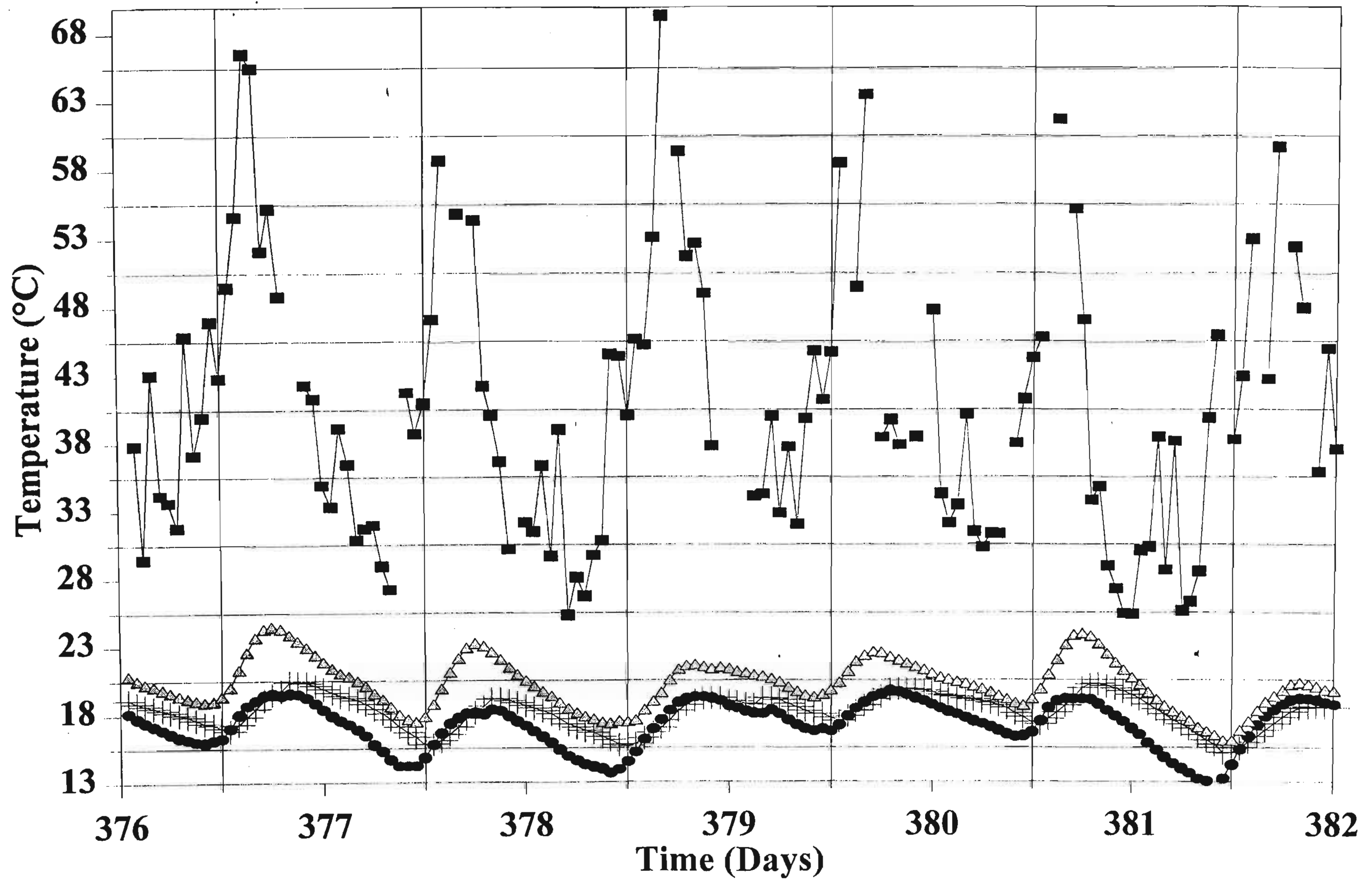

36
33
30
27
24
21
18
15
12
9
Temperature (°C)
334
335
336
337
338
339
340
341
342
343
344
345
346
347
Time (Days)
TC-1
TC-2
TC-3
TC-4



Temperature (°C)
43
40
37
34
31
28
25
22
19
16
13
10
348
349
350
351
352
353
354
355
356
357
358
359
360
361
Time (Days)
TC-1
TC-2
TC-3
TC-4



Temperature (°C)
69
64
59
54
49
44
39
34
29
24
19
14
362
363
364
365
366
367
368
369
370
371
372
373
374
375
Time (Days)
TC-1
TC-2
TC-3
TC-4



Temperature (°C)
68
63
58
53
48
43
38
33
28
23
18
13
376
377
378
379
380
381
382
Time (Days)
TC-1
TC-2
TC-3
TC-4