COMPLEX SOIL-MICROORGANISM-POLLUTANT INTERACTIONS UNDERPINNING BIOREMEDIATION OF HYDROCARBON/HEAVY METAL CONTAMINATED SOIL

BY

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DECLARATION

I hereby declare that the work presented in this thesis, except where indicated, is the result of my own investigation, under the supervision of Prof. E. Senior and Dr. C. A. DuPlessis, Department of Microbiology and Plant Pathology, Department of Agronomy, University of Natal, Pietermaritzburg.

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ABSTRACT

This study evaluated the efficacy of bioremediation as a treatment option for a hydrocarbon and heavy metal contaminated soil. Microbial degradation of hydrocarbons under aerobic, nitrate-reducing and sulphate-reducing conditions was examined. Nutrient supplementation with nitrogen and phosphate as well as aeration seemed to be the most important factors for enhancing biodegradation. From initial batch studies, a carbon:nitrogen ratio of 50:1 was found to be optimal for biodegradation. However, very low carbon to nitrogen ratios were undesirable since these inhibited microbial activity. Manipulation of the pH did not seem to be beneficial with regard to hydrocarbon biodegradation. However, low pH values induced elevated concentrations of leachate heavy metals. Aerobic conditions provided optimal conditions for hydrocarbon catabolism with up to 54% of the original contaminant degraded after 2 months of treatment. Further treatment for up to 20 months did not significantly increase hydrocarbon biodegradation. Under nitrate- and sulphate-reducing conditions, 6% and 31% respectively of the initial contaminant was degraded after 2 months while after a further 20 months, 50% and 42%, respectively were degraded. The addition of soil bulking agents and the use of sparging did not significantly increase biodegradation. Similarly, the addition of inoculum did not influence biodegradation rates to any great degree. The presence of heavy metals up to concentrations of 400 $mg\ell^{-1}$ Mn, 176 $mg\ell^{-1}$ Zn and 94 $mg\ell^{-1}$ Ni did not reduce microbial activity within the soil. During the treatment phase, heavy metal and hydrocarbon migration were limited even under water saturation and low pH conditions. A Biodegradation Index was developed and evaluated and may, potentially, find use as an *in situ* assessment technique for microbial hydrocarbon catabolism. The iodonitrophenyltetrazolium salt assay was also found to be an effective and rapid alternative assay for monitoring bioremediation progress.

CHAPTER 1

BIOREMEDIATION OF HYDROCARBON CONTAMINATED SOILS

1.1 INTRODUCTION

Any major industrial country, or for that matter any country with an industrial base, has the potential to contaminate the environment. This is particularly true when considered in conjunction with production, transportation and storage of products. Of particular concern in the modern world is the question of petroleum and oil hydrocarbons. The sheer volumes of hydrocarbons produced and utilized emphasize their pollution potentials. Polycyclic aromatic hydrocarbons with increasing molecular size have been shown to have concomitant increases in their lipophilicity, environmental persistence and mutagenicity (Miller and Miller, 1981; Jacob *et al.*, 1986). Once present in the environment, some polyaromatic hydrocarbons may be biotransformed to compounds with higher toxicities (Alexander, 1980). Of crude oil constituents, naphthalene, and its methyl substituted derivatives, are amongst the most acutely toxic water soluble substances (Anderson *et al.*, 1974). Benzene, a constituent of many petroleum and oil products, has been classified as a Group A human carcinogen, and has been implicated in nonlymphocytic leukaemia (USEPA, 1987). Of particular concern is the oral ingestion and dermal exposure of children to such contaminants as well as the potential fouling of ground and surface water.

The potential for environmental and hydrocarbon contamination is especially high where such material is produced and stored. In terms of hydrocarbon contamination, deliberate or accidental discharge into the soil accounts for the majority of contamination. Surveys conducted in the USA indicated that the greatest potential for soil pollution is from underground petroleum oil storage tanks. Data from a study by Kovalick (1991) reveal that a significant number of the 7 million storage tanks in the USA are leaking. With increasing industrial growth, it can be assumed that this number will continue to increase. In South Africa, information on such leaks is scarce but it can

be assumed that a similar situation exists. The amount of contamination which is attributed to mismanaged hazardous material disposal practices may, in fact, be significantly greater than in the USA as local legislation to control such activities has been lacking. This has led to illegal disposal practices which are either deliberate or through ignorance. Consequently, the situation in South Africa is particularly worrisome simply because of the lack of information concerning the state of soil contamination.

There exists a number of physical and chemical techniques for the cleanup of contaminated soil. These include: excavation, followed by incineration or chemical and solvent treatment (Electric Power Research Institute, 1988); *in situ* vapour phase stripping of volatiles (Oster and Wenck, 1988); and the extraction of contaminated water for further treatment (Charbeneau *et al.*, 1992). *In situ* chemical treatment involves the immobilization or inactivation of the contaminant with chemical agents which includes: industrial cement; silicate and gypsum mixes; clay minerals; and organic polymers (Lee *et al.*, 1987; Pancoski *et al.*, 1988; Pamukcu, 1993). Such processes may be effective if correctly handled but may prove to be costly if large-scale excavation or long-term treatment is required. Furthermore, such approaches may not result in complete decontamination and may in fact exacerbate the situation by transferring contaminants to surrounding areas (Morgan and Watkinson, 1989).

One of the primary mechanisms by which hydrocarbons in the environment can be eliminated is through biodegradation by naturally occurring soil microorganisms (Leahy and Colwell, 1990). As a result, there has been a growing interest in the use of bacterial biodegradative capabilities for treatment of contaminated soil, either as a separate treatment or in conjunction with one of the other physico-chemical techniques (Morgan and Watkinson, 1989). The principal aim of bioremediation is to optimize environmental conditions, either *in situ* or *ex situ*, so that biodegradation can proceed at optimal rates.

Bioremediation is, in certain cases, safer, cheaper and faster than more conventional cleanup methods. In spite of these advantages, bioremediation has not been

universally accepted as a viable treatment option. Because the technology requires knowledge of disparate fields such as environmental engineering, hydrology and microbiology there is often distrust and misunderstanding regarding its use. Furthermore, processes for evaluating on-going bioremediation schemes are not clear-cut. As a result, there has been a reluctance to use a novel process in favour of more conventional technologies. In the South African context, however, bioremediation does offer a viable technology option, which is also cost effective, which offers relief in a country where ground and surface water resources are scarce.

In South Africa, the development of solutions for environmental pollution problems has been relatively recent. Serious environmental considerations were first given by the South African government in 1972 following the appointment of a pollution subsidiary committee of the Planning Advisory Council in 1971. This subsequently led to the establishment of a permanent Cabinet Committee on Environmental Conservation. In order to advise the Cabinet Committee, a non-statutory South African Committee on Environmental Conservation was established and renamed the Council for the Environment in 1975. This council was given broad control of general environmental conservation until 1980 when the Department of Water Affairs, Forestry and Environmental Conservation was formed. The name of this department was subsequently changed to the Department of Environmental Affairs which acquired jurisdiction over a number of environmental statutes (Schwella and Muller, 1992).

However, it was not until recently that any significant legislation concerning the environment was formulated. This took the form of the Environmental Conservation Act 73 of 1989, and is considered the single most important piece of legislation concerning the environment in this country. The act allows for formulation of policy, establishment of statutory bodies, the defining of protected land and water areas, control of environmental pollution, and the regulation of potentially pollution causing activities. The act was changed by the Environmental Conservation Amendment Act 79 of 1992 which addressed the limitations of the Ministers policy making power (Schwella and Muller, 1992).

Of particular significance is the Constitution of the Republic of South Africa Act 200 of 1993 and specifically the human rights declaration. Section 29 provides that every person has the right to an environment which is not detrimental to his or her health and well being. What is noteworthy is that the right has been granted as an individual right with equal importance to the more traditional human rights (such as right to work, property, justice etc.).

In spite of these implementations the environment has not been particularly well served by environmental law. This stems from the fragmented nature of legislation. Environmental law in this country encompasses a wide set of legal attributes which are found in other areas of conventional law. As a result, implementing legislation is often complicated and an obvious remedy obscured. Furthermore, monitoring specific contravention such as pollution is often difficult and requires trained personnel and prosecutors to present such evidence. An example of inadequate legislation is soil law (Soil Conservation Act of 1946 and 1969). Soil protection is provided in the form of preventing soil loss through erosion (Verster *et al.*, 1992). No provisions are in fact made for soil pollution. Soil pollution often relates directly to groundwater pollution and this is of obvious concern in a country with limited water resources such as South Africa. Prosecution of soil pollution can only be attained indirectly by prosecuting through the Water Act. In such cases, effective and accurate tracing of pollution to source is notoriously difficult.

In spite of these problems, each act which is passed can be expected to address and improve environmental quality to comply with the rights of the individual. Consequently, legislation will improve thus forcing sectors such as industry to achieve greater pollution control. A statement of policy made by the Minister of Environmental Affairs in 1989 is particularly relevant to that Government's environmental approach. In his statement, the minister confirmed a commitment to integrated pollution control. For industry, this asserts that traditional disposal methods, such as excavation and removal elsewhere, may not be acceptable and that treatment at source would be favoured. Furthermore, the commitment to using a system design which includes the best

practicable environmental option (BPEO) signifies that techniques such as bioremediation would be well suited to meet such requirements.

In the following discussion, the principles underlying hydrocarbon biodegradation will first be considered. These will then be consolidated by a discussion of various bioremediation treatment strategies and practices.

1.2 MICROBIAL DEGRADATION OF HYDROCARBON CONSTITUENTS

There are three processes by which microorganisms are capable of metabolising hydrocarbons: fermentation, aerobic respiration and anaerobic respiration (Canter and Knox, 1985). Hydrocarbons present in the environment from spills or disposal are generally complex and consist of hundreds of individual components (Atlas, 1977). Thus, for a particular oil mixture to be degraded, many compounds may have to be catabolized. The chemical properties of these compounds may vary from simple *n*-paraffin, monoalicyclic and monoaromatic compounds, to complex branched-chains and ring structures (Horowitz *et al.*, 1975).

The principal biochemical reactions associated with microbial metabolism of xenobiotics and other synthetically produced organic molecules include acylation, alkylation, dealkylation, dehalogenation, amide or ester hydrolysis, oxidation, reduction, hydroxylation, aromatic ring cleavage, and condensation and conjugate formation (Kaufman, 1983).

1.2.1 Aerobic Degradation

Aerobic respiration involves oxidation-reduction reactions in which molecular oxygen serves as the final electron acceptor. The organic component of the contaminant operates as the electron donor or energy source in heterotrophic metabolism (Gibson, 1977). Oxygen is thus used to decompose hydrocarbons into carbon dioxide, water and other inorganic compounds such as sulphate and nitrate (Freeze and Cherry, 1979;

Pettyjohn and Hounslow, 1983). Aerobic respiration is a more efficient and rapid metabolism than anaerobic respiration (Zitrides, 1983) and, as a result, many bioremediation strategies are made under aerobic conditions.

Metabolic pathways have been elucidated for the degradation of a number of simple aromatic and aliphatic hydrocarbons (Atlas, 1978). The general degradation pathway for an alkane involves sequential formation of an alcohol, an aldehyde and a fatty acid. The fatty acid is then cleaved to release carbon dioxide and a fatty acid which is two carbon units shorter than the parent molecule. The whole process has been classified as a β-oxidation. Enzymatic attack is generally achieved by means of a hydroxylase (mono-oxygenase) system (Britton, 1984). Degradation of certain long-chain alkanes may be via an alternative pathway such as subterminal oxidation (Markovetz, 1971). The degradation pathways for highly branched compounds may proceed by omega oxidation to form a dicarboxylic acid.

The general pathway of aromatic degradation involves a cis-hydroxylation of the ring structure to form a diol. Two hydroxyl groups must be present for enzymatic fission of the ring to occur and these may be either *ortho* or *para*. Bacteria incorporate two molecules of oxygen to form dihydrodiol intermediates. Fungi, in contrast, incorporate one molecule of molecular oxygen to form arene oxides (Gibson, 1977). The ring is then oxidatively cleaved by oxygenases to form a dicarboxylic acid. Degradation of substituted aromatic compounds generally proceeds by initial β-oxidation of the side-chain, followed by cleavage of the ring structure (Atlas, 1978).

1.2.2 Anaerobic Degradation

Anaerobic microbial degradation of hydrocarbons is important in anoxic environments (Sleat and Robinson, 1984). Three groups of microorganisms are believed to be responsible for anaerobic degradation of hydrocarbons in environments which are low in electron acceptors other than carbon dioxide (Berry *et al.*, 1987). These groups are the fermenters, the proton reducers and the methanogens (Boone and Bryant, 1980;

McInerney and Bryant, 1981; McInerney *et al.*, 1981).

Anaerobic degradation of hydrocarbons has been shown to occur under denitrifying, sulphate-reducing and methanogenic conditions. In addition, manganic and ferric ions have been shown to act as final electron acceptors (Berry *et al.*, 1987; Grbic-Galic, 1990). Under anaerobic conditions, only those aromatic compounds with oxygen-containing functional groups (phenols and benzoates) are mineralized since oxygenases are inactive (Zeyer *et al.*, 1986). The aromatic ring may be initially reduced to a substituted cyclohexane before hydrolytic ring cleavage. In some cases, the substituent must be removed or substituted before reduction of the ring can occur. It is unknown which mechanisms are able to deal with rings which have no activating groups to facilitate ring hydration. However, it does appear that there is ring oxidation in some instances which facilitates ring catabolism (Berry *et al.*, 1987).

Although anaerobic catabolism of mononuclear aromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylenes has been demonstrated (Alvarez and Vogel, 1995), very little is known about such degradations. Catabolism of polyaromatic hydrocarbons (PAHs) such as naphthalene under denitrifying conditions has been reported (Mihelcic and Luthy, 1988). Mihelcic and Luthy (1988) suggested that hydroxyl substitution may render PAHs susceptible to anaerobic degradation.

The anaerobic degradation of alkanes is still a topic of debate. Although it has been suggested that alkanes may be anaerobically degraded in conjunction with the reduction of sulphates and nitrates (Shelton and Hunter, 1975), conclusive evidence has still not been reported. Atlas (1991) suggested that such degradation would be of limited significance in the natural environment. There has, however been a pathway proposed for alkene degradation as a result of the observation that methanogenesis increased in response to the addition of these molecules (Schink, 1985). It has also been proposed that an alkane dehydrogenase may be responsible for the reduction of alkanes to alkenes prior to further catabolism (Singer and Finnerty, 1984). This has, however, still to be confirmed.

1.2.3 Fermentation

Fermentation involves the use of organic compounds as both electron donors and electron acceptors (Baker and Herson, 1994). Hydrocarbon degradation proceeds by substrate level phosphorylation as the terminal electron acceptor. The fermentation process occurs in the absence of oxygen and relies on organic compounds as electron acceptors. Fermentation results in a large range of end products such as CO_2, acetate, ethanol, proprionate and butyrate (Speece, 1983).

1.2.4 Co-Metabolism

Hydrocarbons may be catabolized by biochemically-mediated reactions which provide neither energy nor nutrients to the microorganism. This is termed co-metabolism or co-oxidation (Alexander, 1973). The transformation product is still not available as a usable substrate to the organism (Hornick *et al.*, 1983). Co-metabolism requires that two or more substrates are present. One of these is the non-growth substrate, which is neither essential nor sufficient to support microbial metabolism (Perry, 1979), while the other is essential for microbial growth. As a result, the non-growth substrate is incidentally and incompletely biodegraded as a result of a non-specific enzyme with a broad specificity (de Klerk and van der Linden, 1974).

1.3 PHYSICAL AND CHEMICAL FACTORS AFFECTING THE BIODEGRADATION OF HYDROCARBONS

The biodegradation of petroleum hydrocarbons is a complex process which can be affected by a number of physical, chemical and biological factors. The correct manipulation of such factors is essential to obtain conditions which are optimal for bioremediation and high degradation rates.

1.3.1 Chemical Composition of Hydrocarbon Mixtures

Petroleum and waste oil hydrocarbons are complex mixtures of individual components which are generally divided into four classes: the resins (pyridines, quinolines, carbazoles, sulphoxides and amides); the asphaltenes (phenols, fatty acids, ketones, esters and porphyrins); the aromatics; and the aliphatics (Colwell and Walker, 1977). Hydrocarbons differ in their susceptibility to microbial attack and can be generally ranked in the following order of increasing recalcitrance: *n*-alkanes > branched alkanes > aromatics > cyclic alkanes (Perry, 1984).

Saturated hydrocarbons include the labile components most susceptible to microbial attack since double or triple bonds tend to inhibit biodegradation (Zobell, 1946). Branched-chain alkanes are usually less easily degraded than *n*-alkanes, while the presence of straight-chained alkanes appears to inhibit the degradation of branched-chained alkanes (Pirnik, 1977). In the case of quaternary and β-branched compounds steric hinderance of oxidation enzymes results in these compounds being particularly recalcitrant (Britton, 1984). However, a positive correlation between the branch numbers and biodegradation resistance has not been conclusively validated (Singer and Finnerty, 1984). Long-chain aliphatic hydrocarbons are, generally, more easily degraded than short-chain hydrocarbons. Chain lengths of nine carbons or less are difficult to degrade because of their toxicity to microorganisms. The optimal chain length for biodegradation appears to be between 10 and 20 carbons (Baker and Herson, 1994).

Low molecular weight aromatic hydrocarbons may be easily subjected to both evaporation and biodegradation (Kappeler and Wuhrmann, 1978) with the latter attributed to enzymatic attack on the ring structure (Gibson, 1971). High molecular weight polycyclic aromatic hydrocarbons, in contrast, are particularly recalcitrant to microbial attack as they are slowly desorbed and, therefore, less available for biological uptake. Generally, the susceptibility of PAHs to biodegradation is inversely proportional to the ring number (Aronstein *et al.*, 1991). The biodegradation of these compounds is not well understood with little data available, although there have been reports of co-metabolism of these compounds (Cerniglia and Heitkamp, 1989). Generally, the

polyaromatic hydrocarbons appear to be less readily biodegradable than the alkanes.

Cycloalkanes such as hopanes are amongst the most persistent and toxic components of hydrocarbon wastes in the environment (Stirling *et al.*, 1977; Perry, 1979; 1984). There have, however, been reports of degradation by direct oxidation or co-oxidation of both substituted and unsubstituted cycloalkanes with up to six-membered condensed ring structures (Walker *et al.*, 1975a; Perry, 1979).

The degree of substitution affects biodegradation rates. Compounds which contain amine, methoxy, and sulphonate groups, ether linkages, halogens, branched carbon chains and substitutions at the *meta* position of the benzene ring are, generally, persistent (Knox *et al.*, 1968). Persistent cyclic hydrocarbons have been shown to be increasingly susceptible to microbial degradation with the addition of aliphatic side-chains (Atlas, 1978). In addition, linear non-branched compounds are more easily biodegraded than are branched and ring forms (Pettyjohn and Hounslow, 1983).

1.3.2 Physical State of the Hydrocarbons

Hydrocarbons present in bodies of water tend to spread and form a slick (Berridge *et al.*, 1968). Weather conditions may induce the formation of emulsions which increases the area available to microbial attack (Cooney, 1984). The formation of large plates of oil may, however, cause low surface to volume ratios which are unfavourable for microbial degradation (Davis and Gibbs, 1975). Following environmental release, aggregates of weathered and undegraded hydrocarbons may resist microbial catabolism due to their limited surface area (Colwell *et al.*, 1978).

Differences in hydrocarbon degradation in soil and aqueous environments are related to hydrocarbon movement and distribution. Soil systems, typically, contain particulate matter which affects the physical and chemical nature of the hydrocarbon and susceptibility to microbial degradation (Bossert and Bartha, 1984). Terrestrial oil spills are characterised by vertical movement into the soil which may minimize the loss of

volatile hydrocarbons which are toxic to the microorganisms. Adsorption of hydrocarbons to particulate matter may reduce the toxicity of certain hydrocarbon components but may contribute to the formation of recalcitrant molecules (Leahy and Colwell, 1990; Weissenfels *et al.*, 1992).

1.3.3 Hydrocarbon Toxicity

Hydrocarbon toxicity may prevent biodegradation because of inhibitory contaminant concentrations (Pettyjohn and Hounslow, 1983). As a result, the biodegradation of non-toxic target molecules may be delayed or even prevented (Bartha and Atlas, 1977). According to Riser-Roberts (1992), toxicity and degradability may be linked i.e. the most toxic structures may also be the most readily degradable ones. Where *meta*-substitutions are present, resistance to biodegradation may increase with a corresponding decrease in toxicity. In contrast, halogenation in the *para* position increases both phytotoxicity and biodegradability of certain compounds such as phenoxyacetates. Increasing the length of the side-chains may also affect both toxicity and biodegradability.

In the case of alkanes, molecules in the range C_2 to C_6 may be inhibitory as their relatively small size allows them to penetrate into cell membranes. This may also be the case with some of the cycloalkanes of a similar size (Hornick *et al.*, 1983).

The toxicity of PAH's is related to their water solubility (Sims and Overcash, 1983). Low molecular weight hydrocarbons in the aqueous phase are, however, volatilized rapidly and toxicity is limited (Coffey *et al.*, 1977). In the case of alkanes, the vapour phase is more toxic than the liquid phase. Thus, toxicity is, generally, temperature dependent (Bartha and Atlas, 1977).

Although low molecular weight aromatic hydrocarbons are toxic they can be metabolised in low concentrations. Condensed aromatics are less toxic but are degraded at much slower rates. Cycloalkanes, in contrast, are highly toxic and are

utilised as substrates by individual species of organisms only rarely. Degradation is, however, possible by co-metabolism with a mixed microbial metabolism (Riser-Roberts, 1992).

The toxicity of low molecular weight hydrocarbons is due to their ability to destroy lipid-containing pericellular and intracellular membranes (Bartha and Atlas, 1977). Liquid hydrocarbons of the *n*-alkane, *iso*-alkane, cycloalkane and aromatic type with C_{10} and below all share this solvent ability.

1.3.4 Hydrocarbon Concentration

Generally, the rate of degradation of a compound in the environment is governed by the concentration of that compound. The process can be described by Michaelis-Menten kinetics (Pfaender and Bartholomew, 1982). Although degradation of low molecular weight aromatics has been shown to follow such a trend, degradation of high molecular weight molecules are related to aqueous solubilities rather than total substrate concentration (Wodzinski and Bertolini, 1972; Wodzinski and Coyle, 1974; Thomas *et al.*, 1986; Robertson and Button, 1987).

It has been shown that the degradation of long-chain alkanes with low solubilities (0.01 $mg l^{-1}$) occurs at rates exceeding the rates of hydrocarbon dissolution (Thomas *et al.*, 1986). Furthermore, degradation is dependent of the surface area available for emulsification or biofilm attachment (Fogel *et al.*, 1985).

A high concentration of hydrocarbon may cause a decrease in biodegradation because of high toxicity, oxygen limitation and low nutrient availability (Leahy and Colwell, 1990). A correlation to this effect was observed in soil systems contaminated with oil sludge waste (Dibble and Bartha, 1979). A decrease in microbial activity was recorded between 10% (w/w soil) and 15% (w/w soil) contamination and was attributed to inhibition by toxic components of the oil sludge.

1.3.5 Temperature

As is true for other microbial activities, hydrocarbon biodegradation is strongly temperature dependent. Generally, biodegradation rates have been observed to decrease with decreasing temperature, as a result of lowered enzyme activity (Atlas and Bartha, 1972). Higher temperatures tend to increase degradation up to a range of 30°C to 40°C after which the membrane toxicity of hydrocarbons is increased (Bossert and Bartha, 1984). Biodegradation at higher temperatures is possible as thermophilic alkane degrading microorganisms have been isolated (Mateles *et al.*, 1967).

Soil temperature may also have profound effects on the soil matrix and physico-chemical nature of the contaminant. Oil viscosity increases with a decrease in temperature and volatilization of toxic short-chain alkanes is reduced. Water solubilities of these fractions are also increased and lead to a decrease in microbial activity and delayed onset of biodegradation (Atlas and Bartha, 1972). Soil temperature has also been observed to influence soil volume, oxidation-reduction potentials, and water structure within the soil matrix (Paul and Clark, 1989).

Climate tends to select for microbial populations capable of hydrocarbon degradation under specific environmental conditions. This is significant at low temperatures where hydrocarbon-degrading microorganisms have been shown to adapt to low ambient temperatures (Huddleston and Cresswell, 1976; Colwell *et al.*, 1978).

1.3.6 Oxygen

The initial microbial attack of hydrocarbons occurs with oxygenases which require the presence of molecular oxygen. Catabolism of various hydrocarbons has been reviewed by Cerniglia (1992), Watkinson and Morgan (1990) and Smith (1990). Aerobic conditions are, thus, a requirement for hydrocarbon degradation. Oxygen availability in soil is dependent on soil type, water saturation, metabolic rate and substrate concentration (Bossert and Bartha, 1984). Oxygen, in particular, has often been

identified as an important factor that may limit the rate of hydrocarbon degradation in soil (von Wedel *et al.*, 1988; Thomas and Ward, 1989; Leahy and Colwell, 1990). Aeration of surface soils can be achieved by physical manipulations such as tilling and the incorporation of bulking agents and other conditioners. Bulking agents and conditioners improve the soil structure thereby increasing aeration. For contamination below the ground surface, oxygen may be added through the use of blowers, by application of a vacuum, by direct sparging, or by the introduction of water which has been supplemented with air, oxygen or hydrogen peroxide (Baker, 1994).

Emphasis has in the past been placed on aerobic processes since anaerobic degradation has been shown to occur at very slow or negligible rates (Atlas, 1981; Bossert and Bartha, 1984). More recently, however, there has been increasing interest in anaerobic degradation of hydrocarbons. In particular there has been speculation that anaerobic degradation is responsible for the breakdown of a higher proportion of polyaromatic hydrocarbons than initially thought. Under methanogenic conditions, half the initial concentrations of benzene and toluene were mineralized in 60 days, while naphthalene was found to be mineralized in 45 days under denitrifying conditions (Vogel and Grbic-Galic, 1986; Grbic-Galic and Vogel, 1987; Mihelcic and Luthy, 1987; 1988). The anaerobic degradation of aliphatic hydrocarbons is also debatable although there is some evidence to suggest that anaerobic degradation does in fact occur (Schink, 1985).

In the absence of oxygen, hydroxylation of the aromatic ring is thought to depend on water as a source of oxygen (Grbic-Galic and Vogel, 1987). Under denitrifying conditions, nitrate may act as the final electron acceptor (Mihelcic and Luthy, 1988). Similarly, sulphate may also serve as an alternative electron acceptor (Pierce *et al.*, 1975). Reactions of this sort have, typically, been classified as fermentations with partial oxidation and reduction of the substrate liberating carbon dioxide and methane as end products (Grbic-Galic and Vogel, 1987).

Although traditionally dismissed as having an insignificant contribution towards

hydrocarbon degradation, the importance of this process should not be underestimated and should be further investigated to elucidate anaerobic metabolic pathways. Whether mixtures can be mineralized under denitrifying or methanogenic conditions remains to be seen.

1.3.7 Nutrients

Microbial degradation of contaminants such as hydrocarbons requires the presence of certain nutrients for optimal biological growth. The release of hydrocarbons into the environment may result in excessively high carbon/nitrogen and carbon/ phosphorus ratios. High ratios may be due to low inorganic nutrient concentrations and are limiting for microbial growth (Atlas, 1981). Nitrogen and phosphorus may also be limited in soil and enhanced degradation through addition of N-P-K fertilizers has been demonstrated in several studies (Jobson *et al.*, 1974; Dibble and Bartha, 1979). Generally, a C:N:P ratio of 100:10:1 (w/w) should provide adequate nutrients for a bioremediation strategy (Charbeneau *et al.*, 1992). The general evidence collected thus far has, however, suggested that bioremediation enhancement through inorganic nutrient supplements is extremely case specific.

Inorganic nutrient addition may also prove to be ineffective and, in some cases, may inhibit microbial degradation (Morgan and Watkinson, 1989). Morgan and Watkinson (1989) found that phosphate addition in combination with hydrogen peroxide may cause precipitation of insoluble salts thereby decreasing permeability in the biostimulation zone. These findings highlight the inadequacy of chemical analysis to predict the nutrient supplements required. Thus, laboratory and field studies are required for each site as nutrient requirements are site specific.

1.3.8 Salinity

Shiaris (1989) reported a correlation between salinity and the rates of naphthalene and phenanthrene degradation. Ward and Brock (1978) observed a decrease in

hydrocarbon metabolism with increasing salinity (3.3% v/v to 28.4% v/v) in a hypersaline environment. More recently, Rhykerd *et al.* (1995) corroborated this work when they observed decreased mineralization of oil present in NaCl-containing soils. Reduced biodegradation was attributed to a reduction of metabolic rates. This infers that biodegradation of hydrocarbons in hypersaline environments may be slow.

1.3.9 Pressure

High pressure environments, such as those found in the ocean, have been shown to affect the biodegradation rates of hydrocarbons. Schwarz *et al.* (1974a; 1974b; 1975) studied the degradation of tetradecane, hexadecane and a mixed hydrocarbon substrate at pressures of 101kPa and 50650 kPa. Under high pressure, a 40 weeks incubation period was required for degradation in comparison to 8 weeks at low pressures. These findings are of particular significance where oil fractions may settle in deep sea environments leading to the persistence of certain molecules (Colwell and Walker, 1977).

1.3.10 Water Activity

Biodegradation of hydrocarbons in the soil requires water for microbial growth and for diffusion of nutrients and by-products during metabolism. Water activity in soil systems can range between 0 and 0.99 (Bossert and Bartha, 1984). Under conditions of low water activity, microbial growth is limited. As a result, optimal water saturation should be kept between 30% and 80% of field capacity (Dibble and Bartha, 1979; Riser-Roberts, 1992). Excess moisture can be a problem in poorly drained soils or treatment cells as localised anoxic zones may be introduced and oxygen diffusion limited (Riser-Roberts, 1992). In soil systems it is, thus, necessary to maintain correct moisture levels particularly in severely contaminated soil where water holding capacity may be reduced.

1.3.11 Soil pH

Biological activity in soil is greatly affected by pH through the availability of toxicants and nutrients. Soil pH conditions are variable and may range between 2 and 11 (Bossert and Bartha, 1984). The optimum pH for rapid decomposition is between 6.5 and 8.5 (Atlas, 1981). Bacteria and actinomycetes have pH optima around neutral whereas fungi proliferate better under acidic conditions (Dibble and Bartha, 1979). Increasing the pH to 8.5, however, was found to lead to a decrease in microbial degradation rates (Verstraete *et al.*, 1976). For the application and disposal of oil sludge onto soil, Dibble and Bartha (1979) determined a pH optimum of 7.8. Generally, soils tend to be acidic and in many bioremediation projects, acidity may have to be neutralized to raise the pH. This can be achieved through the careful addition of liming agents such as calcium hydroxide, calcium carbonate, calcium magnesium carbonate, calcium oxide or calcium silicate slag (Riser-Roberts, 1992; Baker, 1994).

In addition to maintaining environmental conditions conducive to microbial metabolism, careful control of pH may also serve to immobilize heavy metals in sites polluted with these contaminants (Riser-Roberts, 1992).

1.4 MICROORGANISMS

Surface soils contain large numbers of microorganisms which have the ability to degrade hydrocarbons (Perry and Scheld, 1968). Individual organisms can only metabolise a limited number of hydrocarbons and a mixed population with broad enzymatic capabilities is required to degrade complex hydrocarbon mixtures (Bossert and Bartha, 1984). Species of *Pseudomonas, Arthrobacter, Alcaligenes, Corynebacterium, Flavobacterium, Achromobacter, Micrococcus, Nocardia* and *Mycobacterium* are amongst the most important hydrocarbon-degrading bacteria in soil (Jurtshuk and Cardini, 1971; Bossert and Bartha, 1984).

Spore-forming bacteria such as *Bacillus* species appear to have a negligible role in oil

degradation (Felix and Cooney, 1971). Apart from *Nocardia* and *Mycobacterium*, actinomycetes do not appear to compete well in contaminated soils. However, Kobayashi and Rittman (1982) suggested that actinomycetes may be particularly useful in the treatment of contaminated soil where a composting technique is employed. The roles of algae and protozoa have been poorly documented although a species of alga, *Prototheca zopfi*, was reported to degrade complex hydrocarbon substrates (Walker *et al.*, 1975b). Cerniglia *et al.* (1980) also observed nine cyanobacteria, five green algae, one red alga, one brown alga and two diatoms with naphthalene oxidising capabilities. The extent to which such organisms contribute to hydrocarbon mineralization in soil is still not known.

Methanotrophs, which have a highly specialised metabolism, also exhibit hydrocarbon-degrading capabilities. The metabolic rates are, however, significantly lower than those of aerobic bacteria (Bossert and Bartha, 1984).

Fungi also play an active role in hydrocarbon oxidizing activities in the soil and may be as versatile as bacteria in metabolizing aromatics (Jones and Eddington, 1968). The ability to utilize hydrocarbons occurs mainly in two orders, the *Mucorales* and the *Moniliales* (Nyns *et al.*, 1968). Species of *Cunninghamella, Syncephalastrum, Mucor, Neurospora, Claviceps,* and *Psilocybe* have hydrocarbon degradative capacities (Cerniglia *et al.* 1978; Riser-Roberts, 1992). A white rot fungus, *Phanerochaete chrysosporium* has been shown to degrade a number of complex hydrocarbon mixtures at fairly high rates (Bumpus *et al.*, 1987). Furthermore, this organism has the ability to function in nitrogen-limited environments and may, thus, find practical use for *in situ* cleanup (Bumpus *et al.*, 1987).

It has been noted that microbial activity is generally enhanced in contaminated soils and stimulation of microbial activity has been positively correlated to increased hydrocarbon concentration (Dibble and Bartha, 1979). Pinholt *et al.* (1979) showed that eight months after contamination, the numbers of oil degrading bacteria in soil increased ten-fold to 50% of the total bacterial count while no pronounced increase in fungal diversity was observed.

Jensen (1975) also noted lower bacterial species diversity in oil-contaminated soils than in uncontaminated soils. Populations of *Athrobacter*, *Corynebacterium*, *Brevibacterium*, *Mycobacterium* and *Nocardia* showed positive responses to oil- contamination.

Little is known concerning the relative extent to which bacteria and fungi (including yeasts) participate in hydrocarbon degradation. However, in soil systems, bacteria and fungi are relatively abundant and both groups contribute to hydrocarbon degradation (Bossert and Bartha, 1984). The degree of participation by each group should be a function of the local environmental conditions (Leahy and Colwell, 1990).

1.5 BIOREMEDIATION PROCESS DESIGN

A number of engineered treatment systems are available for the bioremediation of hydrocarbons in soil. The selection of a system should be based on physical, chemical and biological properties of the contaminant, site constraints and local or state requirements. The bioremediation process can be broadly divided into several steps depending on whether the contamination as surface based or subsurface based.

1.5.1 Surface Soil and Sludges

A: Land Treatment

Land treatment is the process of controlled application of waste onto the soil surface and/or the incorporation of the waste or contaminated soil into the upper soil zone. The process relies on the dynamic physical, chemical and biological processes occurring in the soil to immobilize and degrade the waste. The soil/waste mixture is managed in such a way that it enhances the immobilization of the waste by the soil; it stimulates the degradation of the waste by the indigenous microorganisms or inoculum; minimizes volatilization and leaching of the waste out the treatment area; and controls the surface

water runoff (Charbeneau *et al.*, 1992).

The mechanisms of immobilization and degradation include sorption, hydrolysis, photolysis, chemical degradation and biodegradation. The process of volatilization may also contribute to removal of certain low molecular weight hydrocarbons. Biodegradation is affected by the type and concentration of the waste, the presence of waste-degrading organisms, pH, temperature and the availability of oxygen, water and nutrients. Addition of supplements such as carbon and energy sources, and adjustment to optimal pH promote biodegradation (Bossert and Bartha, 1984). Usually, the organisms involved are the indigenous species but organisms with specialised metabolic capabilities may be inoculated where the soil has been sterilized by the presence of toxicant(s). In this case, the toxicant(s) should be removed or detoxified before land treatment can be successful (Thomas *et al.*, 1992).

Land treatment processes can be divided into surface land treatment and on-site land treatment. Both types involve the addition of nutrients, the implementation of tilling to increase the nutrient and oxygen availabilities, and the adjustment of pH and moisture content when needed. Surface soil treatment involves the treatment of contaminated soil in place or incorporation of the contaminant in the surface soil (up to a depth of 45 centimetres of the top soil). The actual treatment zone where attenuation may occur is usually at a considerably greater depth than 45cm (Charbeneau *et al.*, 1992).

On-site treatment involves the construction of a lined and walled holding facility in which the waste is mixed with clean soil or an existing soil/waste mixture. Usually, the construction area has drainage and leachate collection systems. Leachate treatment systems are located on-site to minimize cost of transport to other facilities. Both surface soil land treatment and on site land treatment have been used successfully to bioremediate petroleum hydrocarbons and polyaromatic hydrocarbons (Wang *et al.*, 1990; Testa and Winegardner, 1991; USEPA, 1991). The costs of landfarming are, generally,

low when compared to other treatment alternatives. The treatment time is, typically, between 2 and 6 months (Englert *et al.,* 1993).

B: Composting

Composting exploits the process by which biomass, inorganic by- products and energy, in the form of heat, are produced when organic matter is degraded aerobically. The process requires that the material is biodegradable, has an adequate water content, is present in sufficient quantities to retain heat and is porous enough to allow gas exchange. Free water should be kept to a minimum to allow adequate circulation of oxygen. In certain cases, addition of nitrogen may be required. During successful composting, temperatures as high as 60°C may be achieved. As a result of composting, the waste is converted to a stable material with a decrease in overall mass and water content (Thomas *et al.*, 1992).

Typical bioremediation composting systems include the Windrow, the Beltsville and the "in vessel" systems. The Windrow is an open system in which a biopile is divided into several rows and aeration is achieved by periodically turning the compost pile. The Beltsville, or static pile, is also uncovered but aeration is achieved with an air distribution system under the pile.

The in vessel system involves adding the contaminated soil to a closed or open vessel which is equipped with a temperature controlled aeration system. The material is aerated either by mechanical mixing or through the introduction of air by blowers. (Charbeneau *et al.*, 1992; Thomas *et al.*, 1992).

Soil contaminated with petroleum products is amenable to bioremediation by the composting process. Bulking agents may be added to increase the porosity and facilitate aeration and co-metabolism. Bulking agents include fibrous plant material, wood chips and bark. For recalcitrant material, the waste can be mixed with a highly degradable material

such as manure. The manure acts as the carbon source for the microorganisms while the waste is degraded by co-metabolism (Baker, 1994).

The correct mix of hydrocarbon polluted soil, moisture, nutrients and supplements is critical to achieve a balance between the energy requirements and the bioremediation efficiency. The compost pile must be sufficiently porous to facilitate the passage of air and have a solids content which is appropriate for solids handling equipment. A mix that is too wet will reduce the porosity, hinder the material handling and increase the treatment time.

The compost process may reduce treatment time when compared to landfarming and biopile techniques. Treatment time for organic labile wastes is typically between one to four months with the costs slightly higher than for landfarming (Englert *et al,* 1993).

1.5.2 Liquid/Solids Processes (Slurries)

Liquid/solids contact or slurry phase bioremediation treats hazardous wastes in a closed reactor or open lagoon surface impoundments (Thomas *et al.*, 1992). The process is similar to conventional biological suspended growth treatment. Environmental conditions such as temperature, pH, nutrient and electron acceptor availability can be controlled (Thomas *et al.*, 1992; Baker, 1994). The waste to be treated is suspended and mixed to maximise mass transfer between the microorganisms, nutrients, electron acceptors and waste (Black *et al.*, 1991).

Generally, the bioreactor is oxygenated by means of spargers, compressors, or floating or submerged aerators. Aeration also allows for mixing although mechanical mixing is common. A consequence of mixing and aeration is volatilization. Reactors may, thus, also be equipped with monitoring and trapping systems. Single batch reactors and sequenced batch reactors may be used, either in above ground tanks or lined *in situ* lagoons (Thomas *et al.*, 1992).

The rates of biodegradation in slurry reactors are usually faster than the rates of degradation of the same compound *in situ*. This reflects the greater degree of environmental control and uniform mixing attained in the bioreactors (Baker, 1994). Treatment costs are high due to the engineering requirements (King *et al.*, 1992).

1.5.3 Unsaturated Subsurface Material

Bioventing

Bioventing may be described as the accelerated biodegradation of vapour phase sub-surface contaminants by forcing and/or drawing air through the subsurface region. Soil vacuum extraction has been used for the removal of oily phase contaminants above the water table. A well is placed near the point of contamination but above the water table. A vacuum is then placed on the well to extract the soil air which contains volatile emissions. The emissions are usually disposed to the atmosphere, adsorbed to activated carbon, or treated with a catalytic converter. The bioventing process should not be confused with soil vapour extraction (SVE). Soil vapour extraction is distinguished from bioventing in that the process is of physical/chemical nature. These operations are consequently operated at higher gas extraction rates than bioventing systems.

Before application of a vacuum, the soil air in contact with the contaminants is usually anoxic with little or no aerobic metabolism operative. The vacuum from the extraction well draws oxygenated air through the contaminated soil horizon. After a period of stabilisation, the oxygen concentration decreases with an increase of carbon dioxide emissions, thus indicating the onset of bioremediation (Thomas *et al.*, 1992).

1.5.4 Saturated Subsurface Material

A: Plumes

In cases where the contaminant is soluble in water, a plume forms. Because of low dispersion along flow paths in aquifers, nutrients and electron acceptors are difficult to mix *in situ*. The introduction of uncontaminated water containing supplements merely displaces contaminated water, thus giving the appearance of remediation. The bioremediation of a plume *in situ* requires that the water is pumped from the aquifer, supplemented with requisites for growth, and is returned to the aquifer. As a result of the problems inherent in *in situ* treatment systems, bioremediation of contaminated groundwater is, conventionally, conducted by pumping and then treating in an above ground bioreactor. Where the contaminated vadose zone (the unsaturated soil zone above the water table) undergoes treatment, water percolating through the soil is collected in the aquifer and pumped to the surface for treatment (Baker, 1994).

Plumes containing organics are usually anoxic and contain appreciable concentrations of reduced iron and manganese. Introduction of oxygen and hydrogen peroxide may result in precipitation of hydrated iron and manganese hydroxides which may then foul the well system. Oxygen demand associated with biogenic methane must also be considered in the system design (Thomas *et al.*, 1992).

B: Contaminant Sources

Where a contaminant source is present or trapped below the soil surface, water can be supplied through infiltration wells and galleries to the source. The water moves horizontally away from the infiltration well and joins the flow in any regional aquifers. In the case of infiltration galleries, material is excavated and replaced with gravel to allow uniform distribution of water within the gallery. The flow rate is such that all the native material between the gallery and oily contaminant is saturated. The flow direction is from the gallery down to the aquifer.

Oxygenation of water within the wells can be achieved by air sparging the water column

which can result in concentrations of 10mgl^{-1}. Pure oxygen can be used as an alternative with concentrations of 40mgl^{-1} achievable. Hydrogen peroxide can be tolerated by hydrocarbon degrading bacteria up to concentrations of between 500mgl^{-1} and 1000mgl^{-1} which degrade to oxygen concentrations of between 250mgl^{-1} and 500mgl^{-1}. Because of the greater capacity for oxygen delivery, hydrogen peroxide is often the agent of choice.

Water used for remediation is also normally supplemented with nutrients such as phosphate and ammonia nitrogen. The addition of nitrates serves as an alternative electron acceptor where anaerobic conditions exist. To minimise expenditure, water may be recovered, supplemented with nutrients and electron acceptors and reintroduced into the infiltration wells. Under such conditions biofilm formation in the wells, due to the presence of residual contaminants, may result. Compensation for premature electron acceptor loss may then have to be made.

Generally, the aromatic compounds are preferentially degraded in the presence of solutions containing oxygen and nitrate. Installation time is dependent on system design and treatment strategy. The treatment time may range from six months to several years depending on the extent of contamination and, as a consequence, the costs are substantial (Thomas *et al.*, 1992).

1.6 SEEDING OF MICROORGANISMS

Biological treatment methods for hydrocarbon remediation usually rely upon stimulation and natural selection of indigenous microorganisms present in the site. However, the natural soil flora may lack the metabolic capabilities to degrade certain compounds or emulsify water-insoluble components. Alternatively, they may have the ability but may not be present in sufficient numbers to facilitate cleanup. In such cases it may be necessary to add large numbers of exogenously grown microorganisms (Riser-Roberts, 1992). An effective seeding microorganism must have: the ability to degrade a wide variety of

hydrocarbon components; genetic stability; storage viability; rapid growth following exposure; a high degree of enzymatic activity and growth in the environment; the ability to compete with indigenous microorganisms; non-pathogenicity and an absence of potential toxic metabolite production (Atlas, 1977).

Microorganisms with ability to degrade a wide variety of hydrocarbons have been developed with the aid of gene manipulation. Friello *et al.* (1976) developed a multi-plasmid *Pseudomonas* species capable of degrading aromatic, aliphatic, terpenic and polyaromatic hydrocarbons. Although this would appear to offer an obvious solution for cleanup, there is still concern over the introduction of genetically manipulated microorganisms into the environment (Sussman *et al.*, 1988).

Soil ecosystems are complex, contain high concentrations of organic and inorganic matter, are generally variable in their physical and chemical nature, and growth restrictive to introduced organisms (Bossert and Bartha, 1984). Indigenous organisms adapted to these conditions have been shown to increase in numbers in response to hydrocarbon contamination (Jensen, 1975; Llanos and Kjoller, 1976). Inocula applied under such conditions compete poorly with the highly adapted natural microorganisms (Bossert and Bartha, 1984). In addition, a successful seed microorganism must overcome metabolic limitations due to low concentrations of the target molecule, predation, the presence of inhibitory substances, presence of alternative organic molecules other than the contaminant, and limited movement of inoculum through the soil (Goldstein *et al.*, 1985).

The application of seed organisms appears to have greatest potential in reducing the lag time required for a microbial response. However, even this aspect is debatable under certain conditions. Bioreactors and environments which, to some extent, can be controlled are the best examples of seeding experiments. In such cases, system parameters can be

optimized and competition is reduced (Leahy and Colwell, 1990). Although additional costs are involved in designing and building such systems, some success has been achieved (von Wedel *et al.*, 1988). Perhaps a combination of inoculation with traditional methods such as bioreactor and soil slurry treatments may have merit and is worthy of further investigation, particularly where recalcitrant molecules are concerned.

1.7 CONCLUSION

The world is faced with the task of cleaning hydrocarbon spills whether accidental or intentional. Many of the traditional methods used for treating contaminated soil are either ineffective or do not provide a long-term solution. The use of chemicals in some cleanups has, in fact, exacerbated the problem. The answer, in many cases, lies in the biological treatment of such contamination. Bioremediation practices, combined with on site biodegradation techniques offer very efficient and cost-effective treatment options. Furthermore, the versatility of biodegradation techniques permits their use in conjunction with chemical and physical procedures. A combination of treatments may help to optimize microbial degradation and facilitate total destruction of key contaminants.

In most cases of soil pollution, the indigenous microbial populations are able to degrade the hydrocarbons provided that sufficient nutrients and oxygen are supplied and other environmental factors are modified. Bioremediation is a developing technology and much work is currently being conducted to optimize the requirements for microbial degradation. Key metabolic pathways are being elucidated to predict the efficacy of individual strains and associations. Furthermore, "new" organisms are under development to target a wider range of substrates and recalcitrant molecules. Anaerobic treatments, previously considered as relatively unimportant, are now being explored as an option for hydrocarbon and, in particular, polyaromatic hydrocarbon degradation.

In genetic manipulation may lie the answer to the improvement and enhancement of

hydrocarbon catabolism abilities. However, further development requires detailed knowledge of genetic traits and microbial physiology. Manipulation of ecologically important traits may result in a competitive edge for seed organisms which has previously been lacking. Although the development of speciality organisms has been explored there has, to date, been very little conclusive evidence to support supplementing contaminated sites with these organisms.

From a totalitarian viewpoint, bioremediation is a complex process with interactions between many factors which are yet to be fully understood. Bioremediation work carried out at a laboratory scale has, in many cases, still to be verified *in situ*. However, very few field-scale operations are available for the testing of laboratory data. Other restoration measures may be introduced which give a false impression of the success of biodegradation practices. Furthermore, indirect evidence is sometimes offered as proof of contaminant reduction. However, once methods have been devised which provide conclusive evidence that a specific approach is responsible for contaminant attenuation, full development of the technology may be possible. In conclusion, it must be recognised that in a world which is increasingly environmentally conscious, the development of a technology which appears to offer an environmentally acceptable solution cannot be ignored.

The focus of this study was a site which had been used for 12 years for the disposal of a hydrocarbon and heavy metal contaminated sludge which is a by-product of the coal cracking process. The site is a 4 acre plot located in the Highveld region of the Orange Free State province. The site soil is characterised by a 30% clay and fine silt, and 70% sand and coarse silt composition. Due to a confidential nature of the work, further site details were not made available.

In order to meet new standards which will undoubtedly, be required by pending legislation, several remedial options were considered. Traditional practices are expensive while

bioremediation could well meet the cleanup requirements. The research in this case was initiated to determine the applicability of bioremediation and the environmental risks, and to elucidate the essential microorganism/soil/pollutant interactions underpinning the biotechnology.

The initial step in the site assessment was to confirm the presence of an active microbial population capable of degrading the hydrocarbons. It was also important to identify nutrient, pH and electron acceptor limitations. Because of the presence of heavy metals, there was also the possibility of biodegradation inhibition. Therefore, this aspect was also considered in the initial experiments. These preliminary investigations were carried out in batch systems. Following these determinations, it was important to establish if the hydrocarbons were, in fact, labile under conditions which were similar to those found *in situ*. This work included considering various nutrients and electron acceptors. Soil column experiments were, thus, used to approximate *in situ* conditions and study the effect of various bulking agents, aeration and alternative electron acceptors.

During the degradation process, it was also necessary to predict the fate and transport potential of contaminants in relation to groundwater contamination. These studies were again carried out in soil columns where both hydrocarbon and heavy metal migration were contemplated.

Because the results of hydrocarbon biodegradation treatments are often difficult to interpret, other biodegradation indicators and analytical methods were also explored as alternatives.

CHAPTER 2

MATERIALS AND METHODS

The materials and methods described in this section were used during the course of the study. Other methods, specific to research conducted, are referred to and/or described in the relevant sections in succeeding chapters.

2.1 SOIL AND PINE-BARK PREPARATION

Soil (Appendix Tables A1, A2, A3, A4) and composted (4 months) pine-bark were prepared by air drying for 3 days at ambient temperature prior to sieving through a 2000 μm sieve. Contaminated and uncontaminated soil were supplied from the site and consisted of random samples which were combined.

2.2 COARSE ASH PREPARATION

The coarse ash was prepared as for 2.1. The ash was obtained as a cheap waste product from a source near to the contaminated site.

2.3 HYDROCARBON SLUDGE

Hydrocarbon sludge was obtained from the industrial waste stream of a coal-cracking plant prior to application onto the disposal site. The sludge was transferred to 2 litre glass containers and stored at 2°C in the dark.

2.4 NUTRIENT MEDIA

2.4.1 Medium 1

This medium contained (l^{-1} distilled water): 0.5g KH_2PO_4, 1.5g K_2HPO_4, 2g NH_4NO_3 and

0.2g $MgSO_4.7H_2O$ and was sterilized by autoclaving at 121°C for 15 minutes. The pH of the medium was 7.2.

One m/ trace element solution (50% v/v mixture of trace element solutions A and B) was added to the nutrient medium prior to mixing with the carbon source. Both trace element solutions were filter sterilized (0.2μm filter) and stored at 4°C before use.

Trace element solution A contained:
$FeCl_2.H_2O$, 1.5g; NaCl, 1.5g; $MnCl_2.4H_2O$, 0.197g; $CaCl_2.6H_2O$, 0.238g; $CuCl_2.H_2O$, 0.017g; $ZnSO_4$, 0.287g; $AlCl_3$, 0.050g; H_3BO_3, 0.062g; $NiCl_2.6H_2O$, 0.024g and concentrated HCl, 10m/ per litre distilled water.

Trace element solution B contained:
Na_2Mo_4, 0.0484g; $Na_2SeO_3.5H_2O$, 0.0025g; and $NaNO_3$, 0.0033g per litre distilled water.

2.4.2 Medium 2

This medium contained (l^{-1} distilled water): 3.31g NH_4NO_3, 0.828g KH_2PO_4, 2.48g K_2HPO_4 and 0.33g $MgSO_4.7H_2O$. The pH of the medium was 7.2. No trace elements were added due to the possibility of exceeding threshold toxicity concentrations. Thisstock solution was used undiluted for the 5:1 C:N ratio and diluted to give 20:1, 50:1 and 100:1 ratios. Sterilization was carried out as described in 2.4.1.

2.4.3 Medium 3

This medium contained (l^{-1} distilled water): 1g $(NH_4)_2SO_4$, 0.5g KH_2PO_4, and 0.2g $MgSO_4.7H_2O$. No trace elements were added. The pH of the medium was 4.63.Sterilization was carried out as described in 2.4.1.

2.4.4 Medium 4

This contained (l^{-1} distilled water): 1g KNO_3, 0.5g K_2HPO_4 and 0.2g $MgSO_4.7H_2O$. No trace elements were added. The pH of the medium was 8.06. Sterilization was carried out as described in 2.4.1.

The hydrocarbons present on the surface of soil particles and which were the target molecules for biodegradation provided the carbon source for the four media.

2.5 MICROBIAL INOCULUM

A microbial inoculum was prepared by incubating 50g (dry weight) hydrocarbon contaminated soil in a 1 litre aerobic Erlenmeyer flask which contained 300ml nutrient medium (2.4.1). The flask was incubated in a New Brunswick shaker incubator (70rpm, 30°C and in darkness). Supernatant (200ml) was subsequently transferred to fresh medium (1 litre) and soil (150g) every 2 weeks. The presence of microorganisms was determined by light microscopy.

2.6 SOIL COLUMN DESIGN

Refer to 4.2.1 for details concerning soil column design.

2.7 HYDROCARBON EXTRACTION AND GAS CHROMATOGRAPHY

2.7.1 Soil Extraction

All soil samples were air dried at 35°C for 48 hours in the presence of silica gel desiccation crystals. Extraction solvent (200ml 70% v/v dichloromethane and 30% v/v methanol) was added to 10 g of soil (20g in the case of the ash and pine-bark mixtures). Extraction of the hydrocarbons was with a "Soxtec" 1040 apparatus which refluxed the solvent from the immersed sample. This was followed by a period of washing where the solvent was refluxed and drained through the sample. An 8 hour soxhlet extraction with 12 refluxes was used to extract the hydrocarbons from the soil.

The extract was filtered through a Whatman no.4 filter paper before volume reduction with a Heidolph rotary evaporator. Residual solvent was removed under a stream of nitrogen at ambient temperature (approximately 25°C). The hydrocarbon residues were then each dissolved in 1m/ of hexane before analysis.

2.7.2 Leachate

Soluble hydrocarbons in the leachate (100m/) obtained from the soil columns (4.2.1) were extracted by mixing with 30m/ ether in a 250m/ separating funnel. The ether layer was removed, evaporated (under N_2) and the volume adjusted to 2m/ with *iso*-octane before analysis.

2.7.3 Vapour Traps

The vapour traps (Orbo 32) used for determining the volatile hydrocarbon fractions were placed on the air outlet pipes of the air sparged soil columns. (4.2.2). For hydrocarbon extraction, carbon disulphide (15m/) was passed through the vapour traps and then evaporated to dryness. The residue was diluted to 2m/ with *iso*-octane before analysis.

2.7.4 Glass Wool Liquid Slurry

Hexane (20m/) was added to each 250m/ Erlenmeyer flask (5.2.2) and agitated with a magnetic stirrer and stirrer bar for 2 minutes. The supernatant was transferred to a 400m/ separation funnel. The hexane extraction step was repeated twice and the hexane was separated from the remaining fraction with a separation funnel. The extraction procedure and separation were repeated twice with 200m/ washes of dichloromethane. The hexane and dichloromethane samples were combined and the volume reduced by rotary evaporation (Heidolph rotary evaporator). After evaporation to dryness the samples were resuspended in dichloromethane (4m/) and transferred to 5m/ reactor vials and evaporated down to 0.1m/ before gas chromatography analysis.

Two standard hydrocarbons (0.0056g of a C23 n-alkane and 0.0061g of a C16 pyrene) were first collectively suspended in 15ml hexane before concentration to 0.1ml for analysis.

2.8 HEAVY METAL EXTRACTION

2.8.1 Soil

Metals were extracted from the soil with an ammonium bicarbonate ("Ambic") extraction solution (C. A. du Plessis, personal communication). This was prepared by individually dissolving 197.6g NH_4HCO_3, 37.2g di-sodium EDTA and 3.7g NH_4F in 1000ml distilled water. All of the above were added to a 10 litre container to which 100ml of Superfloc concentrated solution had previously been added. After mixing, the container volume was diluted to 10 litres with distilled water and the pH adjusted to 8.0 with a 2N ammonia solution.

The Superfloc (grade N100) concentrated solution was prepared by dissolving 10g in distilled water and diluting to 2 litres. The 10g of Superfloc powder were added to the solution after a positive vortex had been achieved by stirring. The powder was added very slowly to the edge of the vortex after which the solution was continuously stirred for 2 hours until all the flocculent had dissolved. Stirring was at less than 400rpm to prevent breakage of the long chain molecules which, in turn, could reduce the flocculating power.

Soil extractions were made by placing 2.5g of soil and 25ml of the ammonium bicarbonate solution in 100ml conical flasks. These were shaken for 15 minutes at 180 cycles per minute on a reciprocal shaker. The extracts were filtered through Whatman no. 541 filter paper into glass bottles before analysis by atomic absorption spectrophotometry.

2.8.2 Leachate

Sample preparation: Leachate (4.2.3) samples (5m/) were mixed with 5m/ 2M HNO_3 and allowed to equilibrate overnight at ambient temperature. The samples were then filtered through Whatman no. 1 filter paper and further diluted (20 times) to prevent damage to the atomic absorption spectrophotometer.

2.9 ANALYSES

2.9.1 Soil Physical and Chemical Analyses

Soil physical and chemical analyses of both uncontaminated and contaminated soil were carried out by the Cedara Soil Analysis Laboratory.

2.9.2 Hydrocarbon Analysis

Soil and Leachate

Analysis of hydrocarbons extracted from the soil and leachate was made with a Varian 3600 gas chromatograph fitted with a 30m x 0.25mm capillary column coated with BP5 (5% phenylmethylsilicone) of 0.25µm film thickness. The column temperature conditions were: initial column temperature of 100°C for 5 min followed by a 10°C increase per minute to a final holding temperature of 270°C for 25 minutes. The injector and detector temperatures were both at 250°C and splitless injection (8.5 psi pressure) was used. The carrier gas was helium. The injection volume was 1.5µ/; split ratio, 100:1; and split flow 60m/ min^{-1}. Because of the heterogeneous and complex nature of the hydrocarbon mixture under investigation, it was not possible to quantify the results with standards. However, 1.5µ/ hexadecane were included as an internal standard to allow the respective areas of the peaks to be scaled and quantitatively compared.

Total Hydrocarbon Weight

The total extracted hydrocarbon weight from soil samples was determined after volume reduction by evaporation with a Heidolph rotary evaporator. The hydrocarbon extract was weighed on a 5 decimal point Sartorius scale. After determining hydrocarbon weight, the extract was redissolved and diluted to 20m/ with dichloromethane before proceeding with gas chromatography analysis.

Infrared Analysis

Soil (5g dry weight) was mixed with 5g of anhydrous Na_2SO_4 and placed in a 30m/ glass vial. Carbon tetrachloride (10m/) was added to the vial, which was then sealed. The vial contents were shaken and placed in a sonicating bath. Mixing and sonication were repeated three times. The vials were left to stand at room temperature (approximately 25°C) overnight whereafter the solvent mixture was transferred to another vial which contained 1g of florosil. The florosil had previously been activated by preparing a mixture containing 6% (v/w florosil) in distilled water. The vial contents were mixed and allowed to equilibrate overnight. The solvent was then filtered through glass wool plugged Pasteur pipettes prior to infrared spectrophotometric analysis. Experimental samples and a reference sample (CCl_4 only) were scanned at wavelengths between 3200 cm^{-1} and 2700 cm^{-1} with a Nicolet 5DXC FT-IR Spectrophotometer.

Glass Wool

Hydrocarbon analyses for the glass wool slurry experiments (5.2.2) were carried out under modified conditions to those described for the soil and leachate hydrocarbons.

A Hewlett Packard Series II 5890 gas chromatograph was used. The column temperature conditions were: initial column temperature of 100°C for 5 min followed by a 10°C increase per minute and final holding temperature of 270°C for 25 minutes. The injector and detector temperatures were both 250°C and splitless injection (8.5 psi pressure) was used. The injection volume was 1.5µ/; split ratio, 100:1; and split flow 60m/ min^{-1}. The capillary column used was a HEWLETT® PACKARD HP-SMS

(Crosslinked 5% PhMe silicone) with a length of 30m, internal diameter of 0.25mm, phase ratio of 250 and a film thickness of 0.25µm. The carrier gas was helium.

2.9.3 Heavy Metal Analysis

Leachate and Soil Heavy Metal Analysis

All samples were analysed with a Varian AA-275 Atomic Absorption Spectrophotometer.

Hydrocarbon Sludge Heavy Metal Analysis

Analyses of the hydrocarbon sludge were carried out by the Research and Development Department of Sastech, Sasol.

2.9.4 Scanning Electron Microscopy

All samples were observed with a Hitachi S-570 Scanning Electron Microscope.

Culture Supernatant

Culture supernatant samples were examined after filtration through 0.2µm Millipore filters. The samples were fixed in 3% (v/v) buffered gluteraldehyde for 8 hours and then washed twice in 0.05M cacodylate buffer for 30 minutes. The specimens were then dehydrated by washing with alcohol: 30% (v/v) for 10 minutes, 50% (v/v) for 10 minutes, 70% (v/v) for 10 minutes, 80% (v/v) for 10 minutes, 90% (v/v) for 10 minutes and three washes with 100% for 10 minutes. After preparation by critical point drying, the samples were sputter coated with gold palladium and viewed.

Energy Dispersive X-Ray Analysis

Filter paper (Whatman no. 1) was cut into circular discs which were measured to fit onto viewing stubs. The discs were then soaked in the leachate samples obtained from the soil columns and, subsequently, fixed and critical point dried as described above. Before viewing, the discs were attached with double sided tape onto carbon viewing stubs and were not sputter coated. A control disc (no leachate sample) was also prepared in the same manner as before to correct interference effects during analysis. Energy Dispersive X-Ray Analysis was then employed to detect silica in conjunction with a Hitachi S-570 Scanning Electron Microscope.

Soil Samples

Soil samples were prepared for viewing by direct attachment onto copper viewing stubs followed by fixation (as described above), dehydration, sputter coating and critical point drying.

Glass Wool

Glass wool was fixed, dehydrated, critical point dried and sputter coated as described above. The glass wool was then attached to copper viewing stubs with double sided tape before viewing.

2.9.5 pH Determination (KCl Method)

Soil (10g) was placed in a glass beaker and 25ml KCl (1M) added. The solution was stirred with a glass rod and then allowed to settle for 30 minutes. The electrode of a pH meter (Radiometer Ion-85) was placed in the surface liquid so that a pH reading could be obtained.

2.10 IODONITROPHENYLTETRAZOLIUM CHLORIDE (INT) ASSAY PROTOCOL

Iodonitrophenyltetrazolium chloride was prepared to a concentration of 0.4% (w/v) with

sterilized water and stored as a stock solution at 4°C. For soil assays, 0.5m/ of the INT stock solution was added to a test tube containing a 1g soil sample. The test tube was then incubated at 25°C in the dark for 96 hours. The reaction was stopped by adding 8m/ methanol and sonicating for 15 minutes. The supernatant was then filtered through Whatman no. 1 filter paper and the concentration determined at a wavelength of 490nm with a Milton Roy 801 spectrophotometer.

CHAPTER 3

EVALUATION OF A SOIL MICROBIAL POPULATION FOR HYDROCARBON CATABOLISM AND OPTIMISATION OF TREATMENT CONDITIONS

3.1 INTRODUCTION

Before initiating a bioremediation strategy, it is important to first determine whether an indigenous microbial population capable of hydrocarbon catabolism is present in the soil (King *et al.*, 1992). If a limited indigenous population is found to be present then it may be necessary to include a seeding study as part of the strategy. Toxic soil conditions, as a result of contaminants may result in reduced microbial activity and target molecule biodegradation rates. Under such circumstances an alternative remedial strategy, either as a separate strategy or part of a structured remedial process, may have to considered.

Biodegradation optimization studies also form an important part of the initial assessment (Riser-Roberts, 1992). In most hydrocarbon remediation studies, nutrient availability has been identified as a rate-limiting factor. Nitrogen and phosphorus are often not present in sufficient concentrations due to the high carbon:nitrogen:phosphorus ratio effected by the hydrocarbon contamination. Molecular oxygen, which is a requirement of oxygenase enzymes, is often not present in required concentrations. The soil pH may be either too acidic or alkaline, depending on the particular nature of the soil. Neutral pH conditions are required, not only for the physiological requirements of the microorganisms but also to prevent heavy metal mobilisation. The evaluation and possible manipulation of environmental factors to facilitate optimum conditions are important considerations in the initial study.

The capacity for soil bioremediation can be maximised by the identification of conditions which promote the degradation of the hydrocarbon constituents. Information gained from the above studies is thus important for directing treatments and is

necessary for the selection of further bioremediation strategies.

3.2 EXPERIMENTAL

3.2.1 Electron Microscopy

To visually confirm the presence of a microbial population, soil was first viewed by scanning electron microscopy. Ten grams contaminated soil (2.1) and 100m/ of nutrient medium (2.4.1, Medium 1) were incubated (25°C in the dark) stationary for 30 days in 250m/ Erlenmeyer flasks. Nutrient medium was allowed to cover the soil up to a depth of 3cm to allow sufficient oxygen diffusion. Non-supplemented contaminated soil was also flushed with distilled water (10m/ water to 5g soil) to separate the planktonic from the attached microbial population. The supernatant samples were examined after filtration through a 0.2 µm Millipore filter.

3.2.2 Colony Plate Counts

To compare the relative numbers of microorganisms present in hydrocarbon contaminated soil and uncontaminated soil, the microbial numbers of three soils (hydrocarbon contarninated soil, local Rensburg soil and local Inanda subsoil) were compared. Medium plates, for the purpose of plate counts, were made by mixing 1000g contaminated soil (2.1) with 2000m/ distilled water. The sediment was allowed to settle for two days and the supernatant collected. This fraction, which contained the water soluble hydrocarbons, was mixed with agar ($16g/^{-1}$), autoclaved at 12°1 C for 15 minutes, cooled (approximately 50°C) and aseptically poured into petri dishes. A dilution series (10^{-3} to 10^{7}) of the hydrocarbon contaminated (SAS) and two local uncontaminated soils (Rensburg and Inanda) was prepared in Ringers solution ($1gm/^{-1}$) and aseptically spread in 0.5m/ aliquots onto individual plates with a glass "hockey" stick. The plates were inverted and incubated for 14 days at 30°C. The plates were examined for microbial growth and discrete colonies counted with a standard petri dish counter. To aid statistical accuracy, microbial colonies numbering greater than 30 and

less than 200 were recorded.

3.2.3 Liquid Batch Cultures

Aliquots of supernatant (3.2.1) were used to prepare liquid cultures for the observation of microbial growth by optical density increases. One gram of soil (2.1) was added to 100ml sterile supernatant liquid (autoclaved at 121°C for 15 minutes) and incubated at 25°C in the dark. Aeration was provided by shaking the flasks at 120 rpm on a New Brunswick rotary shaker. The optical density was monitored daily at a wavelength of 590nm with a Milton Roy 801 spectrophotometer.

3.2.4 Carbon:Nitrogen Ratio Optimisation

The C:N ratios were fixed relative to the organic carbon concentration of 2.9% (w/w, Table A.1). A stock solution of Medium 2 (2.4.2), which contained 10% (v/v) inoculum (2.5), was diluted to the required C:N ratio, before adding 100ml to 15g of contaminated soil in a 250ml Erlenmeyer flask. C:N ratios of 100:1, 50:1, 20:1 and 5:1 were prepared. The experiments were made in duplicate and the flasks shaken for 70 days at 120rpm and 30°C (New Brunswick shaker incubator) to ensure maximum contact between the soil, nutrients and microorganisms. The soil was then air dried at ambient temperature, mixed and the hydrocarbon content determined by gas chromatography and infrared spectrophotometry (2.7, 2.9.2).

3.2.5 The Effect of Anaerobic Conditions on Biodegradation

Medium 1 (2.4.1) was introduced and oxygen was excluded by using sealed 250ml Schott bottles and sparging the medium and headspace with oxygen-free nitrogen. Resazurin was used as an indicator of anaerobic conditions. The experiment was made in duplicate and the bottles shaken for 70 days at 120rpm and 30°C (New Brunswick shaker incubator) to ensure maximum contact between the soil, nutrients and microorganisms. The soil was then air dried at ambient temperature, mixed and

analyzed for hydrocarbon content by gas chromatography and infrared spectrophotometry (2.7, 2.9.2).

3.2.6 The Effect of pH on Biodegradation

The medium used for the 5:1 C:N ratio in the aerobic batch experiments (3.2.4) had a pH of 7.2. This was used as the neutral pH status for this experiment with alkali or acidic pH conditions either above or below pH 7.2. Two other pH values were tested, 4.63 and 8.06. For the pH 4.63 medium, only NH_4^+ was used as the N source (2.4.3, Medium 3) while for pH 8.06 medium (2.4.4, Medium 4) only NO_3^- was used as the N source. The respective pH adjusted nutrient media (100ml) were added to 250ml Erlenmeyer flasks which contained 15g of contaminated soil. The experiments were carried out in duplicate and the flasks were shaken (New Brunswick shaker incubator) at 120rpm for 70 days at 30°C to ensure maximum contact between the soil, nutrients and microorganisms. The soil was then air dried at ambient temperature, mixed and hydrocarbon content determined by gas chromatography and infrared spectrophotometry (2.7 and 2.9.2).

3.2.7 The Effect of Surfactant on Biodegradation

The effect of surfactant on biodegradation was also investigated by adding a propylene and ethylene oxide co-polymer surfactant (Merck) to a treatment similar to the 5:1 C:N treatment (3.2.4). The surfactant was prepared to a concentration of 1% (v/v) with nutrient Medium 1 (2.4.1) and the experiment conducted as described in Section 3.2.4.

A control was prepared as described in 3.2.4 with the medium substituted by distilled water.

3.3 RESULTS AND DISCUSSION

3.3.1 Electron Microscopy

Plates 3.1a and b are electron micrographs of the contaminated soil after 30 days incubation with nutrients. The microorganisms were observed to be attached to the soil surfaces. In contrast, somewhat fewer microorganisms were observed to be attached to the non-nutrient incubated soil. The supernatants of both the incubated and non-incubated soils showed the presence of planktonic populations (Plates 3.1c and d). These observations confirmed the presence of hydrocarbon degraders in both treatments.

3.3.2 Colony Plate Counts

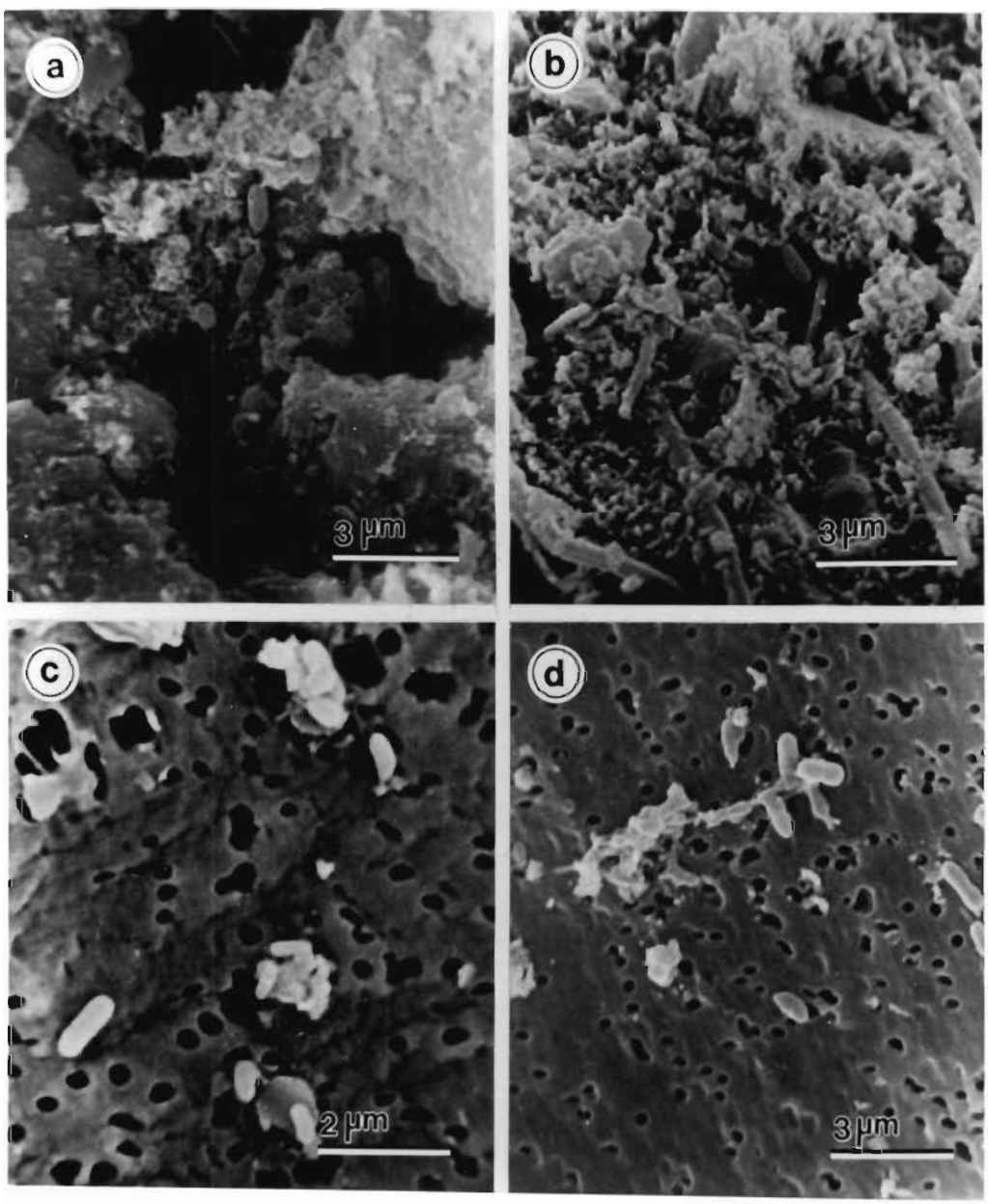
The colony counts for both the contaminated soil and two uncontaminated soils are shown in Table 3.1. At a 10^{-4} dilution, the microbial numbers in the contaminated soil were lower than those found in the Rensburg soil. The difference in microbial numbers were, however, not statistically significant. Microbial numbers in the contaminated soil were, however, higher than were found in the Inanda soil at this dilution. The indigenous population could thus be considered appropriate for *in situ* bioremediation without the need for seeding. These results also indicated that the population present in the contaminated soil appeared to be relatively unaffected by high metal concentrations (A.3) and remained viable.

Table 3.1: Comparison of the number of colony forming units in a contaminated soil and two uncontaminated soils.

Dilution	Hydrocarbon contaminated soil (cfu per gram dry soil)	Rensburg soil (cfu per gram dry soil)	Inanda soil (cfu per gram dry soil)
10^{-7}	<30	<30	<30
10^{-6}	<30	<30	<30
10^{-5}	<30	31	0
10^{-4}	36	179	<30
10^{-3}	>200	>200	>200

Plate 3.1a and b: **Electron micrographs showing microbial attachment to contaminated soil surfaces after 30 days incubation in nutrient medium.**

Plate 3.1c and d: **Electron micrographs of microbial consortia after filtration of column supernatant.**


a
3 µm
b
3 µm
c
2 µm
d
3 µm

3.3.3 Liquid Batch Cultures

Optical density measurements of the liquid cultures were not possible due to the formation of aggregates by the microorganisms. This effect may have been due to low hydrocarbon solubility in the aqueous phase. Soluble heavy metals such as Mn, Zn, Ni and V present in the liquid phase (results not shown) may also have contributed to unfavourable conditions for a planktonic population. Although microbial growth was not measurable by spectrophotometry, the presence of biomass in the supernatant was observed which was consistent with the colony plate counts and electron microscopy observations.

These results suggested that correct manipulation and control of the key environmental factors and addition of nutrients would encourage microbial growth. Thus, the addition of non-indigenous or engineered microorganisms would, possibly, not be necessary. However, due to the time constraints of the study and the relatively slow degradation rates of the complex hydrocarbon mixtures, subsequent experiments were inoculated with indigenous organisms which had been pre-bulked (2.5). This was done to decrease the experimental lag times so that the results could be obtained quickly.

3.3.4 Batch Studies of the Effect of Carbon:Nitrogen ratios, Anaerobic and Aerobic Conditions and Surfactant Addition on Hydrocarbon Biodegradation

Figure 3.1[1] shows the results of the biodegradation of hydrocarbon contaminated soil which was monitored by infrared spectrophotometry. From these results it appeared that the anaerobic conditions as well as the addition of surfactant resulted in lower biodegradation rates. This suggested that oxygen was more effective as an electron acceptor during respiration than nitrate. Thus, for *in situ* bioremediation, aerobic conditions seemed preferable for rapid catabolism of the hydrocarbon mixture (von

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[1] QuattroPro does not support super- and subscript fonts. Sat(SO4) and Sat(NO3) should be regarded as Sat(SO_4) and Sat(NO_3) where shown in the figures.

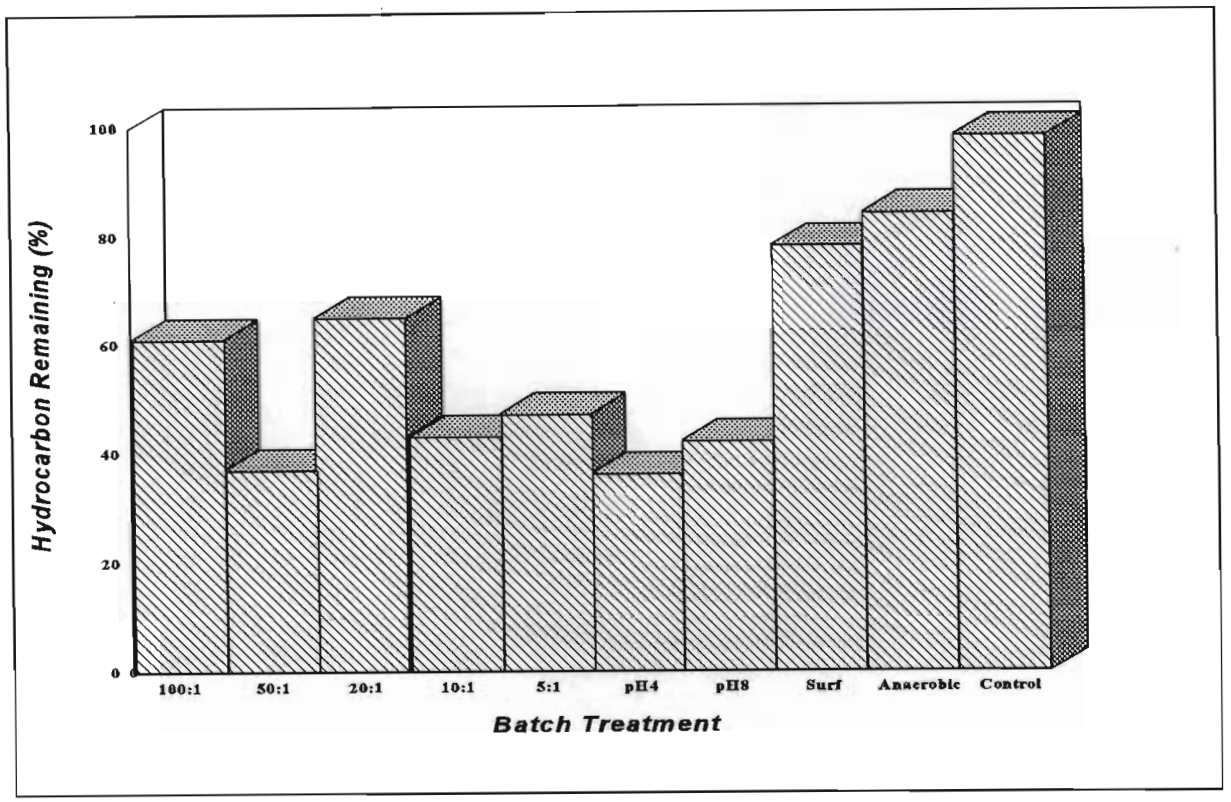

Figure 3.1: Hydrocarbon biodegradation, as determined by infrared spectrophotometry, after treatment under anaerobic conditions, aerobic conditions, pH 4.63, pH 8.06 and in the presence of surfactant and C:N ratios of 100:1, 50:1, 20:1, 10:1 and 5:1.

Wedel *et al.*, 1988; Thomas and Ward, 1989; Leahy and Colwell, 1990). Nitrate may, however, have some potential as a water soluble electron acceptor for controlling contamination at greater soil depths (Mihelcic and Luthy, 1988).

The low biodegradation efficiency observed following the addition of surfactant may have been caused by the toxicity of this particular chemical (propylene and ethylene oxide co-polymer) or the concentration used (3.2.7). Screening for non-toxic surfactants and non-inhibitory concentrations are major tasks, and for this reason further investigations of concentrations and surfactant types were not made. The nitrogen:phosphate ratios used in the medium were fixed since the control and management of soluble phosphate concentrations relative to the soluble nitrogen concentrations are difficult due to chemical interactions between phosphates and soil components. Under *in situ* conditions, it was felt that the C:N ratio would be more easily controlled. The phosphate concentration, even at the lowest C:N ratio used was

calculated to be sufficient to facilitate hydrocarbon catabolism. The different C:N ratios were all, generally, found to have promoted higher biodegradation compared with the anaerobic treatment. Due to the complexity of the contamination more replications and longer time scales could possibly have given more accurate information. The results of the study showed that not all of the hydrocarbon was degraded in any of the treatments during the course of the experiment. Thus, it can be assumed that nitrogen limitation was not a major factor.

With the exception of the 20:1 C:N ratio treatment the following trend was observed. Biodegradation appeared to be linked to the C:N ratio as follows: 5:1 < 10:1 < 50:1. The highest degradation was recorded with a C:N ratio of 50:1. This is comparable to findings by other authors who have recorded optimal degradations at C:N ratios ranging from 9:1 to 60:1 (Dibble and Bartha, 1979; Brown *et al.*, 1983). Degradation was decreased with a C:N ratio of 100:1 and this was possibly indicative of nitrogen limitation. This argument was partly corroborated by the pH 4.63 and pH 8.06 treatments. The media for these treatments contained less nitrogen than the C:N 5:1 treatment and in effect had a higher C:N ratio of 17:1 for the pH 4.63 medium and 33:1 for the pH 8.06 medium. Greater hydrocarbon biodegradation was seen to occur under these conditions than under conditions of possible nitrogen toxicity (C:N ratio of 5:1).

For the pH treatments, NO_{-3} was used as the N source. This was in an attempt to prevent nitrification and, hence, acidification of the medium. The degradation observed with both pH treatments indicated that the hydrocarbon-catabolizing population was capable of withstanding a wide pH range. However, heavy metal toxicity may be of concern under pH conditions which are more acidic than pH 4.63.

CHAPTER 4

SOIL COLUMN BIOREMEDIATION STUDIES

4.1 INTRODUCTION

To examine conditions which are more likely to be found *in situ*, a column study was designed to approximate these. Soil physical effects and hydrocarbon contaminant migration which cannot be accommodated in batch studies could, thus, also be studied. Physical effects such as volatilization, heavy metal leaching, and nutrient, oxygen and microbial migration all affect the bioremediation of hydrocarbon contaminated soil. It is important, therefore, to gain an understanding of interactions between these variables for the purpose of field application.

To maximize the bioremediation process, any potentially toxic components present in the soil should be removed, or their effect on microbial metabolism quantified. The prolonged exposure of the microbial population to such molecules may inevitably lead to a decrease in biological activity and, hence, reduced pollutant biodegradation. The toxic effects of heavy metals on microorganisms have been well documented (Gadd and Griffiths, 1978). Exposure of microorganisms to excessively high heavy metal concentrations may result in the disruption of cell membranes and the denaturation of cellular proteins which may, in some cases, lead to cell death (Foster, 1983).

Soil from the contaminated site under study was challenged with not only hydrocarbon rich sludge but also high concentrations of heavy metals (Table A.3). The continued application of such sludges will, in future, cause elevated metal concentrations, resulting in microbial death. If bioremediation is the treatment of choice, high heavy metal concentrations will reduce the effectiveness of this technology. The second aspect of this component of the study was, therefore, to assess the metal tolerance capabilities of the soil microorganisms by monitoring dissolved oxygen consumption in the presence of increasing metal concentrations. Since heavy metal toxicity is due to

solubilized metals and most microbial activity occurs in the aqueous phase, the toxicity of heavy metals in the aqueous phase was examined.

4.2 EXPERIMENTAL

4.2.1 Soil Columns

Bioremediation studies were carried out in soil columns. The columns were constructed from PVC pipes (internal diameter 4.5cm, length 25cm) with silicone rubber seals. Glass wool was used to prevent soil washout and effluent stream clogging. The soil (2.1) was packed dry by vibration with a Vortex mixer. The soil bulk density for each column was calculated after accurate weighing. The particle densities of the soil, coarse ash and pine-bark were determined by expelling air and measuring water displacement. From the bulk and particle densities the pore volume was calculated. The columns were incubated at ambient temperature (approximately 25°C) and in the dark.

4.2.2 Treatments

Several bioremediation treatment studies were made as described in Table 4.1.

Table 4.1: Bioremediation studies carried out to examine the effects of different treatments on hydrocarbon biodegradation.

Treatment	Column	Description
FC: To determine the effect of field capacity soil saturation with nutrients on bioremediation.	1	Nutrients (2.4.1, Medium 1) and 10% (v/v nutrient solution) inoculum (2.5) were added to the column on a weekly basis and allowed to drain freely.
	2	As for Column 1.

	3	As for Column 1 but no inoculum was added.
FC/2: To determine the effect of soil nutrient supplementation at half the frequency of FC.	4	As for Column 1 but the nutrients and inoculum were added every two weeks.
	5	As for Column 4.
	6	As for Column 4 but no inoculum was added.
Sat(SO_4): Bioremediation under anaerobic conditions was tested with ammonium sulphate as an alternative electron acceptor.	7	The soil was saturated with a nutrient and 10 % (v/v) inoculum solution. Medium 1 (2.4.1) was used but the nitrogen was in the form of $(NH_4)_2SO_4$ ($1g/l^{-1}$). The soil column was supplemented with nutrients by vertical displacement from the base. The column was then allowed to drain freely once a week prior to resaturation with fresh nutrients. Anaerobic conditions were provided by saturation of the soil with the nutrient medium.
	8	As for Column 7.
	9	As for Column 7 but no inoculum was added.
Sat(NO_3): Bioremediation under anaerobic conditions was tested with potassium nitrate as an alternative electron acceptor.	10	This column was treated the same as Column 7 with the exception that nitrogen was in the form of $1g/l^{-1}$ KNO_3.
	11	As for Column 10.

	12	As for Column 10 but no inoculum was added.
Ash: Ash (2.2) was used as a bulking agent to improve soil porosity and soil oxygen holding capacity.	13	Contaminated soil was mixed with coarse ash (2.2) in a 1:1 (w/w) ratio and treated in a similar fashion to Column 1.
	14	As for Column 13.
	15	As for Column 13 but no inoculum was added.
Ash Sp: Ash (2.2) and air sparging was used to improve soil porosity and soil oxygen holding capacity.	16	This column was prepared and treated in the same manner as Column 13. However, after the columns had been freely drained, they were sparged with dry air from the column base. The sparging rate was approximately 50 pore volumes per day. For vapour collection, the column air outlet was equipped with an "Merck Orbo 32" activated carbon vapour trap
	17	As for Column 16.
	18	As for Column 16 but no inoculum was added.
PB: Pine-bark (2.1) was used as a bulking agent to improve soil porosity and soil oxygen holding capacity.	19	This was treated in the same manner as Column 1 with the exception that composted pine-bark (composted for four months) was used as a bulking agent (1:1 w/w ratio). This treatment was initiated 20 days after the other treatments.

	20	As for Column 19 but no inoculum was added.
PB Sp: Pine-bark (2.2) and air sparging was used to improve soil porosity and increase the soil oxygen concentration.	21	This column was prepared and treated in the same manner as Column 19. Air sparging was carried out as for Column 16
	22	As for Column 21 but no inoculum was added.
CI: The viability of improving biodegradation efficiency by supplementation with commercial inoculum was examined.	23	Commercial inoculum (Petrobac®) was prepared (1g/100m/ distilled water) and added to the soil columns. The application times and water content were the same as those for Column 1. Like columns 19-22 this study was also started 20 days after the other treatments.
Surf: A surfactant was also tested to determine wether hydrocarbon biodegradation could be improved by surfactant mediated desorption.	24	Treatment was as for Column 1 but in this case a 1% (v/v) surfactant (Merck propylene and ethylene oxide co-polymer) was added to the nutrient medium. Like Column 23 this treatment commenced 20 days after the other treatments.

The PB (Columns 19 and 20), PB Sp (Columns 21 and 22), CI (Column 23) and Surf (Column 24) treatments were late additions to the research programme and consequently treatment of these columns commenced 20 days after the others. The treatment column supplemented with the commercial inoculum (Column 23) was not supplemented with the indigenous microbial inoculum. The commercial inoculum was revived by dissolving 1g inoculum in 100m/ of nutrient medium (supplied by manufacturer) at ambient room temperature (approximately 25°C).

4.2.3 Analyses

The leachates from all of the columns were collected weekly for analyses and the respective volumes recorded.

Microorganisms

Gram stains and light microscopy were employed to determine the presence of microorganisms in the leachate.

pH

The leachate pH was determined by using a composite sample after the final 50 days of each treatment. These determinations were made, whenever possible, with pH indicator paper. Electrode pH measurements could not be satisfactorily obtained due to the limited leachate volumes.

Heavy Metals

Heavy metal leachate concentrations of manganese and zinc were determined by atomic absorption spectrophotometry (2.8.2, 2.9.3). Manganese and zinc were chosen because of their significant prevalence in the untreated soil (Table A.4). A subsequent heavy metal analysis of the leachate at the end of the experimental period was also made. The leachate was also analyzed for the presence of colloidal material by Si detection on filter paper by energy dispersive x-ray analysis (2.9.4).

Hydrocarbons

After 70 days treatment, one each of the inoculated and uninoculated columns, were destructively sampled for soil analysis. The soils were well mixed and the hydrocarbons

were extracted (2.7.1) for analysis by gas chromatography (2.9.2). Selected leachate samples (from Column 1 and untreated soil) and the activated carbon traps from the vented columns (Columns 16, 17, 18, 21 and 22) were also analysed for the presence of hydrocarbons (2.7.2, 2.7.3, 2.7.4).

Hydrogen Sulphide

The presence of H_2S in the columns was qualitatively determined by placing lead acetate-impregnated filter paper in each column. The filter paper was suspended with "Scotch tape" between the surface of the soil and the top of the column. A black colour was taken as indicative of the presence of H_2S.

4.2.4 Extended Bioremediation Column Treatments

Although destructive sampling was carried out after 70 days treatment, the duplicate columns (Columns 2, 5, 8, 11, 14 and 17) were subjected to further treatment to determine the effects of the various treatments on hydrocarbon attenuation over a prolonged time period. The pine-bark (PB treatment), sparged pine-bark (PB Sp treatment), commercial inoculum (CI treatment) and surfactant (Surf treatment) studies were discontinued.

The columns were treated as before with a total treatment time for the replicate columns of 20 months. At the end of this time period, the columns were destructively sampled. Soil from the different treatments was homogenized by mixing with a mortar and pestle, air dried at ambient temperature, and analysed for metal concentration and hydrocarbon content (2.7.1, 2.8.1, 2.9.2, 2.9.3). The leachates from the columns were analysed for pH (20 months) and weekly heavy metal content (2.8.2, 2.9.3).

4.2.5 Influence of Heavy Metals on Indigenous Soil Microorganisms

Medical oxygen gas (40% O_2 v/v) was used to aerate microbial growth medium

(Medium 1 with trace element solution, 2.4.1) for 10 minutes in 250ml Erlenmeyer flasks which contained 20g hydrocarbon contaminated soil (2.1). A 10% (v/v) inoculum (2.5) was added to each bottle prior to incubation at 25°C. The aeration increased the dissolved oxygen concentration to approximately 25 $mg/^{-1}$. The flasks were incubated at 25°C in the dark on a New Brunswick Rotary Shaker. Oxygen monitoring was made with a Hanna Instruments 9100HI dissolved oxygen meter. A Schott bottle cap was adapted so that the oxygen probe could be fitted and inserted into the culture supernatant for continuous measurement. A sterile control (autoclaved at 121°C for 15 minutes) was used to determine non-biological oxygen diffusion from the system. A mean oxygen consumption rate in the absence of heavy metals for 6 replicates was then calculated.

To establish the effects of heavy metals on microbial activity, the experiment was set up as before, with the initial dissolved oxygen concentrations again raised to 25 $mg/^{-1}$ with 40% (v/v) medical oxygen. Due to the large number of replicates in the experiment, the controlled oxygen concentrations were not continuously monitored. Instead, the flasks were incubated at 25°C for 60 hours after which a single oxygen measurement was taken.

Oxygen was added to a stock culture Medium 1 (2.4.1) which also contained Mn, Zn and Ni in the ratio of 400:176:94. This medium was then further diluted with growth medium to obtain progressively decreased concentrations of the heavy metals. A sterile control experiment was also made by autoclaving the flasks at 121°C for 15 minutes. The media were individually added to 250ml Erlenmeyer flasks until no head space remained. The flasks were sealed with rubber bungs to which an outlet tube was attached to release gas pressure build up. The outlet tubes were immersed in water to prevent air infiltration. The pH of each heavy metal supplemented medium was adjusted to 1.2 with 1N HCl to facilitate solubilization of the heavy metals and eliminate precipitation. The experiment was repeated with the pH values adjusted with 1N HCl to pH 4.5 prior to incubation. The heavy metal concentrations were determined by analysis with a Varian AA-275 Atomic Absorption Spectrophotometer (2.9.3).

4.3 RESULTS AND DISCUSSION

4.3.1 Treatment Results - First 70 Days

Soil Column Leachate Microorganisms

Light microscopy observations and Gram stains showed the presence of microorganisms in the leachate from all the columns. This provided evidence of microbial migration and/or displacement within and from the soil column. This is significant as *in situ* treatment of contamination at a greater depth would not require the use of engineering practices to introduce inoculum into these areas. Provided that sufficient oxygen could be introduced at greater soil depths, it may be possible to displace aerobic microorganisms from the aerobic top soil layer to these areas. This would, as a result, increase the rate of biodegradation through aerobic catabolism. Oxygen delivery to subsurface soil could be achieved by using air sparged or hydrogen peroxide supplemented water. Hydrogen peroxide, in particular, has been found to be suitable for enhancing microbial activity and hydrocarbon catabolism in bioremediation projects (Pardieck *et al.*, 1992).

Leachate pH

Figure 4.1[1] shows the pH values of the leachate collected from the columns after 50 days. Due to the low leachate volumes generated in the FC/2, Ash Sp and PB Sp treatments, the pH values could not be satisfactorily determined. The FC treatment generated leachate which was more acidic than the other leachate treatments. This was

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[1] QuattroPro does not support super- and subscript fonts. Sat(SO4) and Sat(NO3) should be regarded as Sat(SO_4) and Sat(NO_3) where shown in the figures.

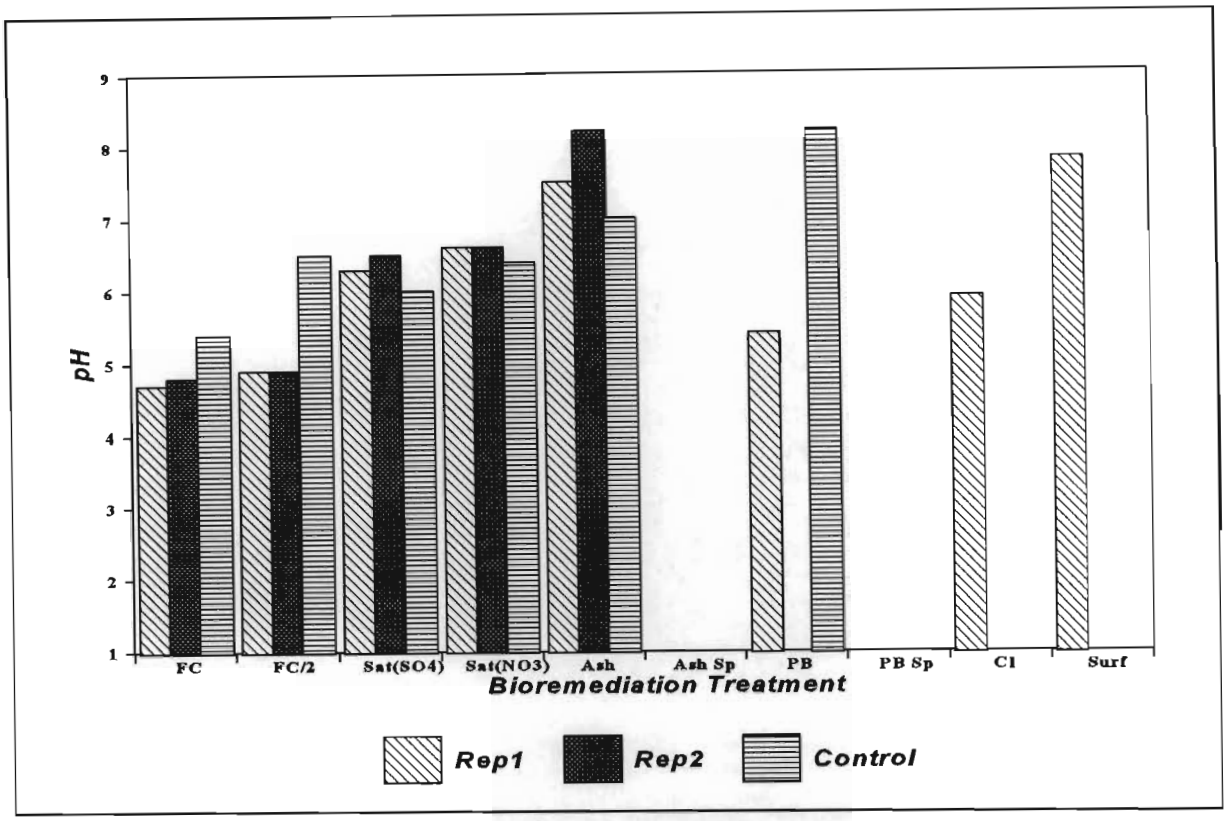


Figure 4.1: pH values of composite soil leachate samples after bioremediation treatments.

thought to result from organic acids produced during hydrocarbon catabolic activity (Zajic, 1964). The pH of the Ash treatment was, in comparison, relatively high. This was attributed to the presence of the ash rather than a possible decrease in microbial activity (it was later ascertained [20 months] that the presence of ash increased the overall soil pH, Table 4.3). The pH discrepancy which was found to occur between inoculated and uninoculated treatments could not be satisfactorily explained. However, in the case of the Sat(SO_4), Sat(NO_3) and the Ash treatments, the addition of inoculum may have resulted in a greater microbial population density which may have metabolised the generated acids more effectively and thus effected a slight pH increase.

Heavy Metals

In general, the pH conditions were found to influence heavy metal mobility and

movement from the hydrocarbon contaminated soil into the leachate. The relatively low leachate pH (Figure 4.1) of the FC treatment was thought to have been a major contributing factor effecting high metal, particularly Mn, concentrations in the leachate (Gadd and Griffiths, 1978). For each treatment only the average values of the duplicate columns and the control column are shown due to the similarities in the results. The manganese concentrations shown in Figure 4.2[2] reflect the volumes of the different leachates. Thus, some of the high concentrations of heavy metals, such as for the FC

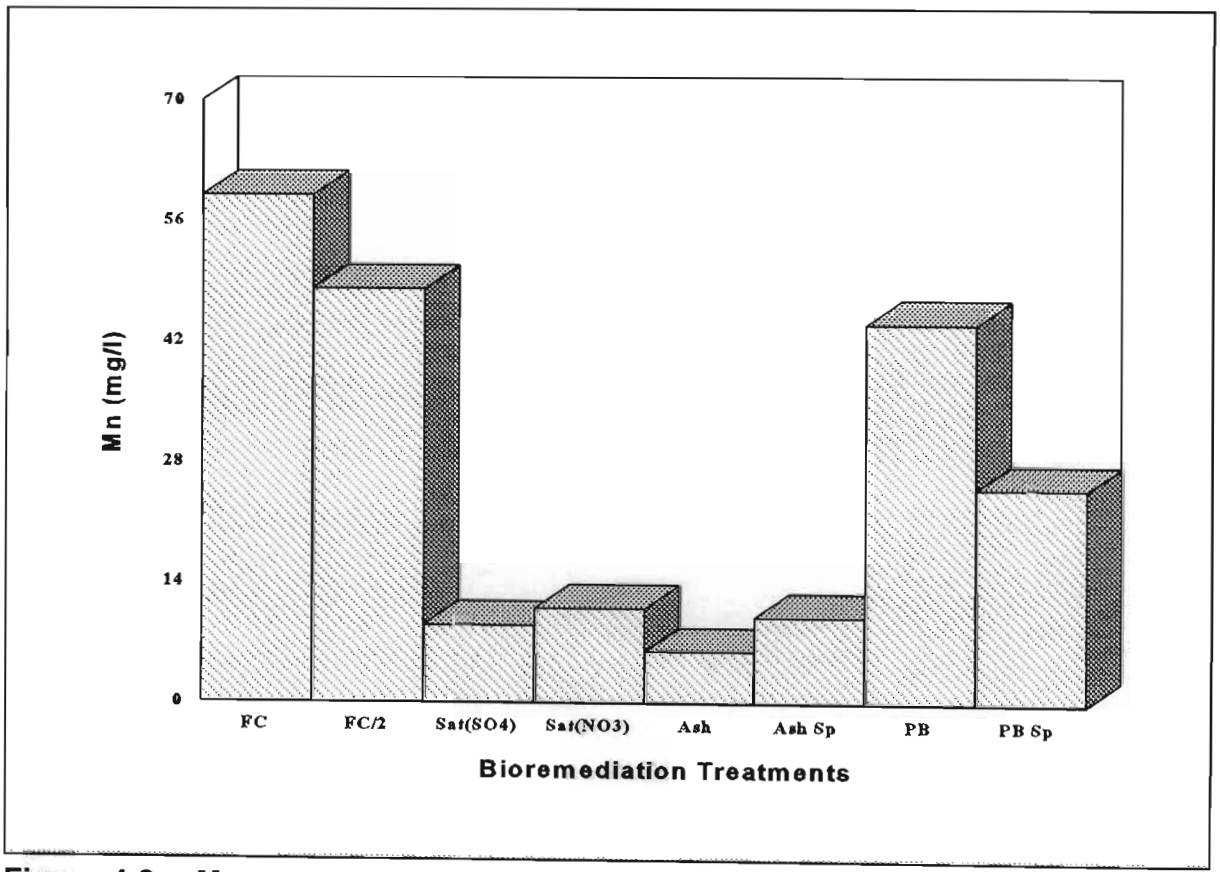


Figure 4.2: Manganese concentrations of the soil column composite leachates for the first 50 days of treatment.

[2] QuattroPro does not support italic and superscript fonts. The concentration unit mg/l should be regarded as mgl^{-1}. Where appropriate, SI units have been used in the text.

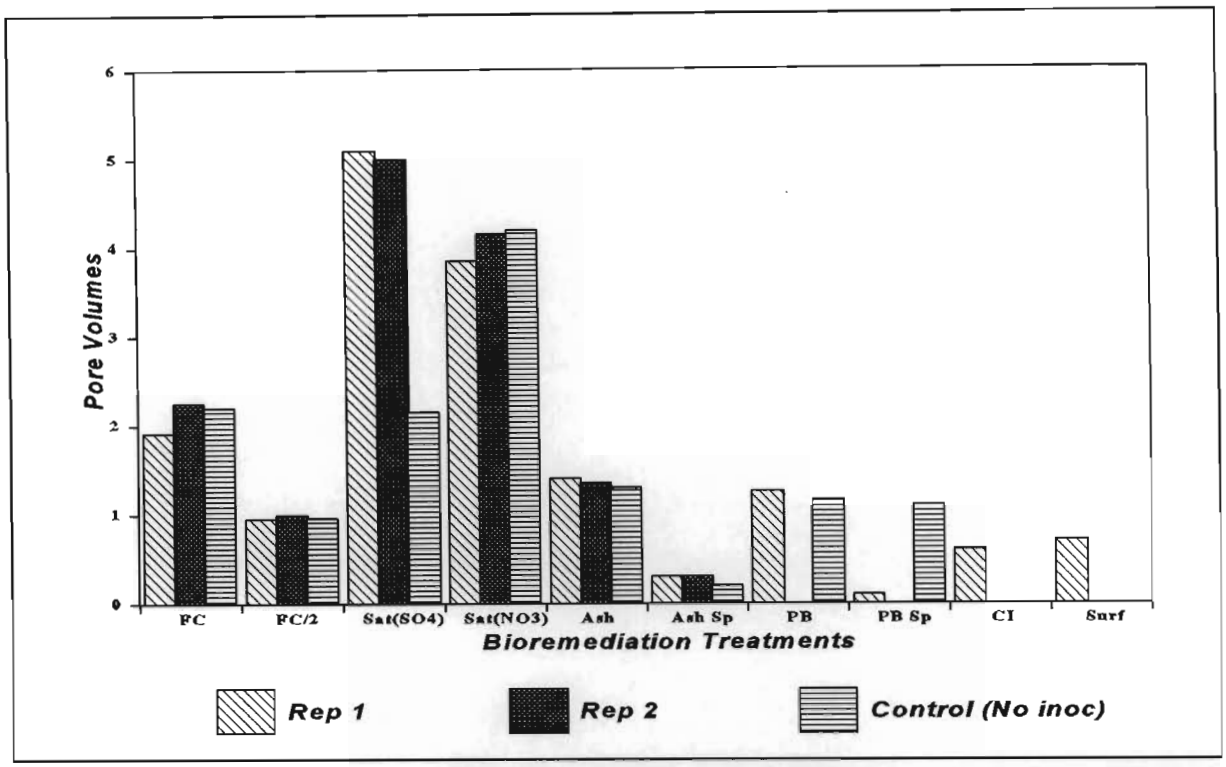


Figure 4.3: Total volumes of liquid collected from the soil columns (expressed in pore volumes) [Pore volume = Total volume collected from column (m*l*) / Total pore volume of soil in column (m*l*)].

and FC/2 treatments, were slightly skewed by the fact that these treatments resulted

in lower volumes of leachates than other treatments e.g. Sat(SO_4) and Sat(NO_3) and would, therefore, be more concentrated (Figure 4.3). The metal concentrations were, thus, adjusted according to the pore volumes of the columns (Table 4.2). Figure 4.4 shows the Mn concentrations adjusted for the average pore volumes flushed through the columns.

Table 4.2: Bulk density and porosity of soil in the columns.

Treatment	Column	Bulk Density (Kg cm^{-3})	Porosity % (v/v)
FC	1	1.03×10^{-3}	49
	2	1.03×10^{-3}	51
	3	1.01×10^{-3}	50

FC/2	4	1.02×10^{-3}	51
	5	1.05×10^{-3}	52
	6	1.05×10^{-3}	53
$Sat(SO_4)$	7	0.98×10^{-3}	49
	8	1.02×10^{-3}	51
	9	1.03×10^{-3}	52
$Sat(NO_3)$	10	1.00×10^{-3}	50
	11	1.01×10^{-3}	49
	12	1.01×10^{-3}	49
Ash	13	1.04×10^{-3}	48
	14	1.05×10^{-3}	48
	15	1.02×10^{-3}	49
Ash Sp	16	1.11×10^{-3}	44
	17	1.09×10^{-3}	45
	18	1.01×10^{-3}	49
PB	19	0.99×10^{-3}	50
	20	1.02×10^{-3}	47
PB Sp	21	1.00×10^{-3}	50
	22	1.01×10^{-3}	49
Commercial Inoculum	23	1.02×10^{-3}	50
Surf	24	1.01×10^{-3}	50

Figures 4.2 and 4.4 indicate that the FC treatment produced leachate which contained high concentrations of Mn even after correcting for pore volume flux. This could be attributed to the low pH conditions (Figure 4.1) which were possibly facilitated by the generation of organic acid intermediates (Zajic, 1964) from microbial catabolism. High metal concentrations in the PB treatment may have been facilitated by the presence of chelating agents from the composting pine-bark and organic acids from hydrocarbon

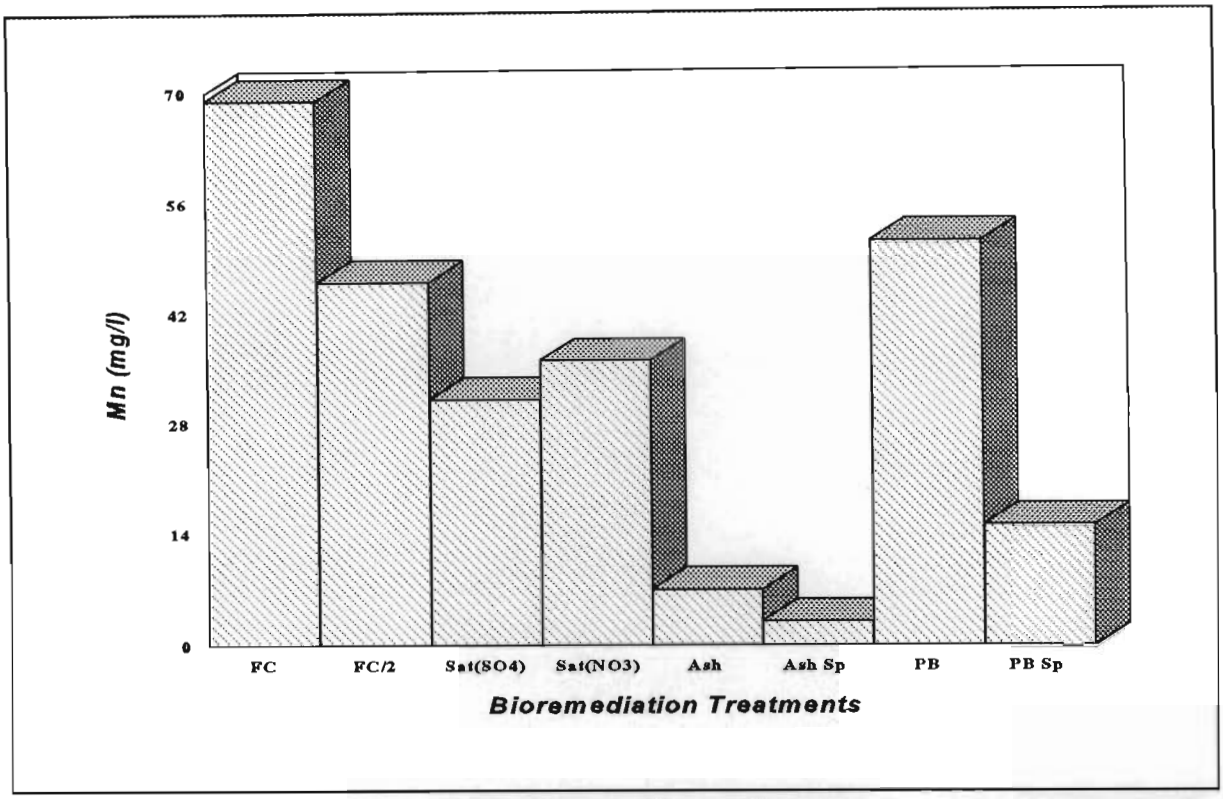


Figure 4.4: Manganese concentrations of the soil column composite leachates for the first 50 days of treatment and after accounting for pore liquid flux.

degradation. The relatively aerobic conditions present in the Ash, Ash Sp and PB Sp treatments may have resulted in oxidised non-mobile phase Mn. This would account for the low Mn concentrations in the leachates. Similarly, the FC/2 treatment conditions were relatively aerobic in comparison to the FC treatment as the soil macropores were not saturated as often.

The low Mn concentrations recorded for the anaerobic treatments [Sat(SO_4) and Sat(NO_3)] were ascribed to reduced metal mobility due to alkaline conditions and complexation as metal sulphides (Gadd and Griffiths, 1978). Hydrogen sulphide was detected by the blackening of lead acetate filter paper. Further confirmation of metal sulphide formation was the formation of gold coloured iron pyrite (FeS_2), particularly on the soil surface of the Sat(SO_4) treatment column.

The zinc concentrations recorded for the first 50 days of treatment are shown in Figure

4.5 and Figure 4.6. The initial zinc concentration for the Ash Sp treatment was high (Figure 4.5) but was found to be lower after correction for pore volume flux (Figure 4.6). The high Zn concentrations for the Sat(NO_3) treatment could not be satisfactorily explained. The corrected Zn concentration of the FC treatment was found to be high and was possibly related to acidic conditions.

Figure 4.7 and Figure 4.8 show the non-adjusted leachate concentrations for Mn and Zn at the end of the 70 day treatment period. Unfortunately, the sparged columns did not produce volumes which were sufficient for analysis. The high metal concentrations of the FC and FC/2 were found to remain high over the total treatment period. However, the Zn concentrations of the Sat(SO_4), Sat(NO_3) and Ash treatments were high even though the conditions were not particularly acidic. This implied that Zn mobility was not pH dependent to the same extent as Mn.

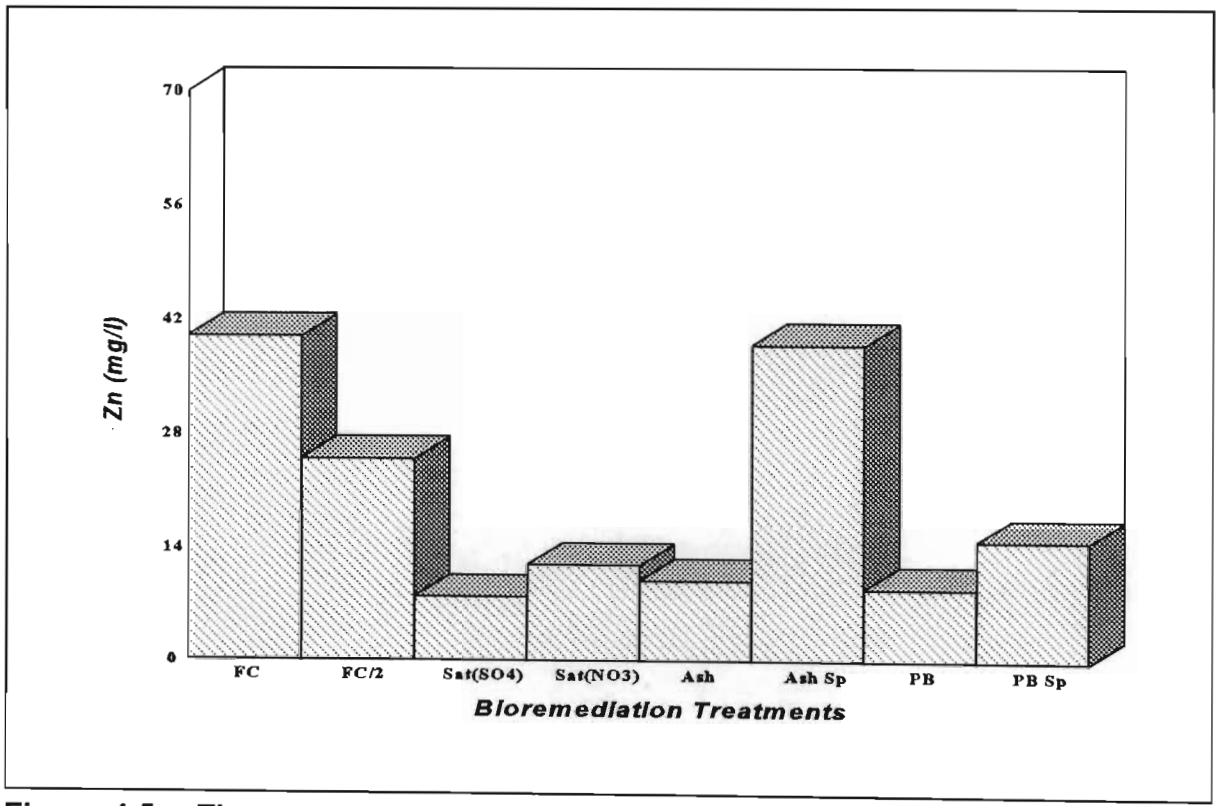


Figure 4.5: Zinc concentrations of the soil column composite leachates for the first 50 days treatment.

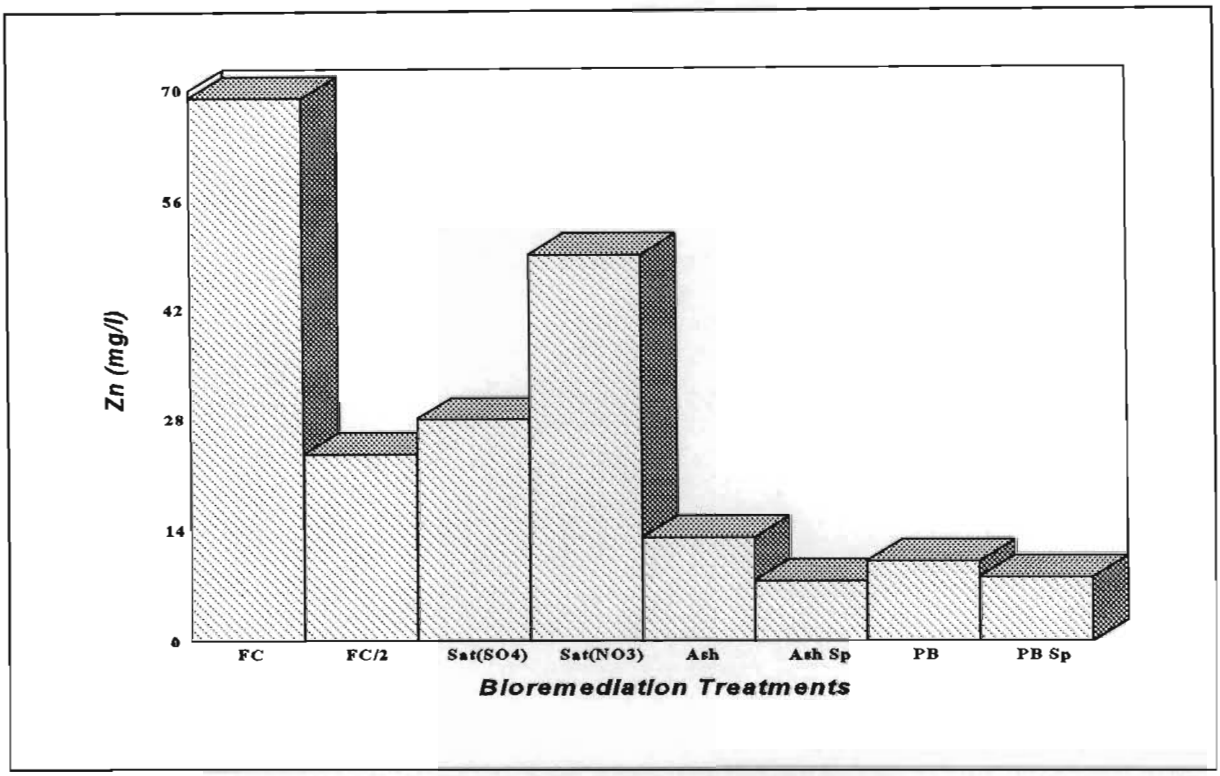


Figure 4.6: **Zinc concentrations of the soil column composite leachates for the first 50 days of treatment and after accounting for pore liquid flux.**

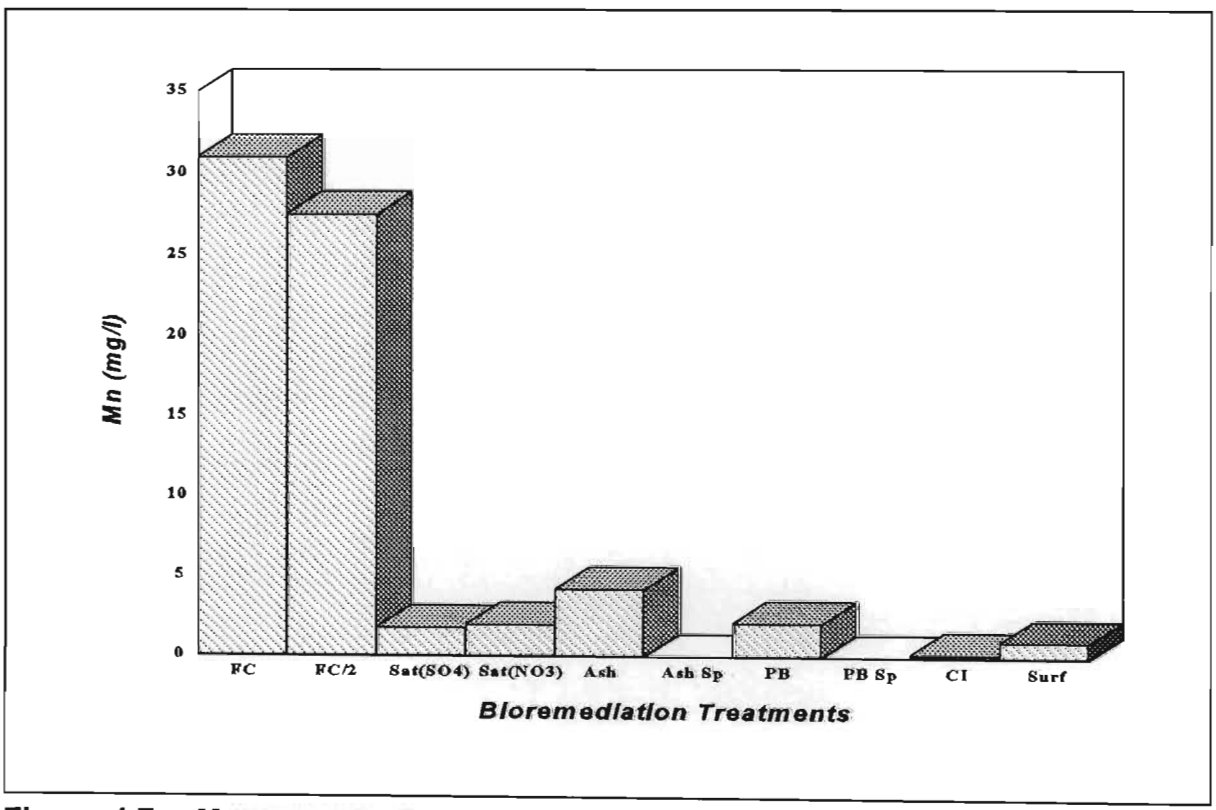


Figure 4.7: **Manganese concentrations of the soil column leachates after 70 days treatment.**

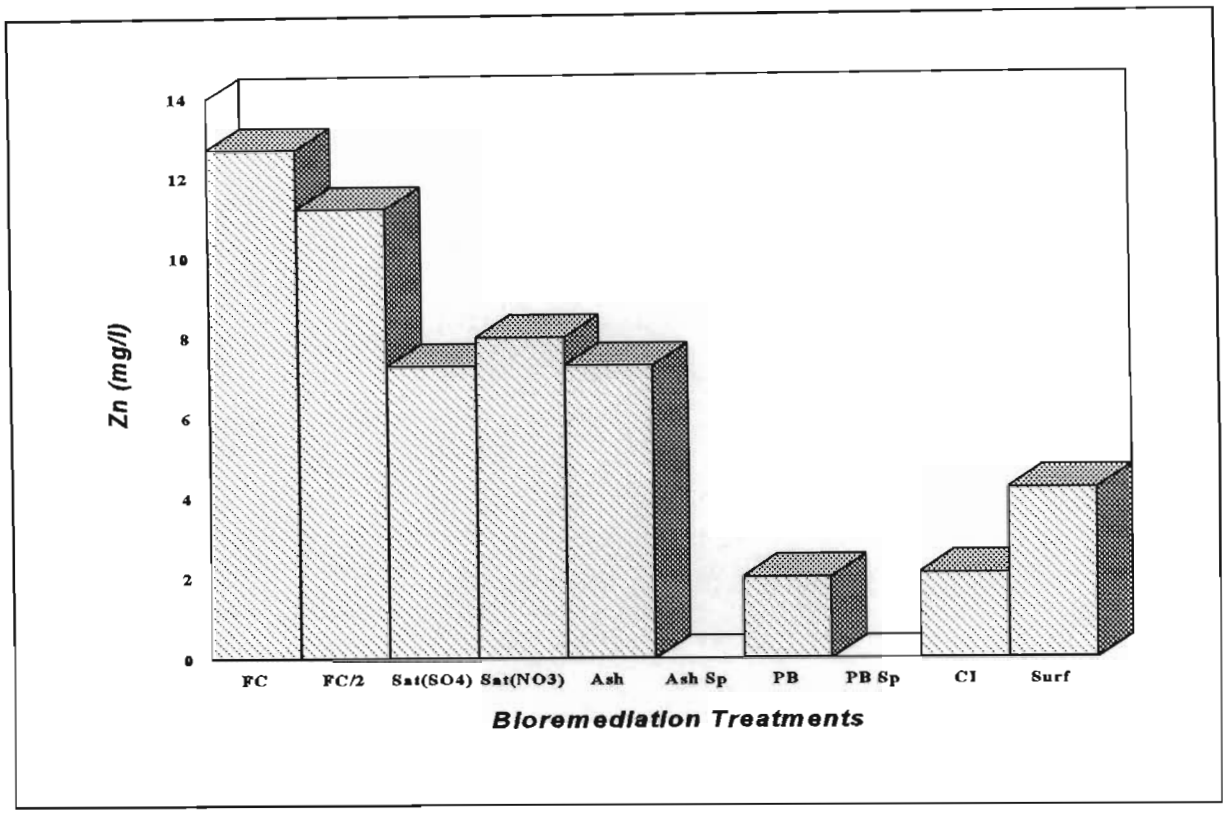


Figure 4.8: Zinc concentrations of the soil column leachates after 70 days treatment.

It must be noted that for the Surf and CI treatments, very low concentrations of Mn and Zn were detected after the 70-day treatment period. The CI column was treated in a similar fashion as the FC column except that the inoculum and nutrients were specific for the requirements of the commercial inoculum. The nutrients and enzymes were supplied in a dried form on wood chips. This supplied medium and inoculum may have contained components which precipitated and/or complexed with the metals which resulted in lower Mn and Zn leachate concentrations. The microbial/enzyme mixture of the inoculum may also have facilitated conditions which were not excessively acidic (Figure 4.1) and thus limited metal mobility. Similarly, alkaline conditions in the Surf treatment could be offered as an explanation for the low metal concentrations recorded.

Hydrocarbons

Hydrocarbon degradation as assessed by infrared spectrophotometry is shown in

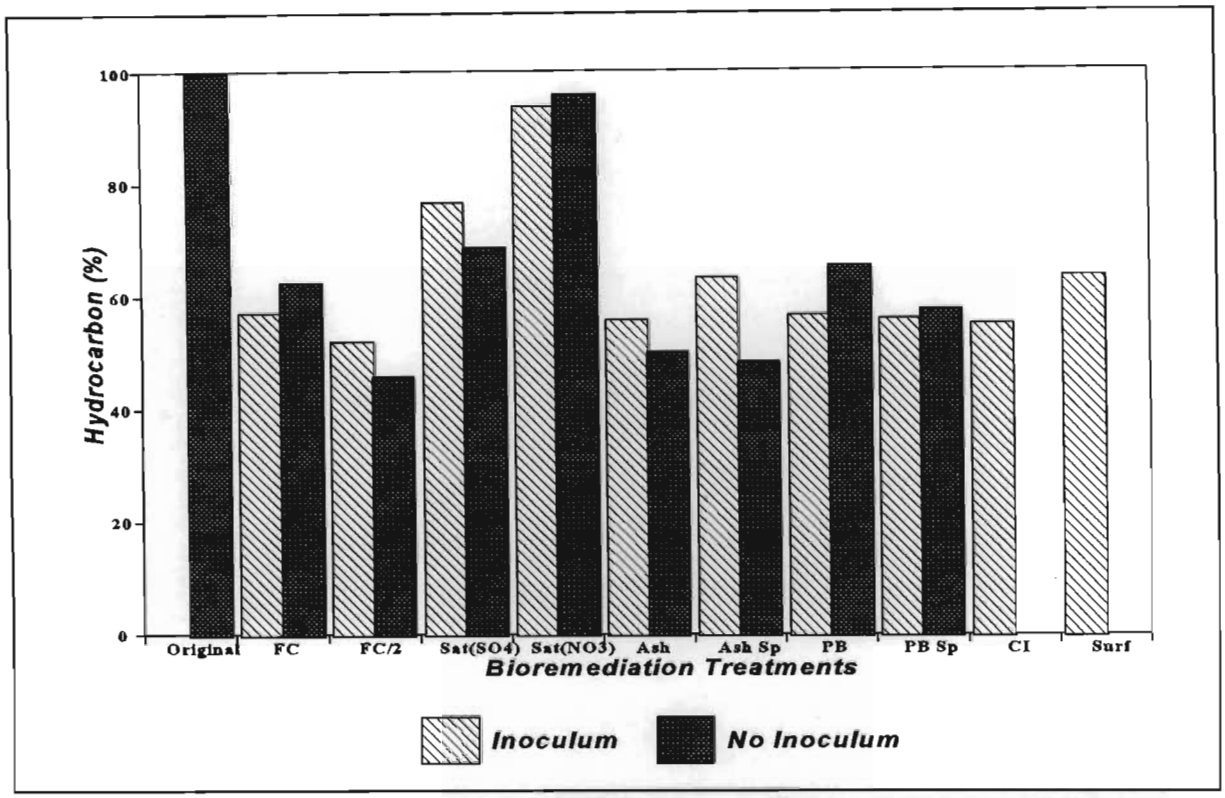


Figure 4.9: Hydrocarbon (% w/w of original contamination) remaining in the soil after various bioremediation treatments over a 70-day period.

Figure 4.9.[3] As in any bioremediation treatment system, the apparent degradation of hydrocarbons may have been significantly influenced by hydrocarbon volatilization and leaching. Although low molecular weight hydrocarbons are more susceptible to degradation than the high molecular weight molecules, they are also more soluble and thus prone to leaching. Volatilization of these molecules is also more likely to occur due to their lower boiling points relative to the high molecular weight hydrocarbons. It was, thus, important to consider the volume of liquid displaced from the soil (Figure 4.3) as well as volatilization due to soil venting. Hydrocarbon leaching was, however, thought to influence hydrocarbon removal to a limited extent. This was corroborated by the observation that the columns which had the greatest number of pore flushes [Sat(SO_4) and Sat(NO_3)] showed the least amount of hydrocarbon removal. Likewise, the absence of significant hydrocarbon concentration differences between the sparged and non-sparged bulked treatments indicated that hydrocarbon volatilization did not significantly

[3] For ease of interpretation, hydrocarbon disappearance has been expressed as percentage loss of original hydrocarbon concentration before treatment.

contribute to hydrocarbon loss. Since leaching and volatilization did not appear to be major factors, hydrocarbon loss was attributed to microbial activity.

Inoculum

The addition of inoculum did not appear to significantly promote biodegradation (Figure 4.9). In some treatments, hydrocarbon degradation was actually found to decrease with the addition of inoculum. These results implied that the indigenous population was capable of hydrocarbon degradation once growth-limiting factors had been eliminated. Furthermore, the addition of inoculum under field conditions would not be required during the initial phase of treatment. The apparent variation between the various inoculated and non-inoculated treatments could not be satisfactorily explained. The variations were, however, minimal and were considered to be insignificant.

Water Content and Anaerobosis

The liquid content at field capacity appeared to have a limited effect on biodegradation. Figure 4.9 shows that degradation with treatment FC/2 was slightly higher than the FC treatment. This could be explained by the fact that the FC treatment had reached relatively saturated conditions compared to FC/2. As a result, certain zones of anaerobosis may have caused lower biodegradation rates. Anaerobosis as a result of saturation was indeed observed to lower hydrocarbon catabolism. The "anaerobic" treatments of Sat(SO_4) and Sat(NO_3) were the least effective for hydrocarbon degradation. It is important to note that the ideal conditions for bioremediation would preclude soil conditions which are relatively dry. A compromise between saturated conditions and conditions inhibitory to microbial metabolism because of low water activity should, thus, be maintained for optimal bioremediation. An optimal moisture content of between 30% and 80% of field capacity was reported by Dibble and Bartha (1979) and Riser-Roberts (1992).

Although a decrease in hydrocarbon catabolism was observed for the "anaerobic"

treatments, this was not indicative of reduced microbial activity. The soil population was, in fact, found to be relatively active as was indicated by the production of H_2S as described previously (4.3.1). Biodegradation at reduced rates under anaerobic conditions was thus confirmed since the formation of H_2S was indicative of sulphate reduction in the presence of an organic carbon source (Grbic-Galic, 1990).

Bulking Agents

The Ash and Ash Sp treatments effected relatively high hydrocarbon degradation during the 70 day treatment period (Figure 4.9). This was ascribed to the greater porosity and, hence, aeration of the soil (Hay and Kuchenrither, 1990). The greater soil porosity was not, however, reflected as a lower bulk density (Table 4.2), possibly due to the greater particle density of the coarse ash. Although air sparging reduced soil moisture, this was not found to adversely affect microbial catabolism.

Analysis of the carbon vapour traps revealed the presence of hydrocarbons of relatively low molecular weights and low boiling points. However, the concentrations of these molecules were low and did not appear to contribute significantly to the overall hydrocarbon attenuation.

Although the pine-bark (PB) and sparged pine-bark (PB Sp) treatments effected significant removal of the hydrocarbons, the decreases were not as great as the corresponding Ash treatments. This could possibly be explained by the 20 day incubation time difference. The results of both the ash and pine-bark treatments did suggest that aeration was an important factor in hydrocarbon degradation.

Leachate Hydrocarbons

It was found that the leachates of all the columns contained soluble hydrocarbons (Figure 4.10, only FC treatment shown). Not only were water soluble hydrocarbons present but also high molecular weight (>C20), relatively insoluble, hydrocarbons were

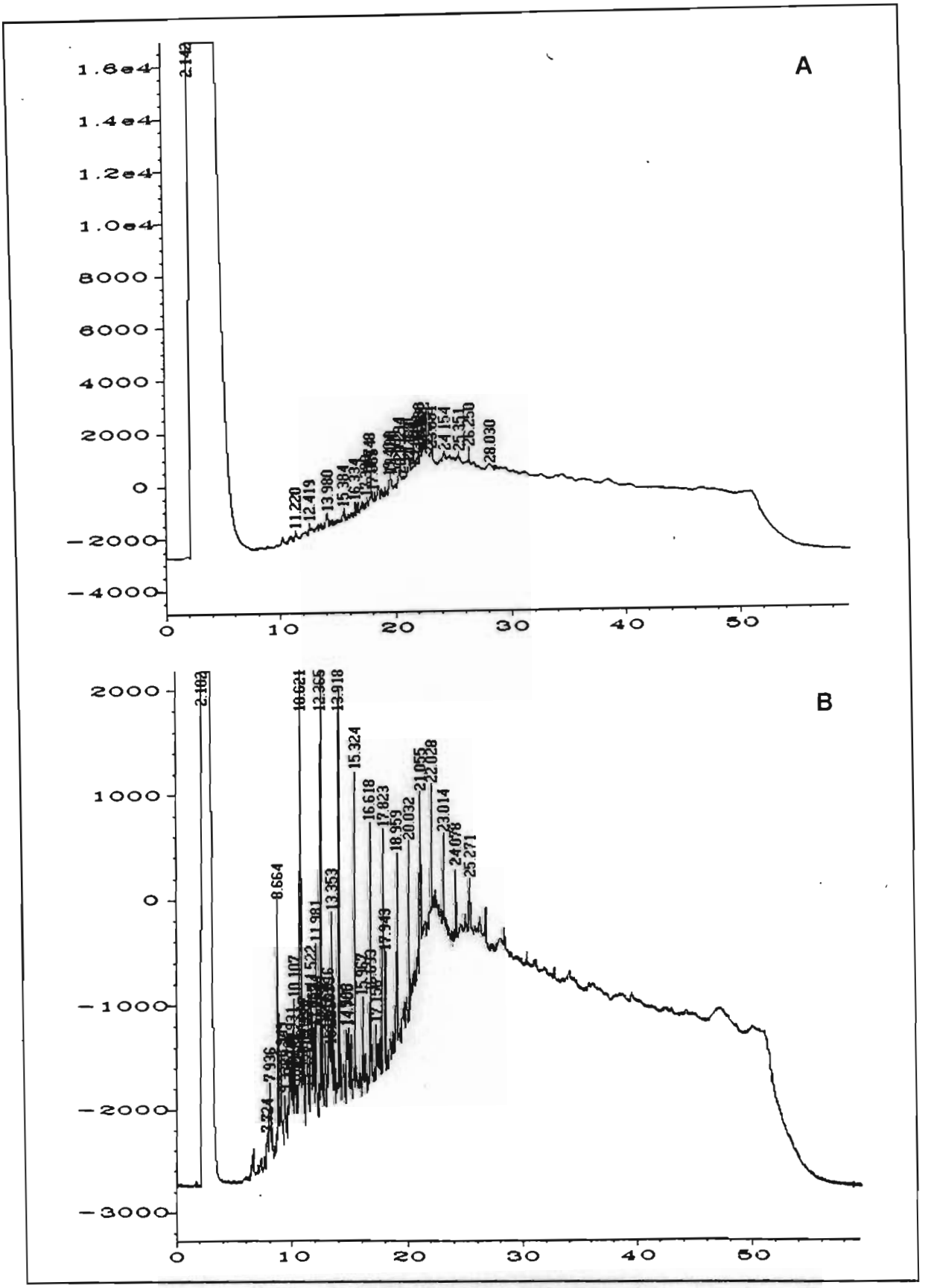


Figure 4.10: Comparison of chromatograms of soil column leachate (FC treatment) (A) and an extract from the untreated contaminated soil (B).

detected in the leachates. Examination of the leachate insoluble fractions offered some explanation for this apparent anomaly. Biosurfactants and the slight solubilities of the high molecular weight hydrocarbons may have accounted for their presence. It is more likely, however, that the hydrocarbons had attached to colloidal material in the leachate as a result of their hydrophobic nature (Pettyjohn and Hounslow, 1983). The presence of colloidal material, such as microorganisms, was confirmed by the observation of bacteria in the leachate as described earlier. From the preliminary results (not shown) obtained by energy dispersive X-ray analysis, Si was also found which implied the presence of soil mineral colloidal material (Brady, 1984). The possibility of biogenic Si was precluded since colloidal material selected by electron microscopy for EDX analysis was observed to be free from microbial biomass. This indicated that the hydrocarbons had migrated as a result of attachment to these colloidal materials. Initially, this was thought to have negative implications for *in situ* bioremediation. However, in a natural soil system, both microorganisms and colloidal material would be retained at greater depth because of the filtration effect of the soil (i.e. decreased average pores sizes with increased depth). The dangers of hydrocarbon migration in the leachate as a result of attachment to colloidal materials would, thus, appear to be limited in *in situ* treatments. It was, however, difficult to quantify the concentration of leachate hydrocarbons and thus soil hydrocarbon reduction due to leachate loss. This aspect would, however, require monitoring and further assessment onsite.

4.3.2 Protracted Treatment (20 Months)

Following continued evaluation of bioremediation over an extended time period, the surfactant (Surf), pine-bark (PB) and commercial inoculum (CI) treatments were abandoned. As previously discussed (Chapter 3), the addition of surfactant was found to inhibit hydrocarbon biodegradation. The use of surfactant in the initial column studies was also not beneficial in improving hydrocarbon catabolism compared with the other treatments. It was thus felt that further evaluation of surfactants was not justified during the course of this study. The use of pine-bark as a bulking agent was considered to be impractical since the contaminated site was situated a considerable distance from

readily available sources of this material. Furthermore, manipulating pine-bark with construction handling equipment may prove to be difficult. The option of seeding with commercial inoculum was not considered to be viable because of cost and the fact that there was little, if any, advantage gained from inoculation.

Heavy Metals and pH

Figure 4.11 shows the leachate pH results after 20 months for the various bioremediation treatments. A similarity between the pH trends observed after 70 days (Figure 4.1) and those obtained after a treatment time of 20 months was observed for all the treatments. The FC/2 and FC treatments were, again, found to have the lowest leachate pH values after the respective treatment times. Of the anaerobic treatments, Sat(SO_4) maintained a consistent pH although the pH for Sat(NO_3) was observed to increase from 6.6 to 8.2. Under conditions where nitrate is the sole nitrogen source, nitrate assimilation may occur. During the course of assimilation, nitrate is reduced to

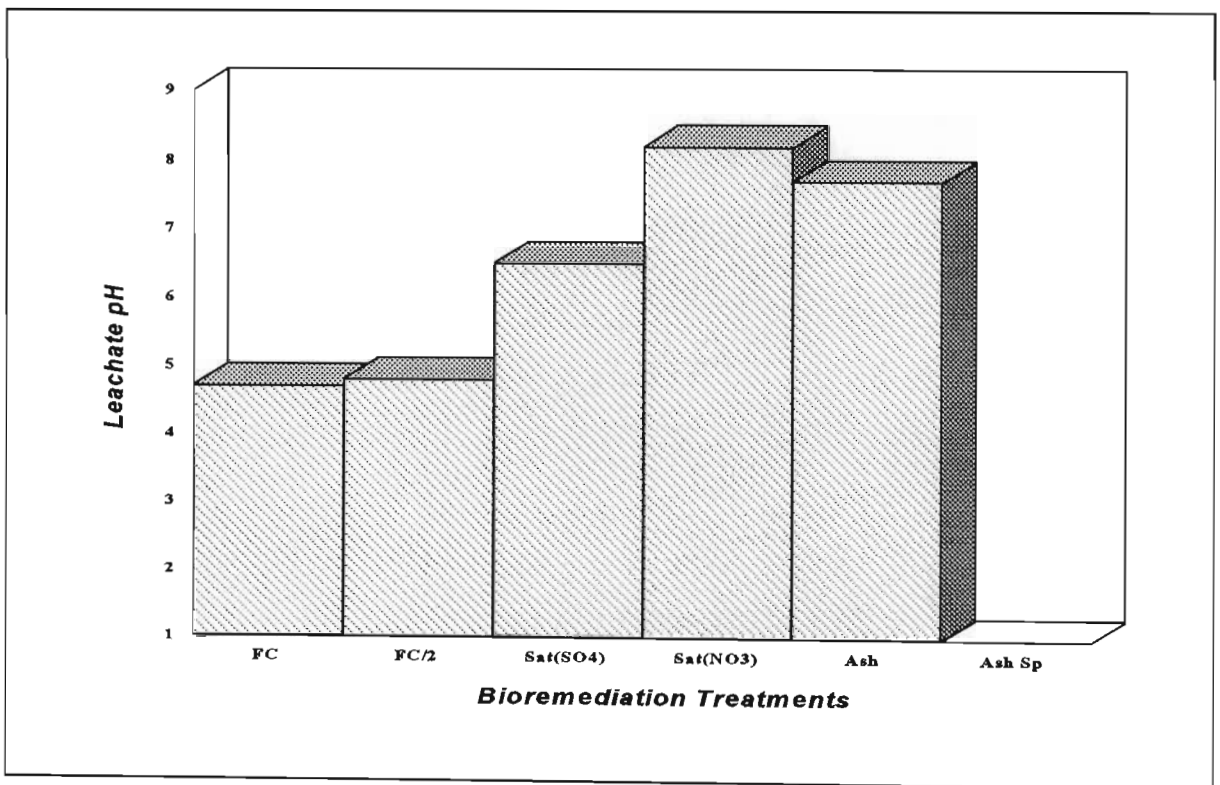


Figure 4.11: Leachate pH for various bioremediation treatments after a 20 month treatment period.

ammonium, which may have resulted in an elevated leachate pH after an extended treatment time. Furthermore, dissimilatory nitrate reduction is a proton consuming process which will increase the pH (Schlegel, 1990). The Ash treatment was also observed to maintain a high pH during the extended treatment period. This was probably because the fly ash has a relatively high pH which acted as a buffering agent in the soil.

The presence of manganese in the leachate samples was found to correlate with the 70 days treatment and Mn mobility again appeared to be pH dependent (Figure 4.12). The treatments with low to moderate acidity (FC, FC/2, Sat(SO_4) showed the greatest potential for Mn migration. The treatments which produced relatively alkaline conditions tended to immobilize Mn in the soil. This trend was again observed with regards to zinc (Figure 4.13). Variations between the FC and FC/2 treatments with respect to Mn and Zn could not be explained. The soil pH as determined by the KCl method (Table 4.3,

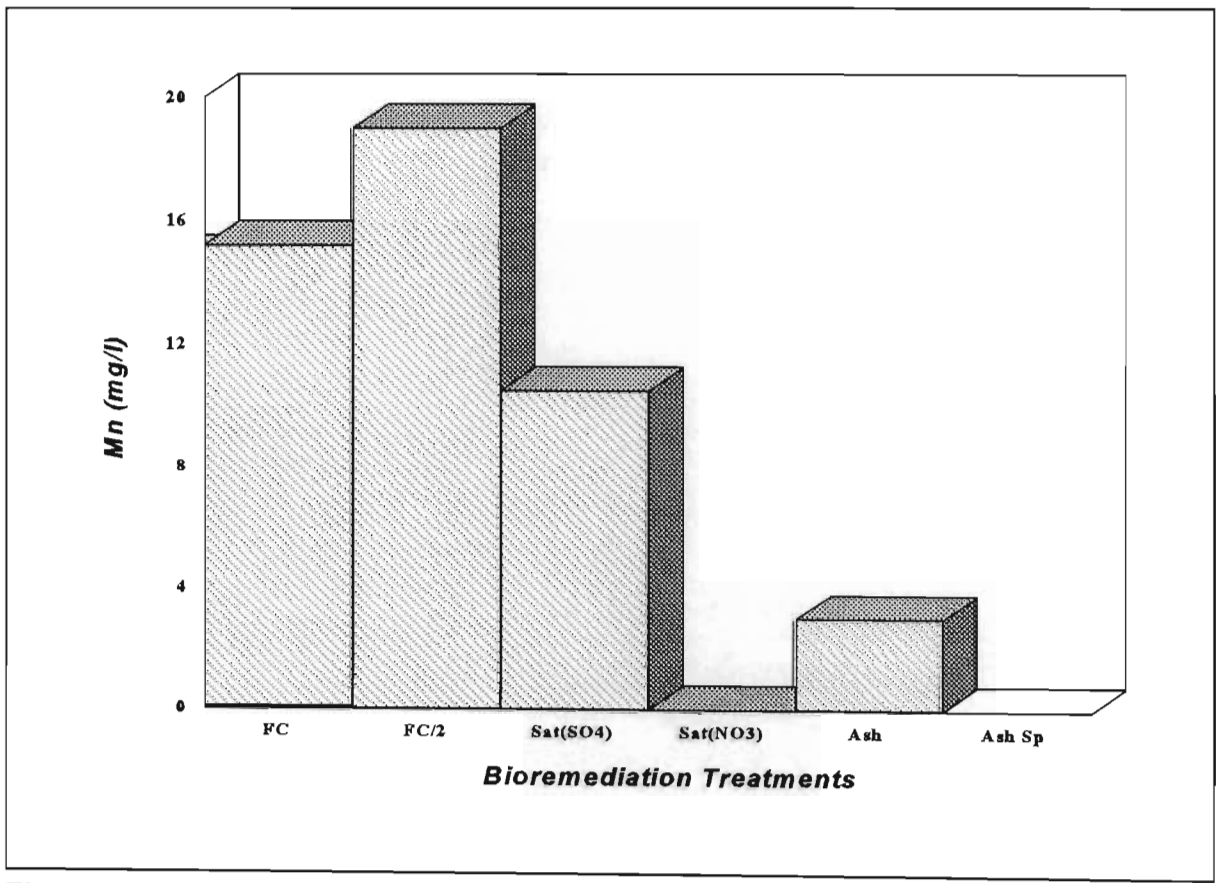


Figure 4.12: Manganese concentrations of corrected (adjusted for pore liquid flux) soil column leachates after 20 months treatment.

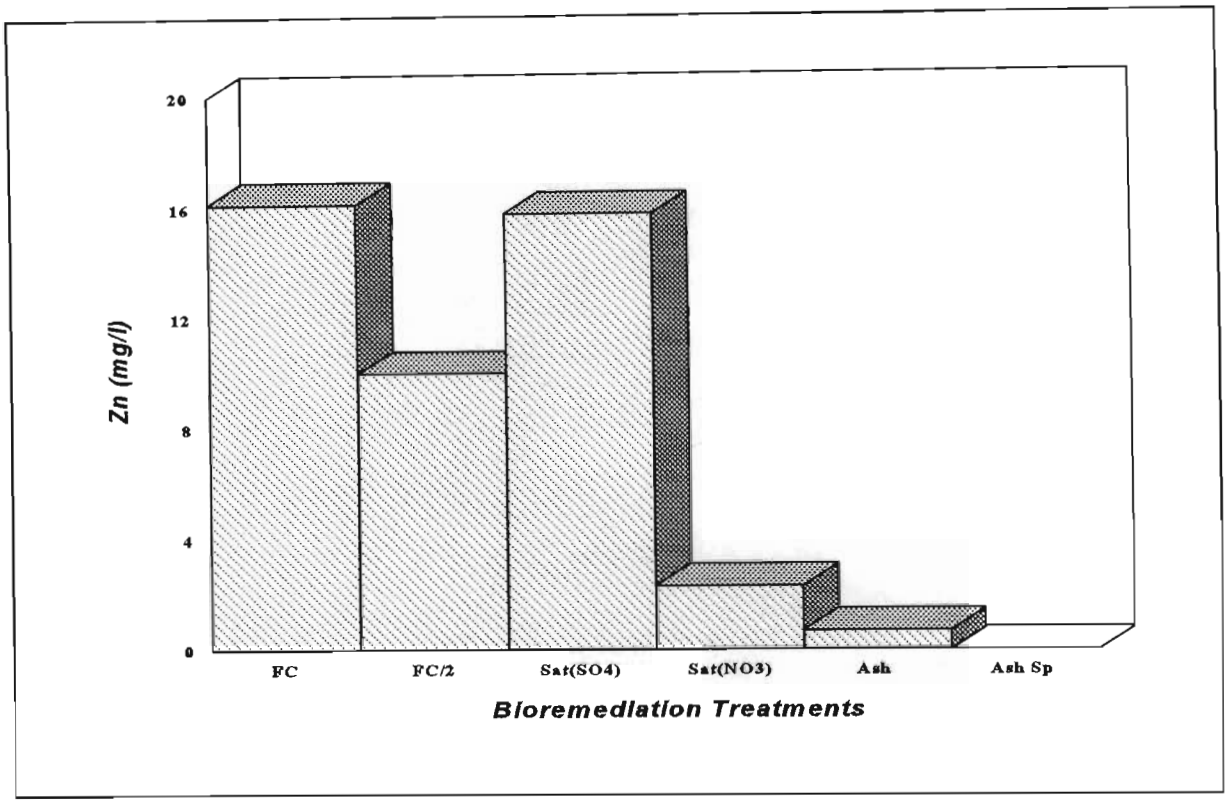


Figure 4.13: Zinc concentrations of corrected (adjusted for pore liquid flux) soil column leachates after 20 months treatment.

Figure 4.14), was found to follow a similar trend to that of the leachate pH for the different treatments. Table 4.3 gives the heavy metal concentrations present in the soil after the 20 month treatment period. The losses of Mg and Ca from the contaminated soil were found to be greatest although these metals pose only low environmental risks (Brady, 1984). Zinc was the only metal of significance which was found to be removed in high concentrations during the treatment period. This implied that leaching was an important mechanism for Zn removal from this particular soil type. Manganese concentrations in the Sat(SO_4) and Sat(NO_3) treatments were found to be higher than the initial contaminated soil concentrations after treatment and these could be attributed to the extraction procedure. The Ambic extraction procedure (2.8.1) is a partial extraction procedure which extracts the biologically-available metals only and is not a measure of the total metal concentrations. The anaerobic conditions resulting from the Sat(SO_4) and Sat(NO_3) treatments may have effected reduction of unavailable Mn^{4+} to available Mn^{2+}, thus giving rise to elevated Mn concentrations.

The mobility and loss of heavy metals appeared to follow a general trend of a decreased pH followed by an increasing loss of heavy metals from the soil (Table 4.3).

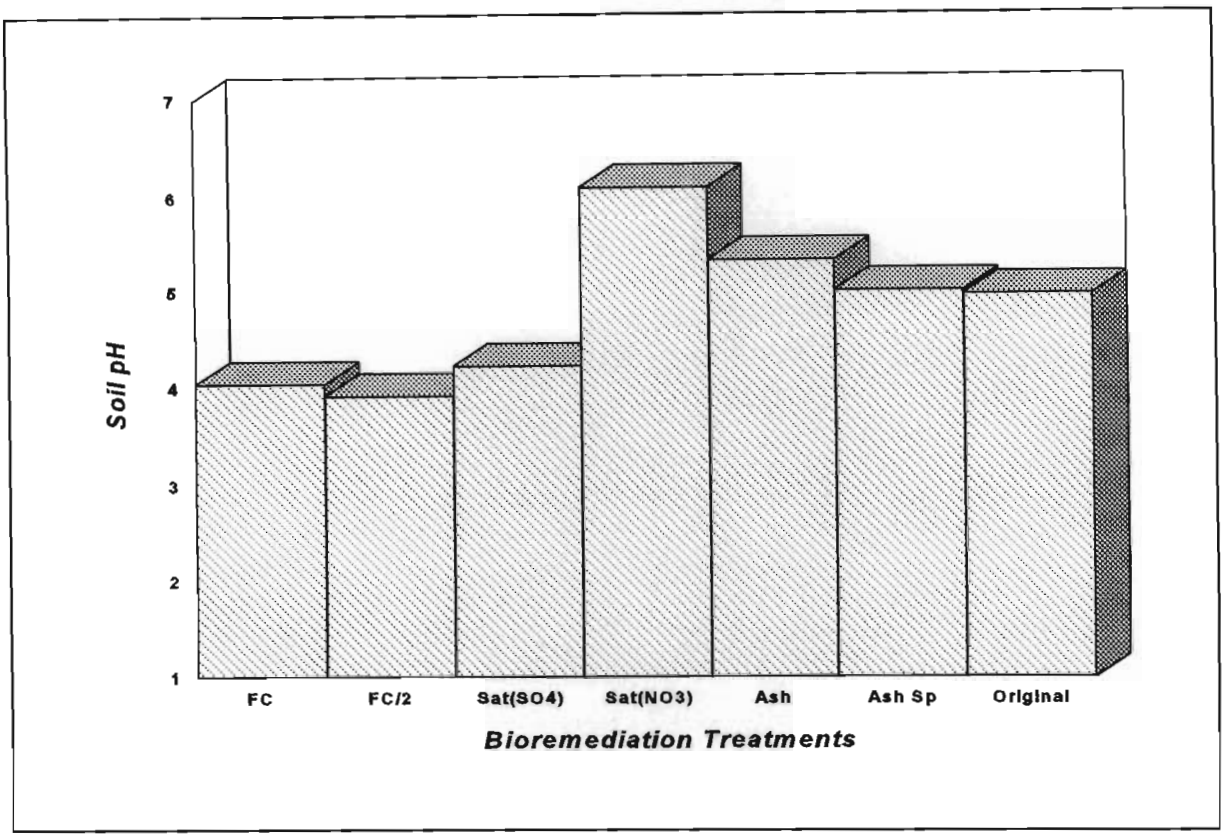


Figure 4.14: Soil pH of the treatment columns after a 20 month treatment period.

Table 4.3: Soil pH and residual metal concentrations of the soils after extended treatment (20 months).

Treatment	pH (KCl)	Mn	Zn	Ca	Mg	Cd	Ni
		$mgkg^{-1}$					
FC	4.04	81	30	932	183	0.2	15.8
FC/2	3.91	86	38	1007	157	0.2	15.9
Sat(SO_4)	4.22	116	48	670	174	0	19.0
Sat(NO_3)	6.08	105	43	940	212	0.1	14.0
Ash	5.32	72	39	1530	175	0.1	15.1
Ash Sp	4.99	61	29	1180	148	0.1	12.4
Untreated (control)	4.95	83	119	2170	489	0	15.7

This has serious implications for potential groundwater pollution, particularly as microbial hydrocarbon metabolic pathways such as fermentation may induce acidic conditions. Bioremediation under such circumstances would have to be regulated to

maintain suitable pH conditions.

Hydrocarbons

A comparison of the hydrocarbon losses after 70 days and 20 months treatment are shown in Figure 4.15. No significant changes in hydrocarbon losses were observed for the Ash, Ash Sp and FC/2 treatments. After 70 days, the FC/2 treatment showed greater hydrocarbon attenuation than the FC treatment. However, after 20 months, hydrocarbon loss of the FC treatment was found to have improved when compared to the FC/2 treatment. This indicated that the optimal conditions for biodegradation would be favourable under a relatively high soil moisture content.

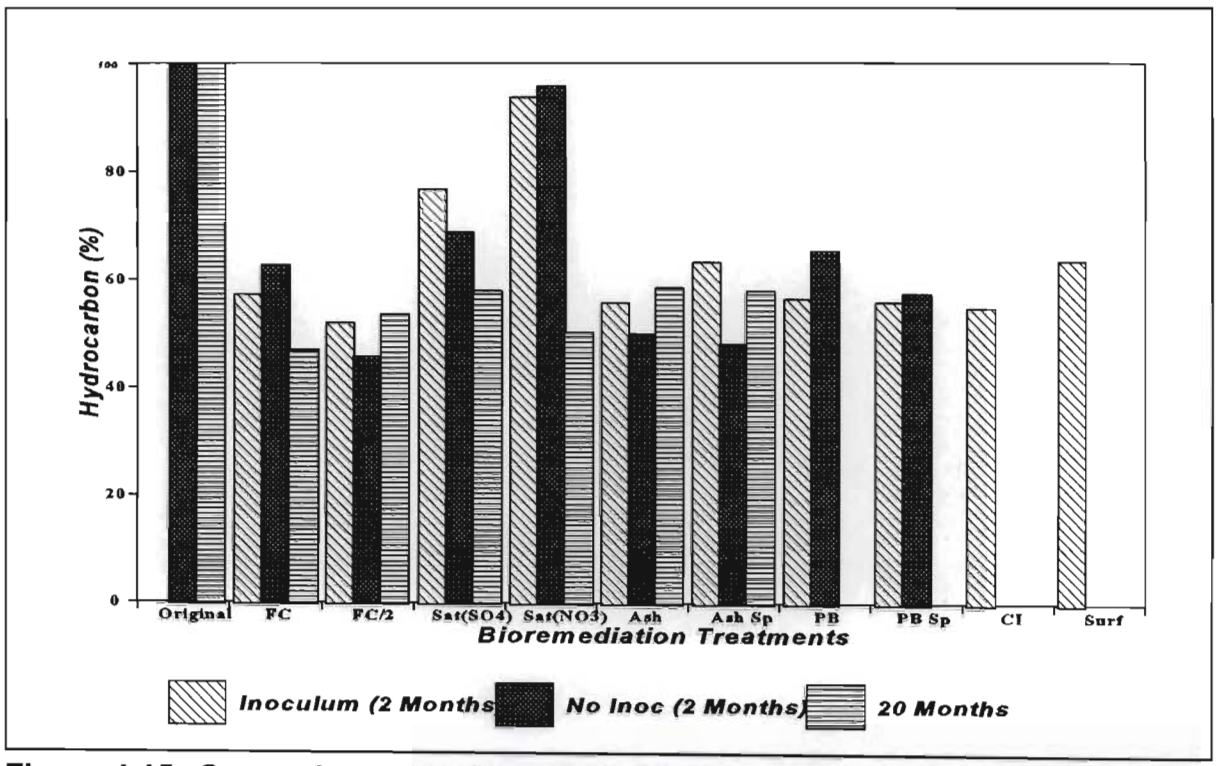


Figure 4.15: Comparison of hydrocarbon (% w/w of original contamination) loss after 70 days and 20 months treatment as determined by infra-red spectrophotometry.

Extended treatment of soil under anaerobic conditions showed interesting results. After 70 days, the Sat(SO_4) and Sat(NO_3) treatments were the least effective in facilitating hydrocarbon biodegradation. After 20 months, however, the efficiency of both

treatments was found to have improved. Hydrocarbon losses in these treatments were found to have occurred to the same extent as the aerobic treatments. The implication was that anaerobic hydrocarbon biodegradation increased with treatment time.

Although infrared spectrophotometry (2.9.2) showed that hydrocarbon removals of up to 52% (FC treatment) were obtained, these figures are optimistic in their estimation. The infrared method is biased towards the lighter fraction hydrocarbons. As this fraction is more labile than the heavier hydrocarbons, the analytical results would tend to be skewed towards higher biodegradation figures. A more accurate estimation of total hydrocarbon loss was obtained by hydrocarbon gravimetric measurement (2.9.2). From these analyses (Figure 4.16), as well as gas chromatography traces (Figure 4.17), it

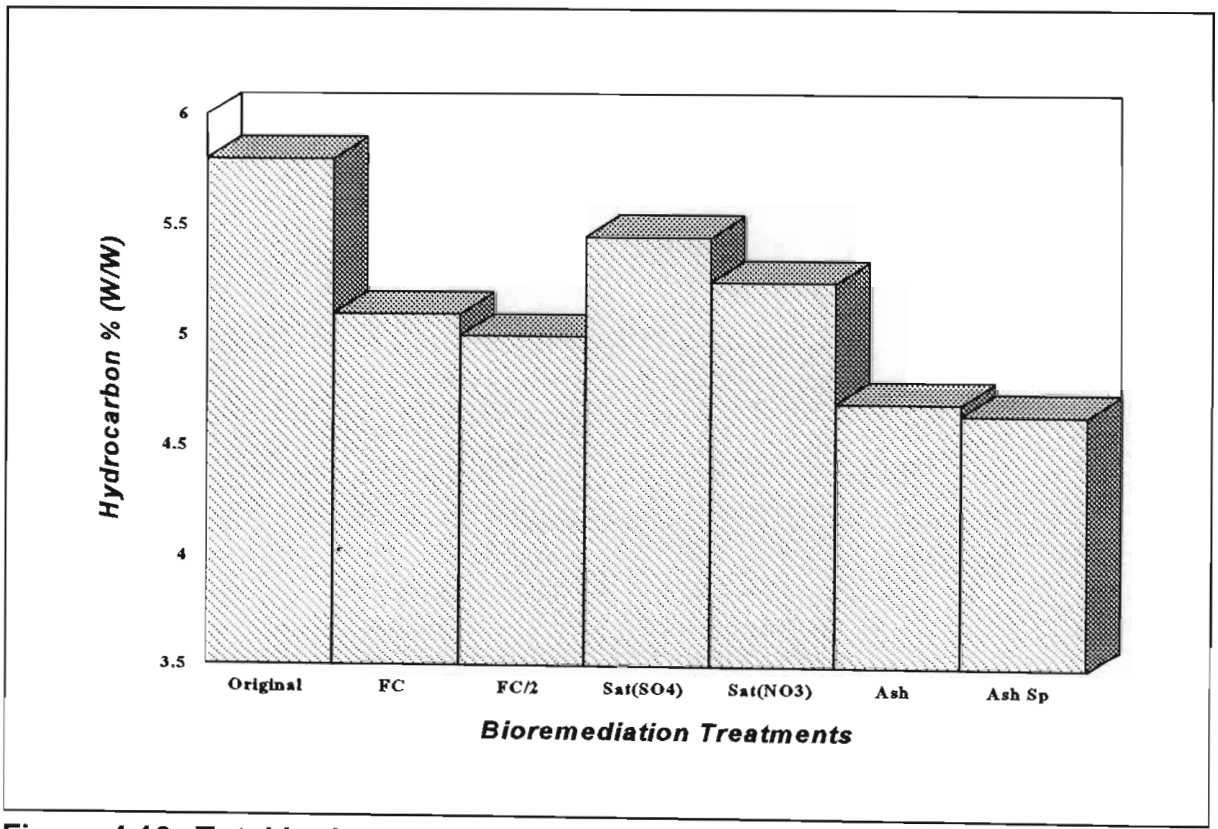


Figure 4.16: Total hydrocarbon content of soils after a 20 month treatment period.

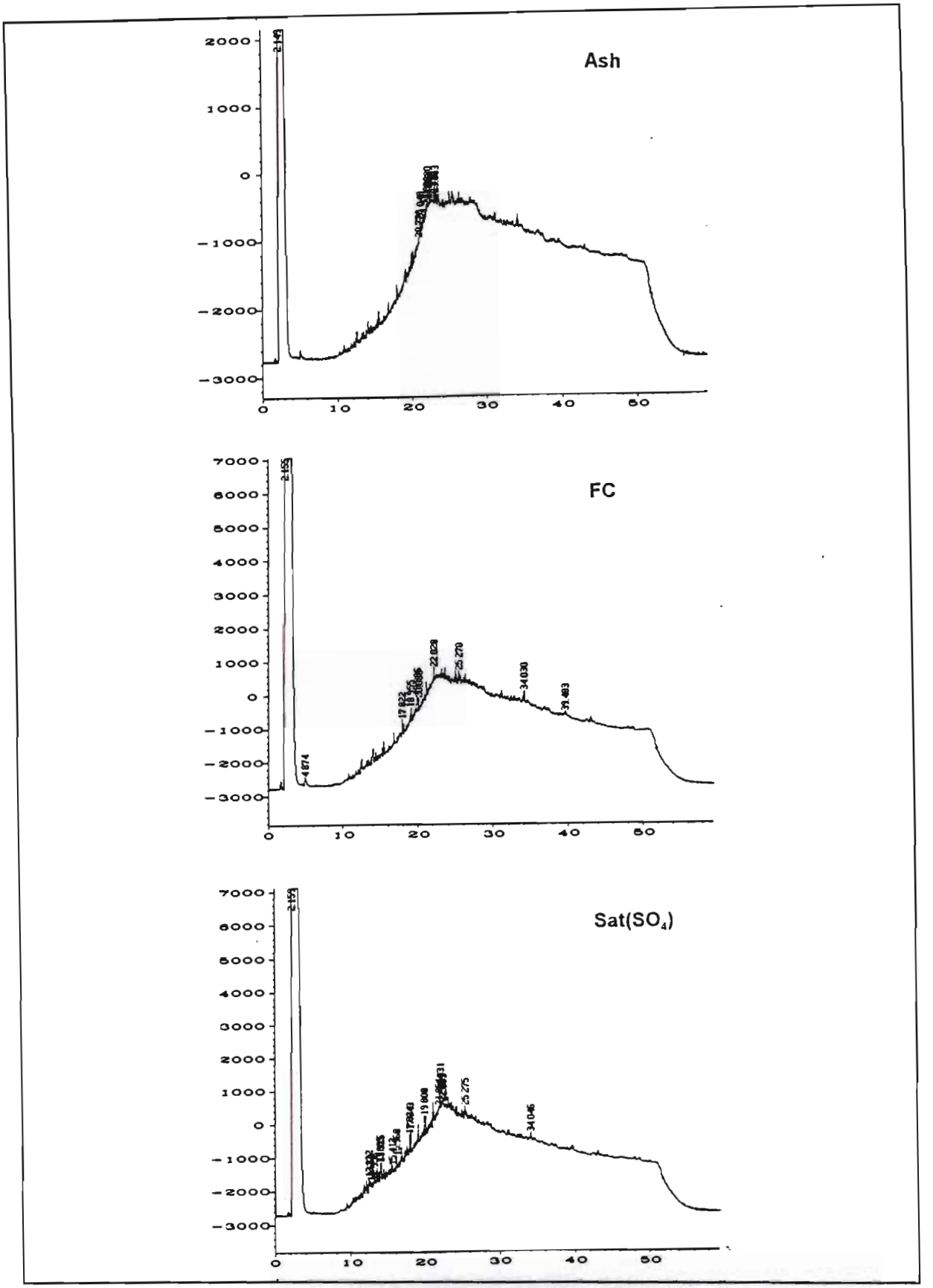


Figure 4.17: Selected gas chromatography traces [Ash, FC, Sat(SO_4)] obtained for various treatments after 20 months (not all traces are shown since differences between some traces were not detected).

was evident that a considerably smaller fraction of the total hydrocarbons was in fact attenuated by the various treatments than had first been estimated. A comparison of the chromatogram obtained for the original (untreated) contaminated soil and the same soil after 20 months treatment showed the disappearance of individual peaks, although an unresolved hydrocarbon "hump" still remained. It could be concluded that individual components of the petroleum mixture were biodegraded but that the majority of the unresolved hydrocarbons remained.

From Figure 4.15, it is clear that during the initial treatment period (70 days), the aerobic treatments and particularly those treatments which improved soil aeration (Ash and Ash Sp), had the greater beneficial effects on hydrocarbon biodegradation. However, the proportion of hydrocarbons degraded under anaerobic conditions was greater after 20 months. Thus, anaerobic degradation appeared to become the main mechanism of hydrocarbon removal. This may be explained by the rapid aerobic degradation of the light molecular, labile hydrocarbons during the first two months of treatment. At this point it was likely that aerobic degradation slowed when recalcitrant molecules predominated. Anaerobic catabolism is a slower and less rapid process and would account for hydrocarbon degradation over a longer time period i.e. between 2 and 20 months

The inclusion of ash as a soil bulking agent was found to be beneficial for hydrocarbon degradation. However, the ash had relatively high heavy metal concentrations which could increase the soil metal concentration. Since the buffering capacity of ash is unknown, the potential for groundwater contamination under low pH conditions is, thus, a factor which must be considered before ash incorporation.

4.3.3 Influence of Heavy Metals on Indigenous Soil Microorganisms

The ratio of Mn:Zn:Ni was kept constant in all of the flasks and was the same ratio as found in the contaminated soil (Table A.4). Although other heavy metals were present in the soil, due to the time constraints of the study, exhaustive testing of all the metal

species was not possible. Manganese, Zn and Ni were, thus, used as indicator metals since they were present in the highest concentrations in the soil and/or available in a readily soluble form (Table A.4).

The mean oxygen consumption rate of 6 replicates (after correction for non-biological diffusion of 0.075 $mg\,l^{-1}h^{-1}$) was 0.101 $mg\,l^{-1}h^{-1}$. This was taken as the oxygen consumption rate for the soil under the conditions described in 4.2.5 in the absence of inhibitory substances such as elevated metal concentrations. A decrease in this oxygen consumption rate was thus taken to be indicative of microbial inhibition.

In the presence of heavy metals and no pH adjustment (Figure 4.18), microbial activity decreased in response to increases in heavy metal concentrations. However, the observed effects, could also be attributed to the low pH conditions which were required

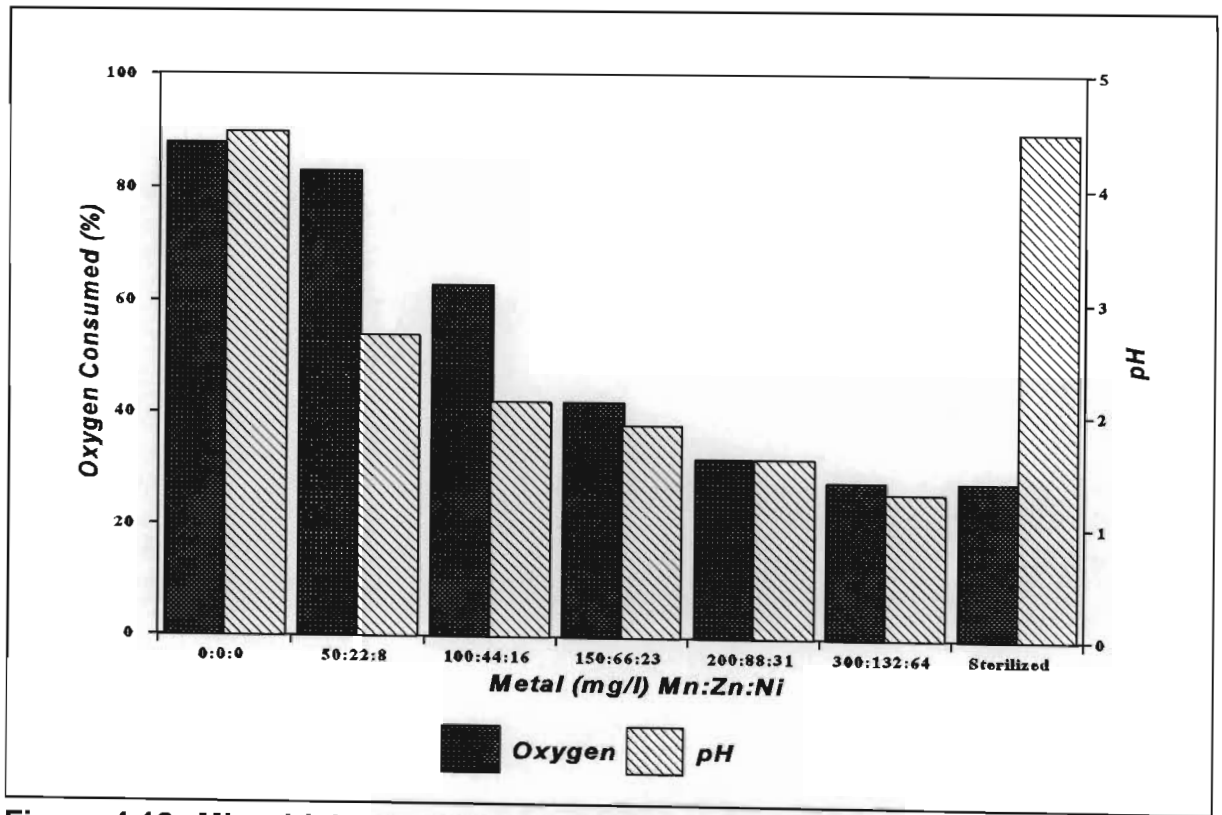


Figure 4.18: Microbial oxygen consumption in the presence of different Mn, Zn and Ni concentrations and no pH adjustments.

to maintain the heavy metals in solution (the laboratory standard solutions of Mn, Ni and Zn were all maintained in strong acidic solutions to prevent precipitation and this contributed to low pH conditions). After adjusting the pH to 4.5 (Figure 4.19), the increased heavy metal concentrations appeared to have little effect on microbial activity, since the oxygen consumptions were comparable with the 0:0:0 treatment. The pH adjustment did, however, result in heavy metal precipitation of solubilized cations (Figure 4.20 A-C) possibly as insoluble hydroxides or oxides (Gadd and Griffiths, 1978). The presence of phosphate in the growth medium may also have contributed to the precipitation effect (Sadler and Trudinger, 1967).As a result, the metal concentrations were not as high as when initially applied.

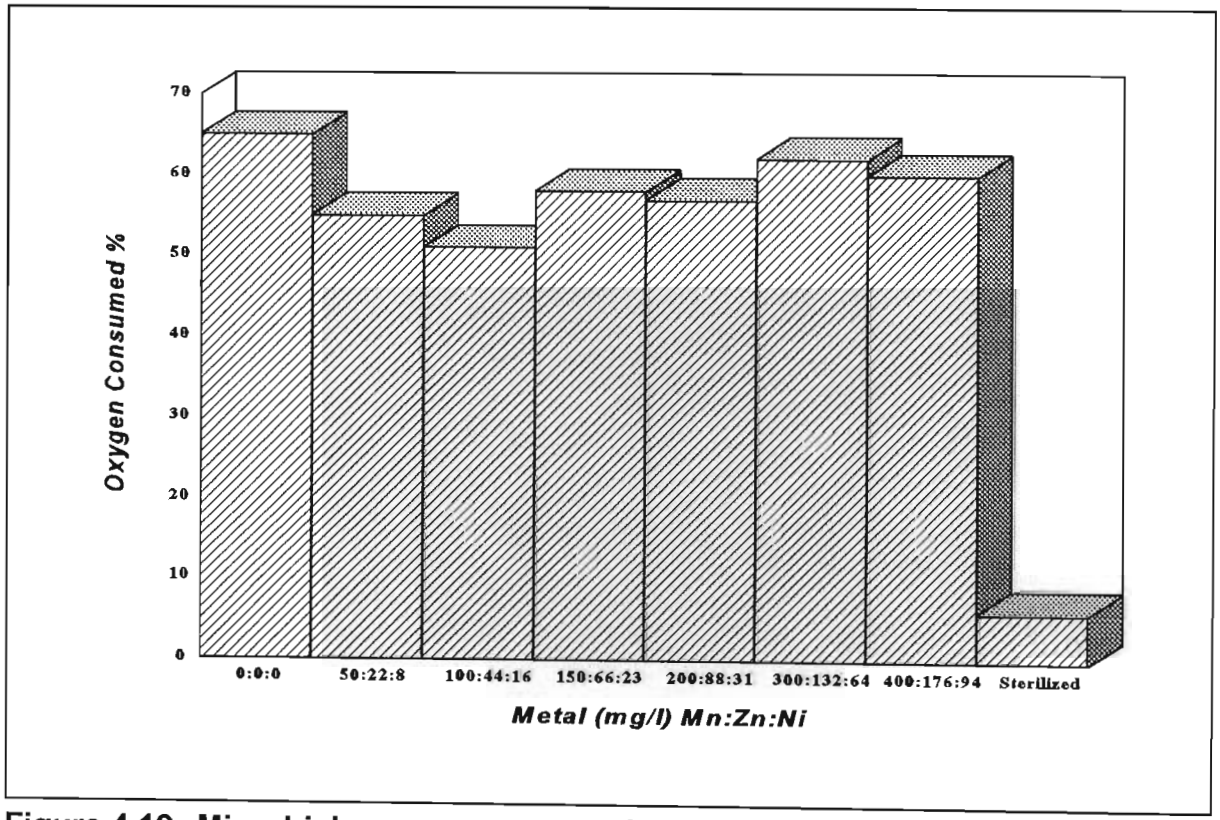


Figure 4.19: Microbial oxygen consumption in the presence of different Mn, Zn and Ni concentrations and pH poised at 4.5.

With respect to metal toxicity in the contaminated soil, microbial activity did not appear to be adversely affected by concentration ratios ≤ 327:100:50.9 ppm Mn:Zn:Ni (Figures 4.19, 4.20 A-C). Thus reduced microbial activity could rather be attributed to acidic conditions rather than the elevated metal concentrations. Therefore, providing that soil

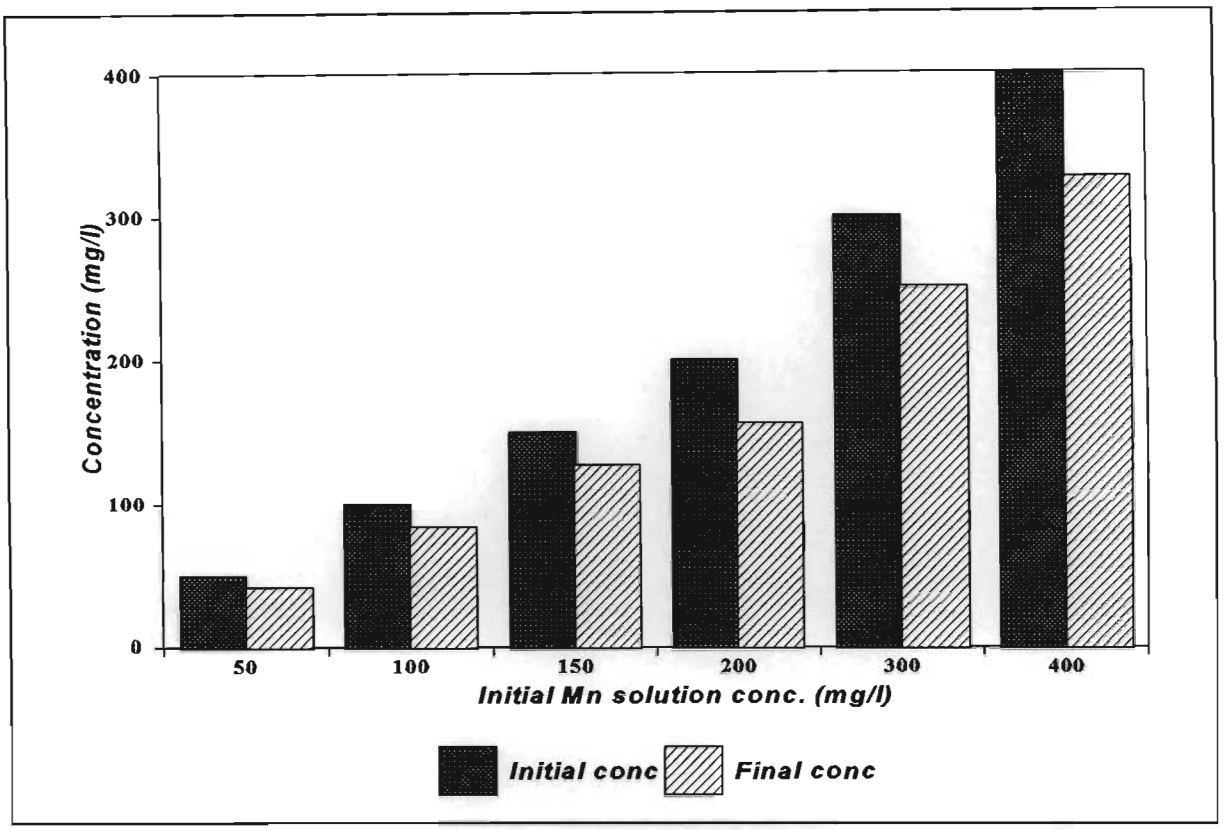


Figure 4.20A: Mn concentration reduction due to precipitation after adjusting the medium pH to 4.5.

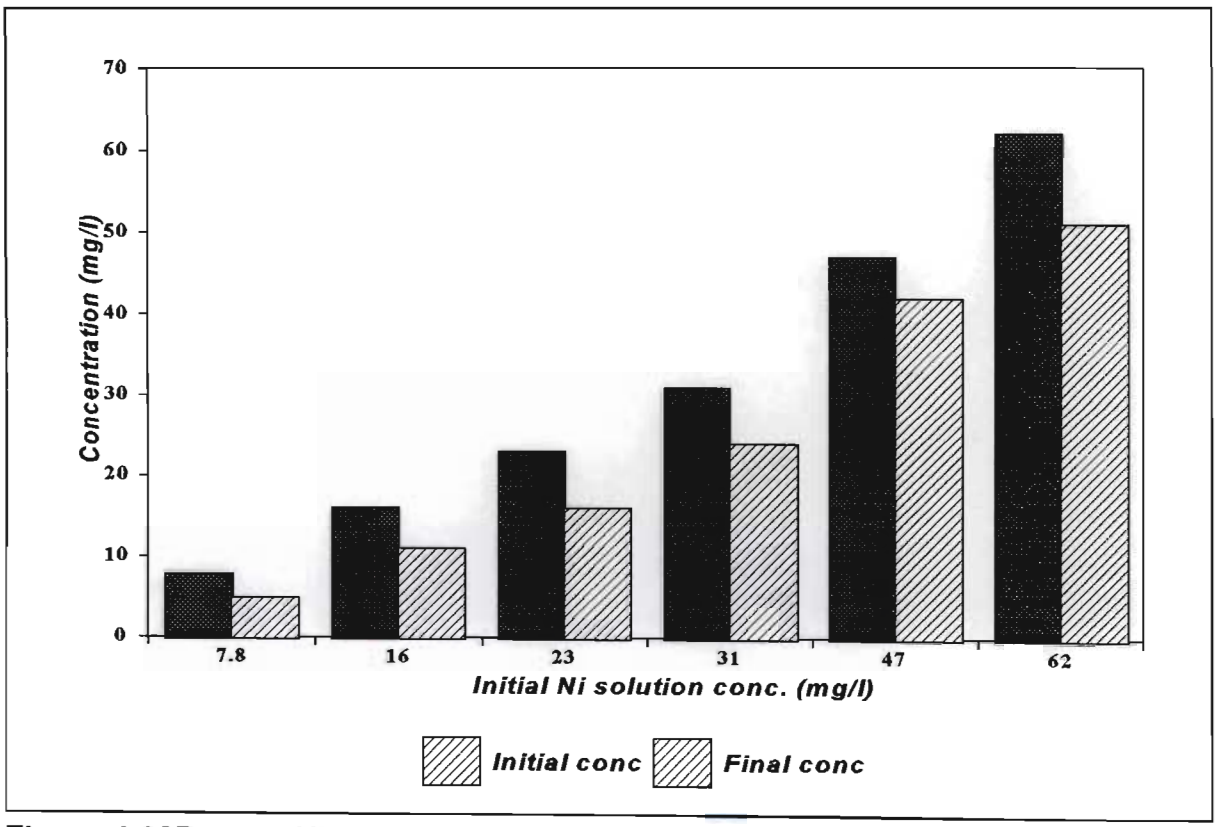


Figure 4.20B: Ni concentration reduction due to precipitation after adjusting the medium pH to 4.5.

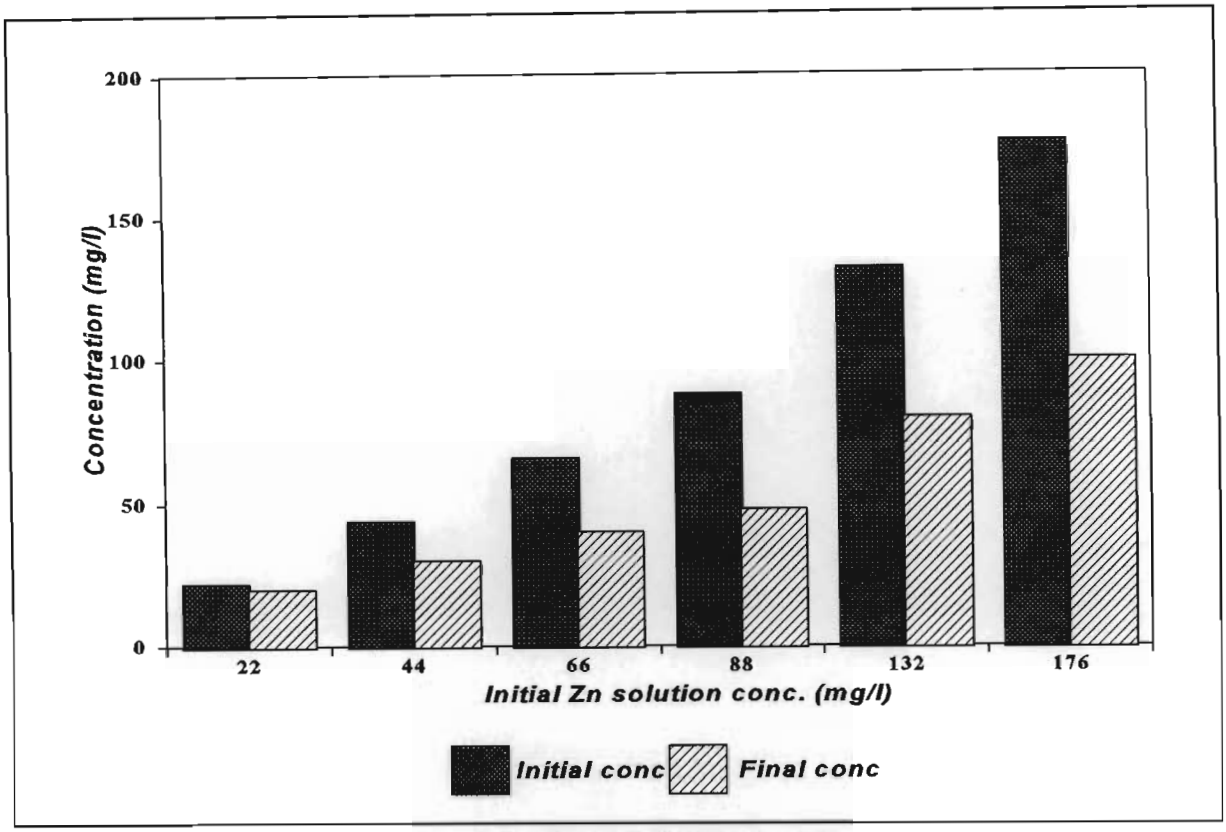


Figure 4.20C: Zn concentration reduction due to precipitation after adjusting the medium pH to 4.5.

pH conditions are maintained at ≥ 4.5, there is little danger of the sludge applied heavy metals reaching microbial inhibitory concentrations. The heavy metal concentrations which were detected in the leachate after the bioremediation treatments (4.3.1, 4.3.2) were, in all instances, found to be within the tolerance limits for microbial activity. Although it is possible that metal concentrations may increase above the maximum tested ratio of 327:100:50.9, this is unlikely to occur. Since the sludge is rich in organic molecules, reduced bioavailability of a large proportion of the heavy metals through precipitation, chelation and complexation should result (Benes *et al.*, 1976). Also, the buffering capacity of the pre-compromised soil should minimize solubilization and mobilization of the heavy metals. It should be noted, however, that the continuous application of hydrocarbon sludge diminishes the adsorptive capacity of the soil (Chapter 6). To maintain suitable conditions for *in situ* bioremediation it is necessary to continually monitor the acidity of the soil and, if necessary, lime application should be considered. Such a treatment would also be beneficial to maintain pH conditions between 6 and 8, which are optimal for biodegradation.

The issue of heavy metal contamination raises the question of which remedial action to follow. Traditionally, the presence of metals motivates the microbiological bioremediation approach (King *et al.*, 1992). This assumes that the natural microbial population is compromised by heavy metal toxicity and is incapable of effective xenobiotic biodegradation. Bioremediation is consequently facilitated and enhanced with the introduction of selected or engineered microorganisms which are resistant to the adverse effects of heavy metals. However, there is no conclusive evidence of increased hydrocarbon biodegradation rates compared with the natural population. Despite the additional expense, there is no guarantee of adaptation and survival of the introduced organism(s).

The soil contamination examined in this study extended to several metres below ground level and thus excavation methods of treatment could prove expensive. The alternative option of bioremediation over a protracted time period would necessitate augmentation of the natural population with nutrients and oxygen. Although the treatment would be prolonged, the costs would be lower. The results obtained in this study suggested that the natural population was, through selection, metal tolerant which thus eliminated the need to introduce engineered microorganisms. The intrinsically high tolerance of natural populations to heavy metals following exposure has been reported (Angle *et al.*, 1993).

In conclusion, although bioremediation as a treatment does not necessarily facilitate the removal of heavy metals, groundwater contamination is unlikely to occur in the short term. To fully determine the threat of groundwater pollution, further work must be carried out to determine the depth to groundwater, soil permeability and other factors contributing to metal migration. However, for the interim, maintaining the pH near neutral, should limit the toxicity and mobility of heavy metals into groundwater and allow suitable conditions for biodegradation to prevail.

CHAPTER 5

DEVELOPMENT AND EVALUATION OF A NOVEL BIODEGRADATION INDICATOR

5.1 INTRODUCTION

The majority of methods commonly employed for the analysis of hydrocarbon contaminated soil and water were proposed for use by the United States Environmental Protection Agency (EPA). However, during initial development, these methods were not specifically designed for the analysis of hydrocarbon contaminated soil and water and consequently have not been fully evaluated with regards to their efficiency (Potter, 1993). The relative composition of the components present in hydrocarbon mixtures may change once released into the environment. The processes responsible for changes of this nature include volatilization, dissolution and biological and abiotic degradation. Each process may influence certain compounds or group of compounds and the rates of change are usually a function of the environmental conditions. As a result, analytical methods need to have a broad scope and where target compound analysis is involved, selectivity and specificity are prerequisites (Potter, 1993).

Total petroleum hydrocarbon detections by EPA methods 413.1 and 418.1 (USEPA, 1974) have problems specifically related to their use. Where gravimetric techniques are employed, volatile compounds may be lost in the solvent concentration step. Similarly, certain hydrocarbon constituents may remain attached to soil particles as a consequence of low solvent solubilities. Infrared procedures, which are commonly used to estimate the total hydrocarbon content, measure the presence of CH_2 groups in the contaminant mixture at a wavelength of 2930 cm^{-1}. As a result, the methods generally have moderate to poor sensitivity for certain aromatic molecules. The response obtained from infrared spectrophotometry is a function of the relative amounts of aromatic and aliphatic

hydrocarbons the sample contains and the wavelength setting (Potter, 1993).

Another problem associated with conventional hydrocarbon analysis is the use of standards. Hydrocarbon mixtures which are used for instrument calibration usually have constant molecular compositions (the ratio of aromatics to aliphatics), whereas the relative compositions of various hydrocarbon products and samples may be highly variable. As a result, uncertainty may be introduced into measurements of degradation (DeAngelis, 1987).

Accurate hydrocarbon analysis is often linked to mass spectroscopy. This type of analysis requires sophisticated instrumentation which is costly and often unavailable in South Africa. In addition, the analysis of extracts from contaminated soils by gas chromatography is a difficult task since the chromatograms often show poor separation and resolution of the various key components.

In order to minimise and eliminate some of the difficulties associated with total hydrocarbon analysis, the validity of developing an index of hydrocarbon biodegradation was investigated.

5.2 EXPERIMENTAL

5.2.1 Column Treatments

Bioremediation studies were carried out in soil columns. The columns were constructed from PVC pipes (internal diameter 4.5cm, length 25cm) with silicone rubber seals. Glass wool was used to prevent soil washout and effluent stream clogging. Dry soil (2.1) was packed by vibrating with a Vortex mixer. The soil columns were incubated at ambient temperature (approximately 25°C) in the dark.

5.2.2 Treatments

Several bioremediation treatment studies were made as described in Table 5.1.

Table 5.1: Bioremediation treatments during the course of the column study.

Treatment	Column	Description
FC: To determine the effect of field capacity soil saturation with nutrients on bioremediation	1	Nutrients (2.4.1, Medium 1) and 10% (v/v nutrient solution) inoculum (2.5) were added to the column on a weekly basis to allow free drainage.
	2	As for Column 1 but no inoculum was added.
FC/2: To determine the effect of soil nutrient supplementation at half the frequency of FC.	3	As for Column 1 but the nutrients and inoculum were added every two weeks.
	4	As for Column 3 but no inoculum was added.

Sat(SO_4): Bioremediation under anaerobic conditions was tested with ammonium sulphate as an alternative electron acceptor.	5	The soil was saturated with nutrients and 10% (v/v) inoculum. Medium 1 (2.4.1) was used but the nitrogen was in the form of $(NH_4)_2SO_4$ (1gl^{-1}). The soil column was supplemented with nutrients by vertical displacement from the base. The column was then allowed to drain freely once a week prior to resaturation with fresh nutrients. Anaerobic conditions were provided by saturation of the soil by the nutrient medium.
	6	As for Column 5 but no inoculum was added.
Sat(NO_3): Bioremediation under anaerobic conditions was tested with potassium nitrate as an alternative electron acceptor.	7	This column was treated the same as Column 5 with the exception that nitrogen was in the form of 1gl^{-1} KNO_3.
	8	As for Column 7 but no inoculum was added.
Ash: Ash (2.2) was used as a bulking agent to improve the soil porosity and soil oxygen holding capacity.	9	Contaminated soil (2.1) was mixed with coarse ash (2.2) in a 1:1 (w/w) ratio and treated as for Column 1.
	10	As for Column 9, but no inoculum was added.

Ash Sp: Ash (2.2) and air sparging was used to improve soil porosity and soil oxygen holding capacity.	11	This column was prepared and treated in the same manner as Column 9. However, after the columns had been freely drained, they were sparged with dry air from the column base. The sparging rate was approximately 50 pore volumes per day. For vapour collection, the column air outlet was equipped with an "Merck Orbo 32" activated carbon vapour trap.
	12	As for Column 11, but no inoculum was added.
PB: Pine-bark (2.1) was used as a bulking agent to improve soil porosity and soil oxygen holding capacity.	13	This was treated in the same manner as Column 1 with the exception that composted (composted for four months) pine-bark was used as a bulking agent (1:1 w/w ratio). This treatment was, however, initiated 20 days after the other treatments.
PB Sp: Pine-bark (2.1) and air sparging was used to improve soil porosity and the soil oxygen concentration.	14	This column was prepared and treated in the same manner as Column 13. Air sparging was carried out as for Column 11
	15	As for Column 14, but no inoculum was added.

After 70 days, the soil from all columns was destructively sampled. Each soil was well mixed with a mortar and pestle and the hydrocarbons extracted (2.7.1) for analysis by gas chromatography and infrared spectrophotometry (2.9.2).

5.2.3 Determination of a Biodegradation Index in a Soil Free System

A portion of the thick hydrocarbon sludge (2.3) was immobilized by placing a 1m/ sample between two layers of glass wool in a 100m/ nutrient solution (2.4.1, Medium 1) in a 250m/ conical flask.

Ten replicate samples with 10% (v/v) inoculum (2.5) were incubated in the dark on a New Brunswick rotary shaker incubator (70 rpm) at 30°C. One incubation flask was removed every 2 weeks and, after sampling, the contents were frozen until gas chromatography analysis could be made (2.7.4, 2.9.2). Upon sampling, the glass wool was removed and viewed by electron microscopy for the presence of microbial populations (2.9.4). Supernatant samples were also removed for Gram staining and examination by light microscopy.

5.3 RESULTS AND DISCUSSION

5.3.1 Biodegradation Index

A Biodegradation Index was developed from measurement of gas chromatogram peak areas following various bioremediation treatments. The Biodegradation Index is based on estimating the relative degradation of lower boiling point to higher boiling point hydrocarbons. Gas chromatograms are shown in Figure 5.1A-C of the original contaminated soil, FC (Column 2) and Sat(SO_4) (Column 6) treatments respectively. After bioremediation, a distinctive change was observed in the characteristic hydrocarbon "hump", i.e. the hydrocarbon molecules with lower boiling points (low number of carbons)

were catabolized to greater extent than compounds with higher boiling points (high carbon numbers). As a consequence, the chromatogram area associated with the light fraction hydrocarbons was reduced relative to the chromatogram area of the heavier hydrocarbons. The Biodegradation Index is then calculated from these changes.

The Index was formulated as:

BI (Biodegradation Index) = $(A1/A2)_x/(A1/A2)_o$

where $(A1/A2)_o$ is the area ratio of the untreated contaminated sample (Figure 5.1A); and $(A1/A2)_x$ is the area ratio of a sample which has undergone treatment (Figure 5.2). A BI ratio of 1 would, therefore, indicate that no biodegradation relative to the original soil contamination had occurred. Conversely, decreasing BI values indicate increased hydrocarbon biodegradation. The area ratio (A1/A2) was determined by a vertical line coinciding with the retention time of a selected aliphatic or aromatic hydrocarbon of a

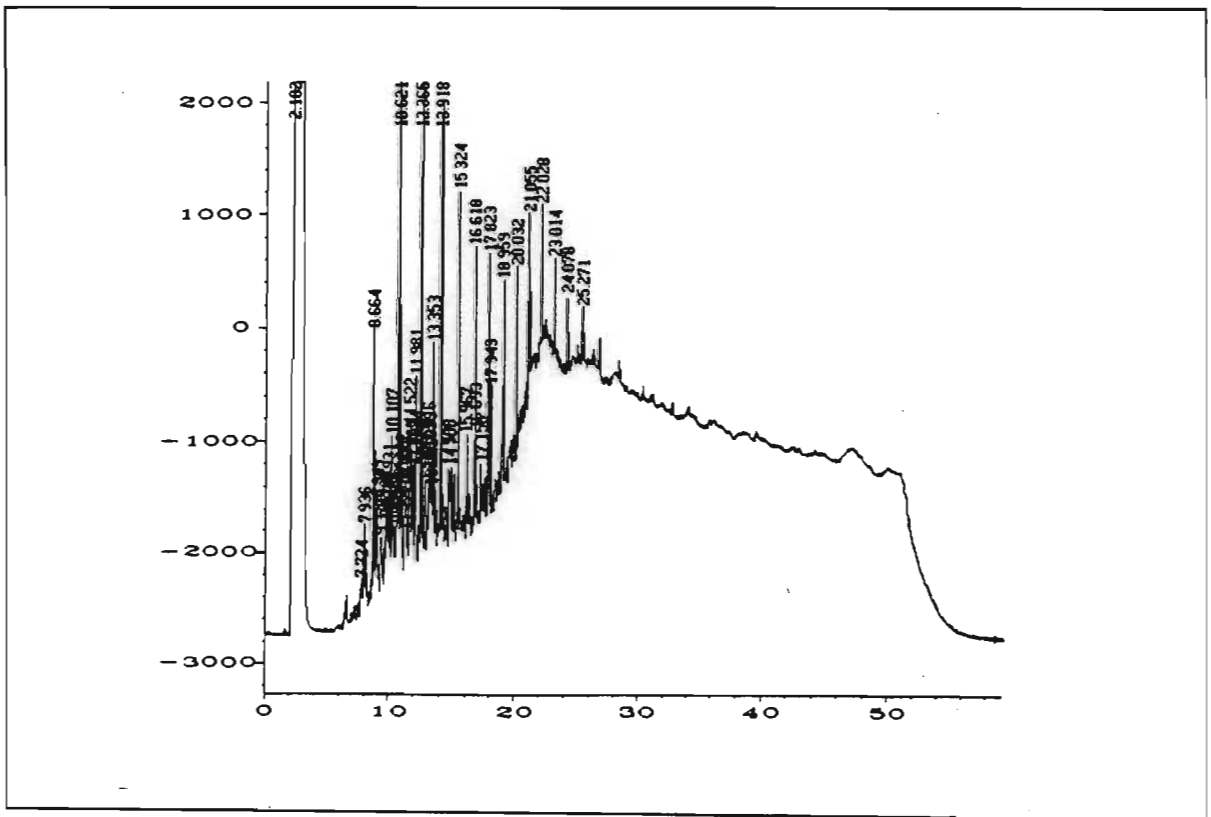


Figure 5.1A: Gas chromatograph of extract of untreated contaminated soil

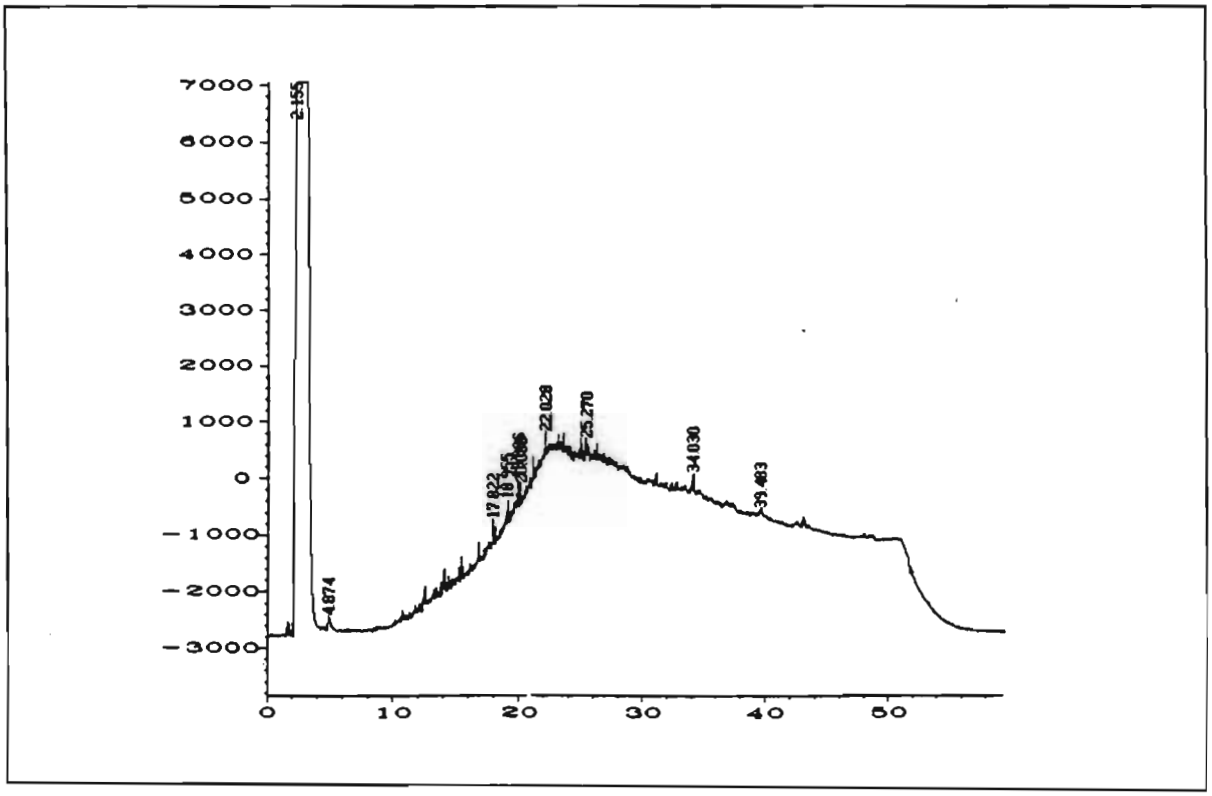


Figure 5.1B: Gas chromatograph of extract of FC treated contaminated soil (Column 1).

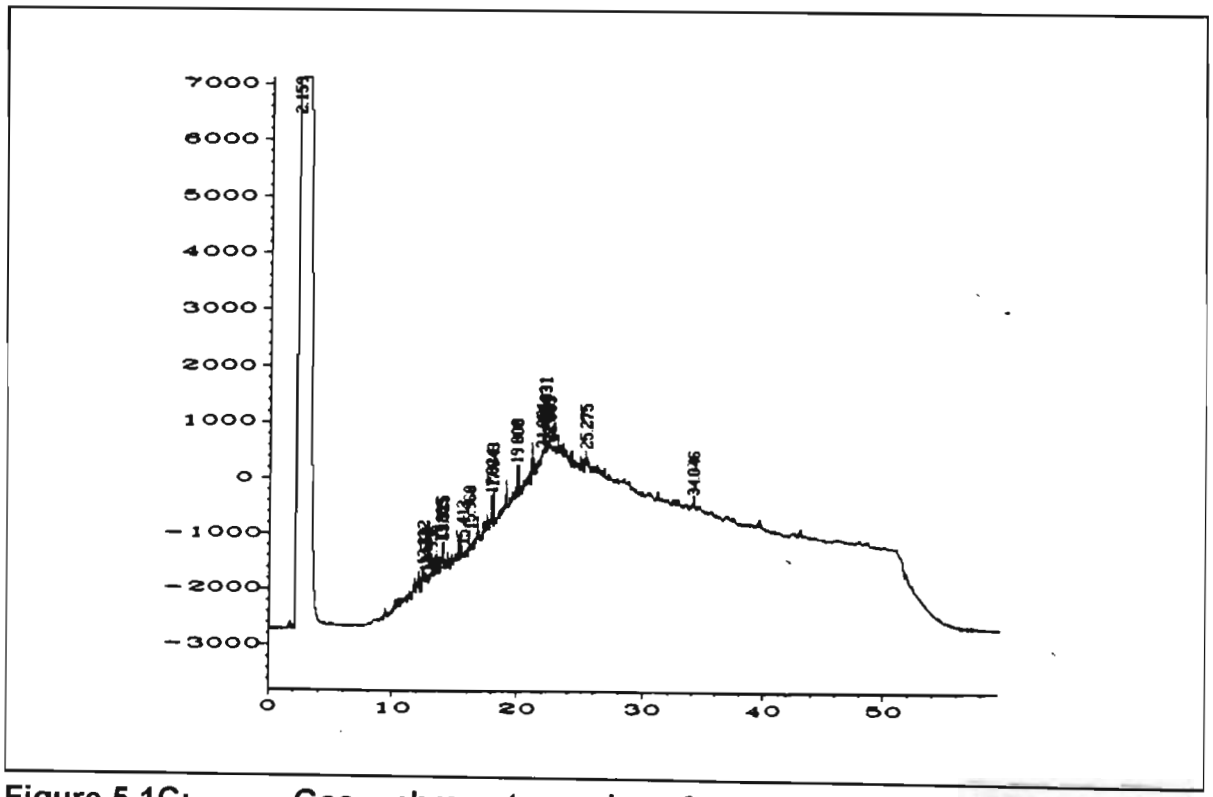


Figure 5.1C: Gas chromatograph of extract of Sat(SO_4) treated contaminated soil (Column 5)

known carbon chain length or number (Figure 5.2). Ideally, the selected molecule should have a carbon number of C20 as several studies have shown that hydrocarbon molecules larger than C20 are less biodegradable than molecules with a carbon number lower than 20 (Block *et al.*, 1990; Cerniglia, 1993; Douglas *et al.*, 1993). Indeed, this observation has been used as the basis of degradation monitoring by determining the pristane/phytane branched alkane to normal alkane (C17-C18) ratios (Kennicutt, 1988). The exact selection of the marker compound may vary depending on the composition of the hydrocarbon mixture and the analytical conditions and techniques which are used.

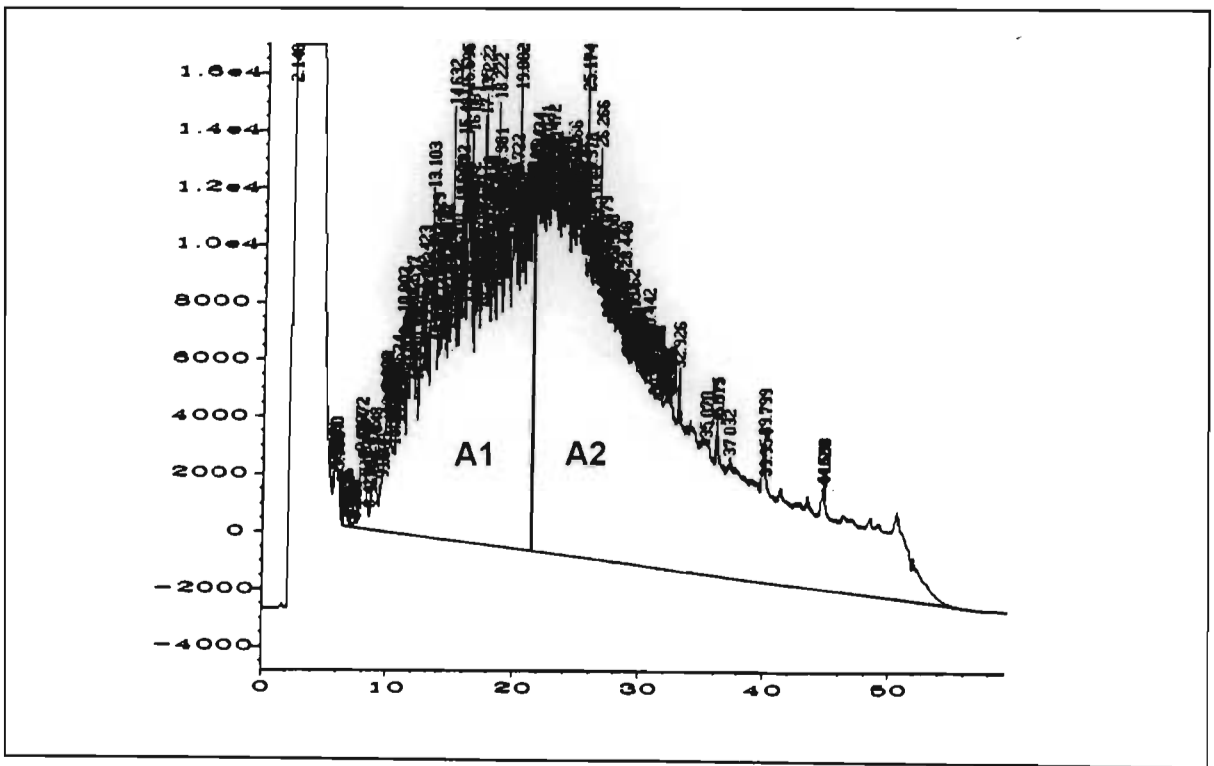


Figure 5.2: Gas chromatogram showing the derivation of the Biodegradation Index.

5.3.2 Comparison of Hydrocarbon Degradation as Assessed by the Biodegradation Index and Infrared Analysis

The application and validity of the Biodegradation Index was tested on a contaminated soil which had undergone bioremediation. For the column studies, a retention time coinciding with a C20 alkane (21.347 minutes) was used to determine the Biodegradation Indexes for the various treatments. A comparison of bioremediation efficiency, as determined by the Biodegradation Index and EPA Method 418.1 (infrared spectrophotometry; USEPA, 1974) is shown in Figures 5.3 and 5.4. Both analytical methods revealed similar biodegradation trends for both the inoculated and uninoculated treatments. Statistical correlation (r-squared as determined by standard regression output) between the two methods was, however, found to be weak to moderate (0.63 for inoculated treatments and 0.75 for uninoculated treatments). This did not necessarily invalidate the Biodegradation Index as there may have been several contributory factors. Detection sensitivity may have varied because total hydrocarbon detection methods (such as infrared spectrophotometry) are less sensitive to high molecular weight hydrocarbons because of the extraction procedures required for analysis (Baugh and Lovegreen, 1993). Furthermore, the apparent hydrocarbon concentration obtained by infrared analysis may have been subjected to fluctuation between duplicate samples because of non-uniform hydrocarbon distribution within the soil. This problem was eliminated in the Biodegradation Index determination as the change of lower molecular weight hydrocarbons relative to the higher molecular weight hydrocarbons was compared and not the total hydrocarbon concentration. The Biodegradation Index should thus be independent of fluctuating contaminant concentrations. Similar low correlations between the results obtained from different hydrocarbon analytical methods have also been previously reported (Block *et al.*, 1990). If these problems are taken into account then the similarities in biodegradation trends as determined by both analytical methods (Biodegradation Index and infrared) assume greater significance. It can, thus, be intimated that the biodegradation trends as assessed by the Biodegradation Index are a close approximation to the results and trends obtained by infrared spectrophotometry, and that the Biodegradation Index may be a valid method for monitoring hydrocarbon degradation.

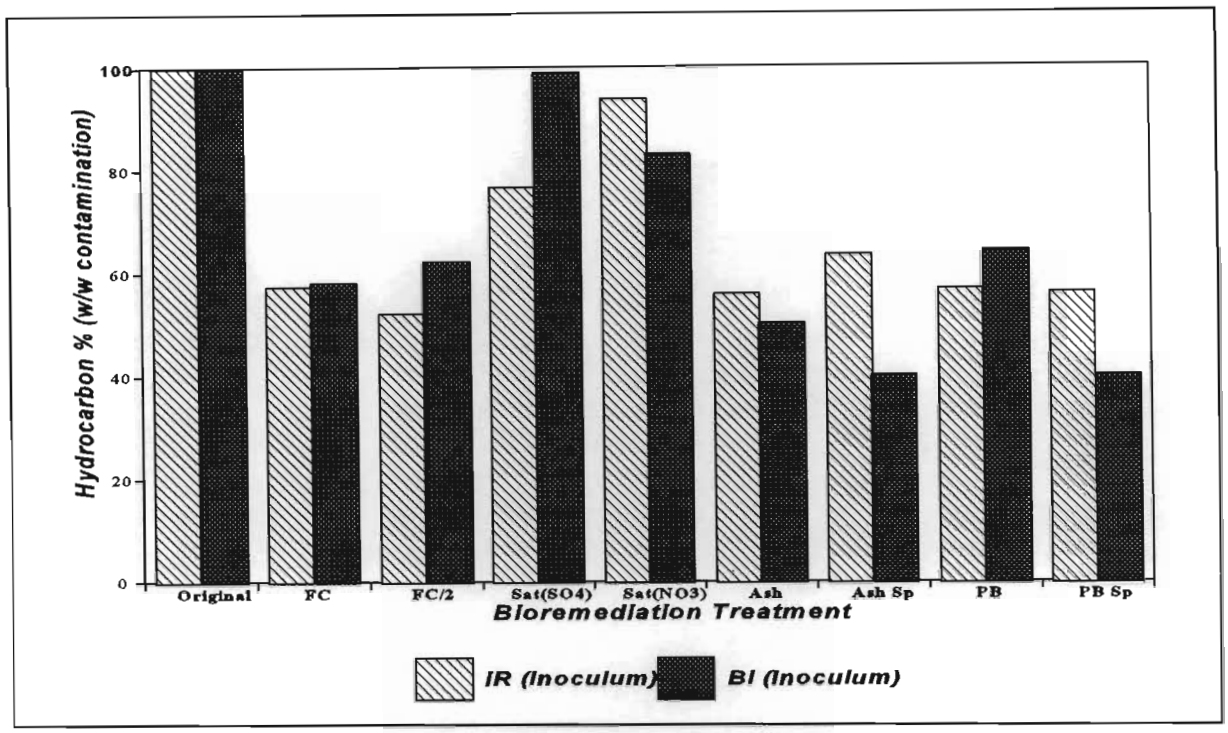


Figure 5.3: Comparison of soil hydrocarbon degradation as determined in different inoculated remediation treatments by the Biodegradation Index (BI) and infra-red spectrophotometry (IR) analysis.

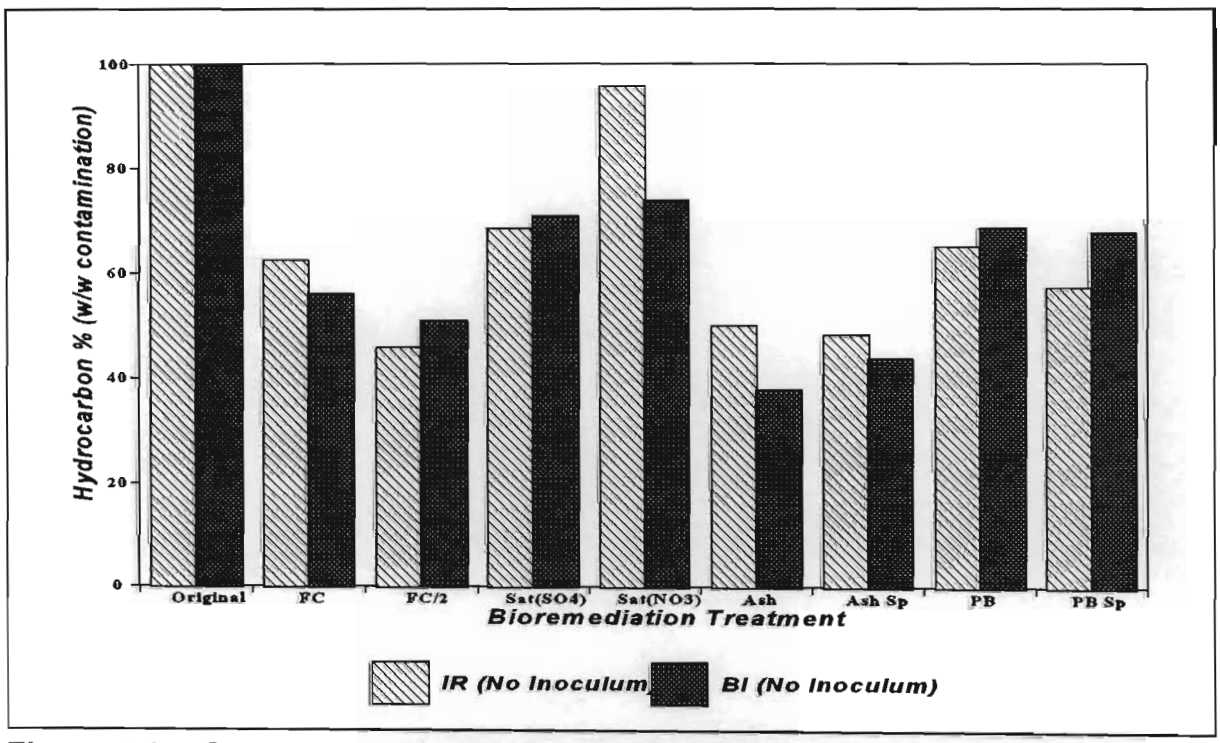


Figure 5.4: Comparison of soil hydrocarbon degradation for uninoculated soil treatments as determined by the Biodegradation Index (BI) and infra-red spectrophotometry (IR).

5.3.3 Biodegradation Index Following Soil Free System Treatments

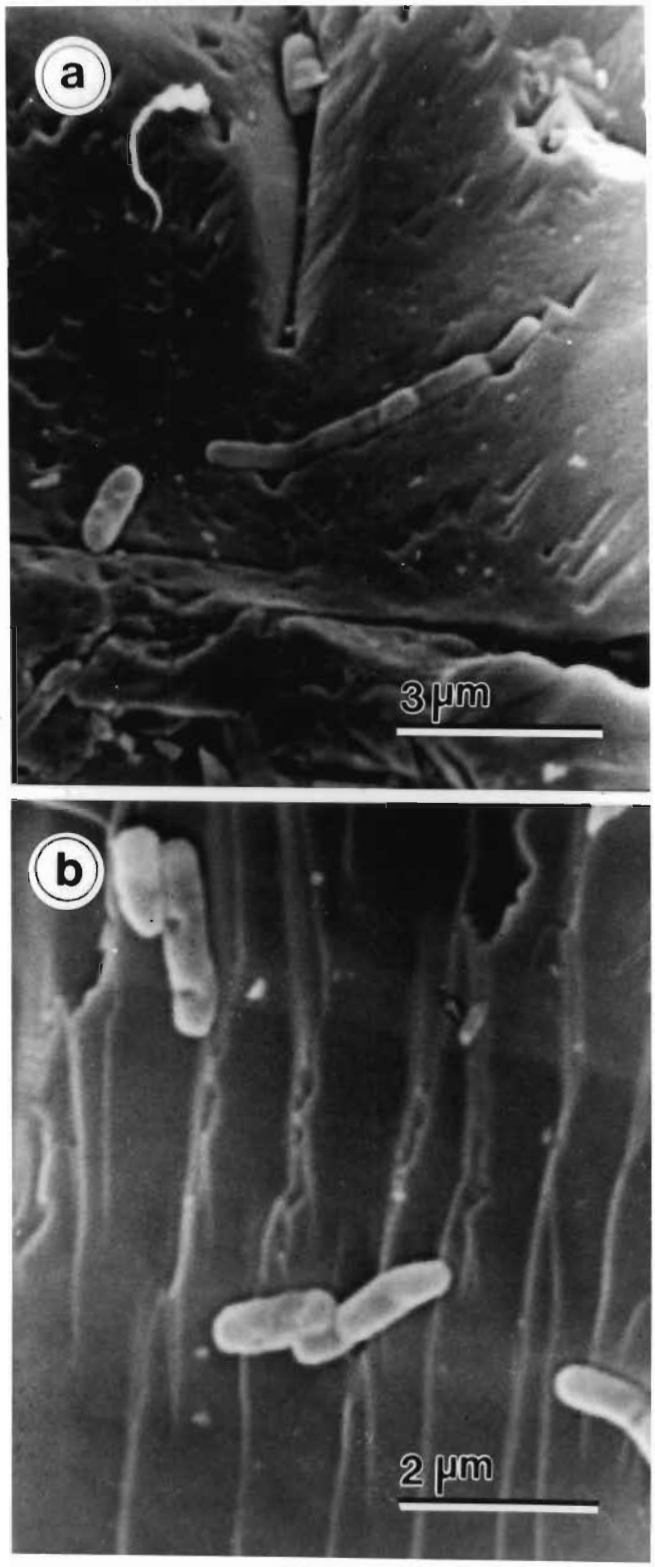
To further investigate the validity and usefulness of the Biodegradation Index, a study was made in a soil-free system. Because of the variability of hydrocarbon distribution in soil, the results obtained from soil analyses are often questionable. In addition, natural organics present in soil may affect the accuracy of the data obtained. To eliminate these potential problems, the Biodegradation Index was tested with hydrocarbon sludge (2.3) which was subjected to biodegradation in the absence of soil over several weeks.

Examinations of the glass wool and culture supernatant by electron and light microscopy showed the presence of microorganisms in both cases (Plate 5.1). As the flasks were incubated in the dark with the hydrocarbon as the sole carbon source, the results were taken to be indicative of hydrocarbon degradation. However, previous studies (Chapter 4) indicated that the hydrocarbon mixture was semi-recalcitrant which suggested that biodegradation (in this case) was slow.

Another factor which required attention was the uncertainty associated with determining the best elution time (associated with a specific hydrocarbon) which was required to calculate the Biodegradation Index. It was thus important to broadly establish the best time interval which could be further explored with more sophisticated equipment and exhaustive analysis. The Biodegradation Index was thus calculated with elution times associated with two marker hydrocarbons. Because biodegradability, generally, decreases with increasing hydrocarbon molecular weight, the best selected molecule would, theoretically, contain the highest carbon number i.e. C30. However, the hydrocarbon sludge used in this study did not include sufficient hydrocarbons in the > C30 range. Thus, a polynuclear aromatic hydrocarbon (pyrene C16, elution time 22.259 minutes) and a C23 alkane (elution time, 23.324) were selected as the test hydrocarbons to determine the area ratio.

Figure 5.5 shows the Biodegradation Index obtained for the hydrocarbon sludge after an

Plate 5.1: Electron micrographs showing microbial consortia on glass wool.


a
3 µm
b
2 µm

extended biodegradation treatment time while the gas chromatograms are shown in Figure 5.6. If the Index obtained after 13 weeks of treatment is considered as a possible spurious error then a trend can be observed. The Biodegradation Index decreased during the first 16 weeks which correlated with the expected pattern of labile hydrocarbon catabolism. However, between weeks 18 and 20, the Biodegradation Index indicated a reversal of the light to heavy hydrocarbon ratio to values which were similar to those determined at the start of the treatment. This observation was, therefore inconsistent with the natural progression of hydrocarbon catabolism where the labile (low boiling point) hydrocarbons would be expected to decrease with increased treatment time. A possible explanation may be gained by considering biodegradation for the whole hydrocarbon mixture profile. Initially, lower weight hydrocarbons were degraded, which resulted in a smaller measured area relative to the heavier hydrocarbon fraction and hence decreasing Biodegradation Index values (first 16 weeks of treatment). However, with increased treatment time, the biodegradation of selected heavier and long-chain hydrocarbon fractions would have resulted in fragmentation of these compounds over an extended time period. It is theorised that the presence of the resultant shorter chain compounds would, as a result, have skewed the area ratios towards the initial ratios and thus gave an Index which suggested little biodegradation after weeks 18 and 20. Whether this trend would change with extended treatment time to reflect the expected course of biodegradation is uncertain and could, perhaps, be determined by further studies over protracted time periods or with a readily labile hydrocarbon mixture. The use of contaminated hydrocarbon sludge during this study was not ideal as it had earlier been shown to be relatively recalcitrant (Chapter 4). However, it should also be noted that the hydrocarbon sludge was used for the purpose of providing continuity for the entire study.

To obtain a Biodegradation Index which was both accurate and reproducible, it was important to establish which hydrocarbon should be used in the area ratio determinations. In this study, the Index was calculated with, first, pyrene and then a C23 alkane. However,

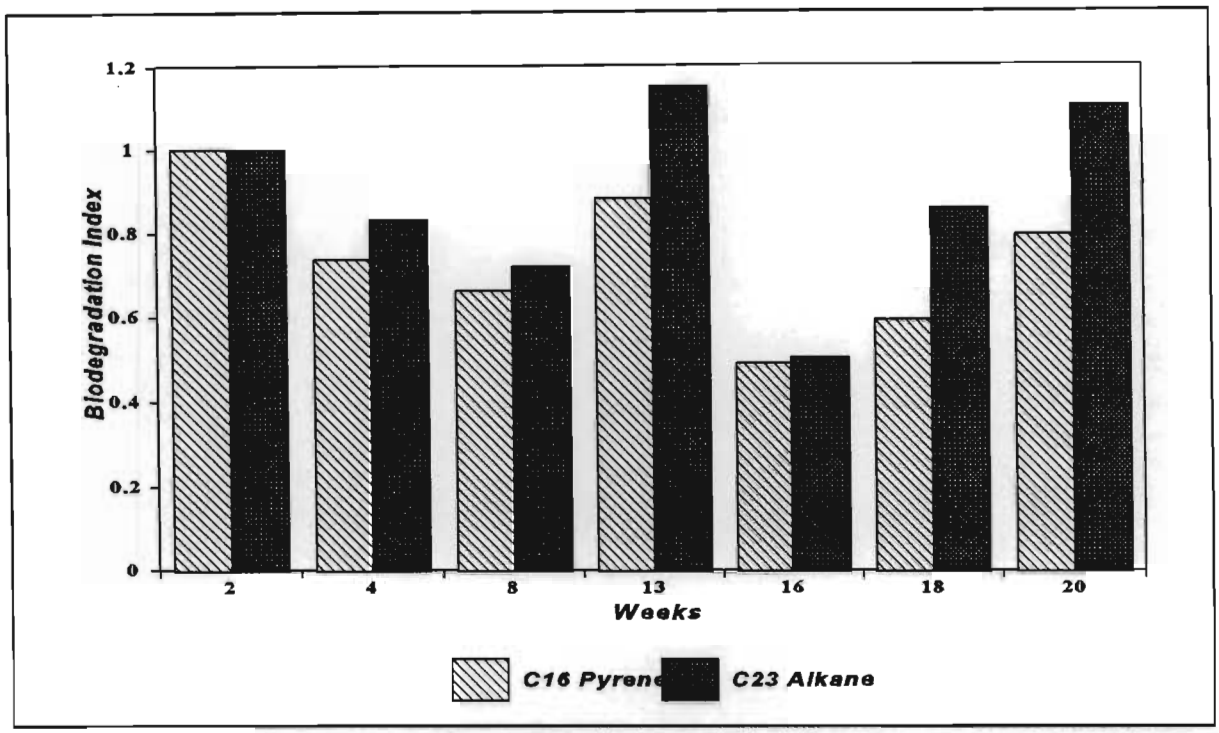


Figure 5.5: Comparison of hydrocarbon sludge degradation as determined by the Biodegradation Index with a C16 (pyrene) and a C23 alkane hydrocarbon.

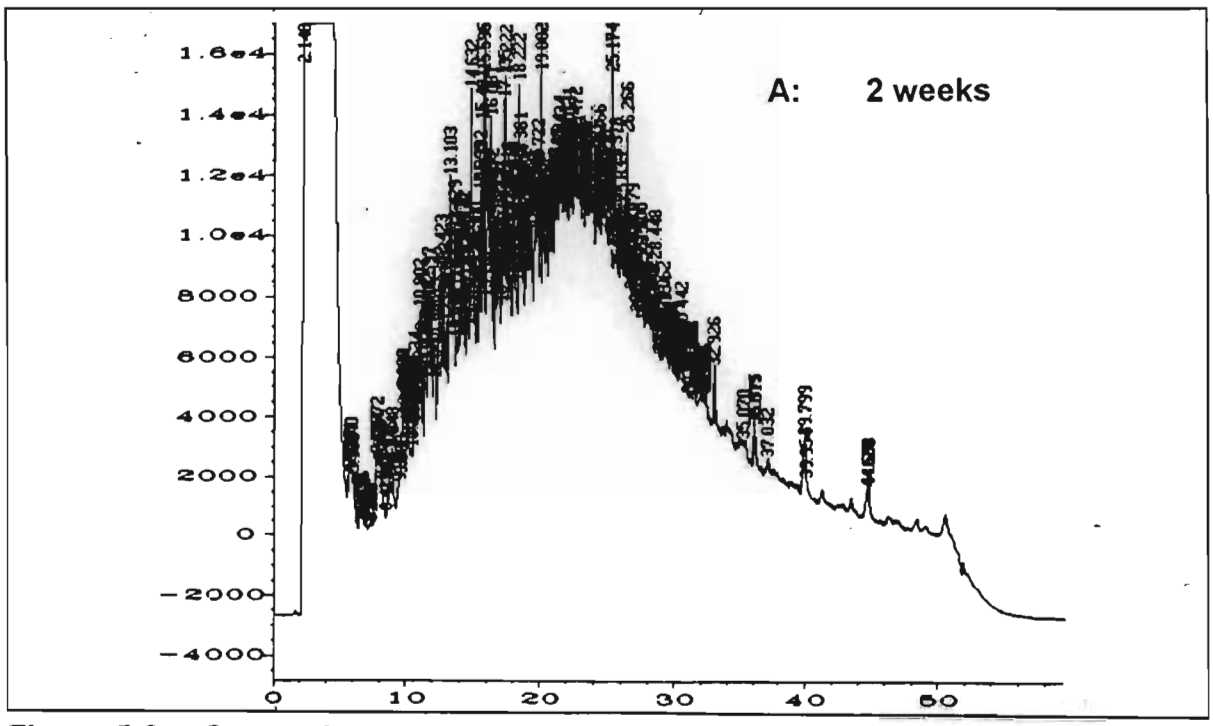


Figure 5.6: Comparison of gas chromatograms of hydrocarbon sludge after treatment of 2 (A), 4 (B), 8 (C), 13 (D), 16 (E), 18 (F) and 20 (G) weeks.

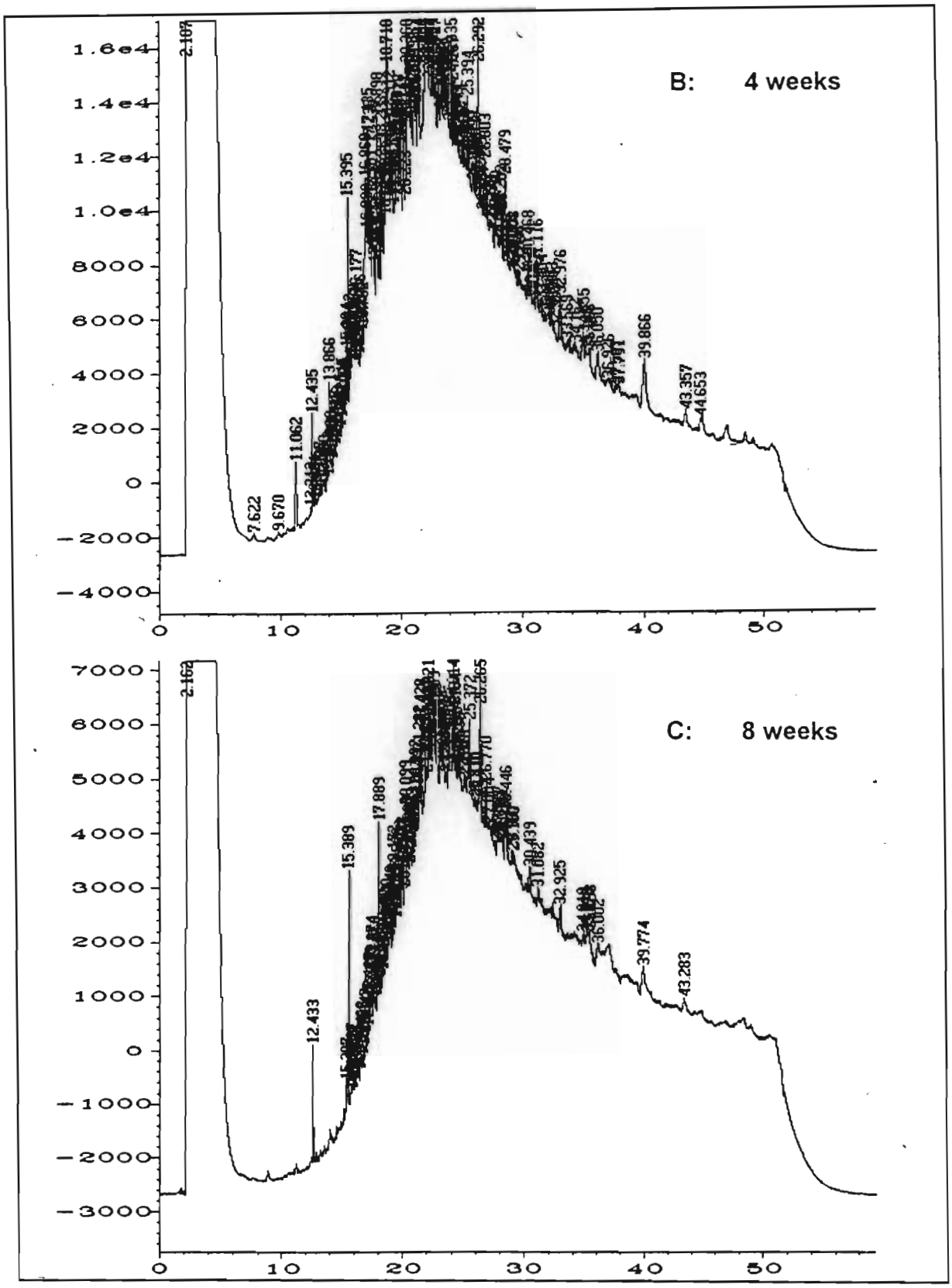
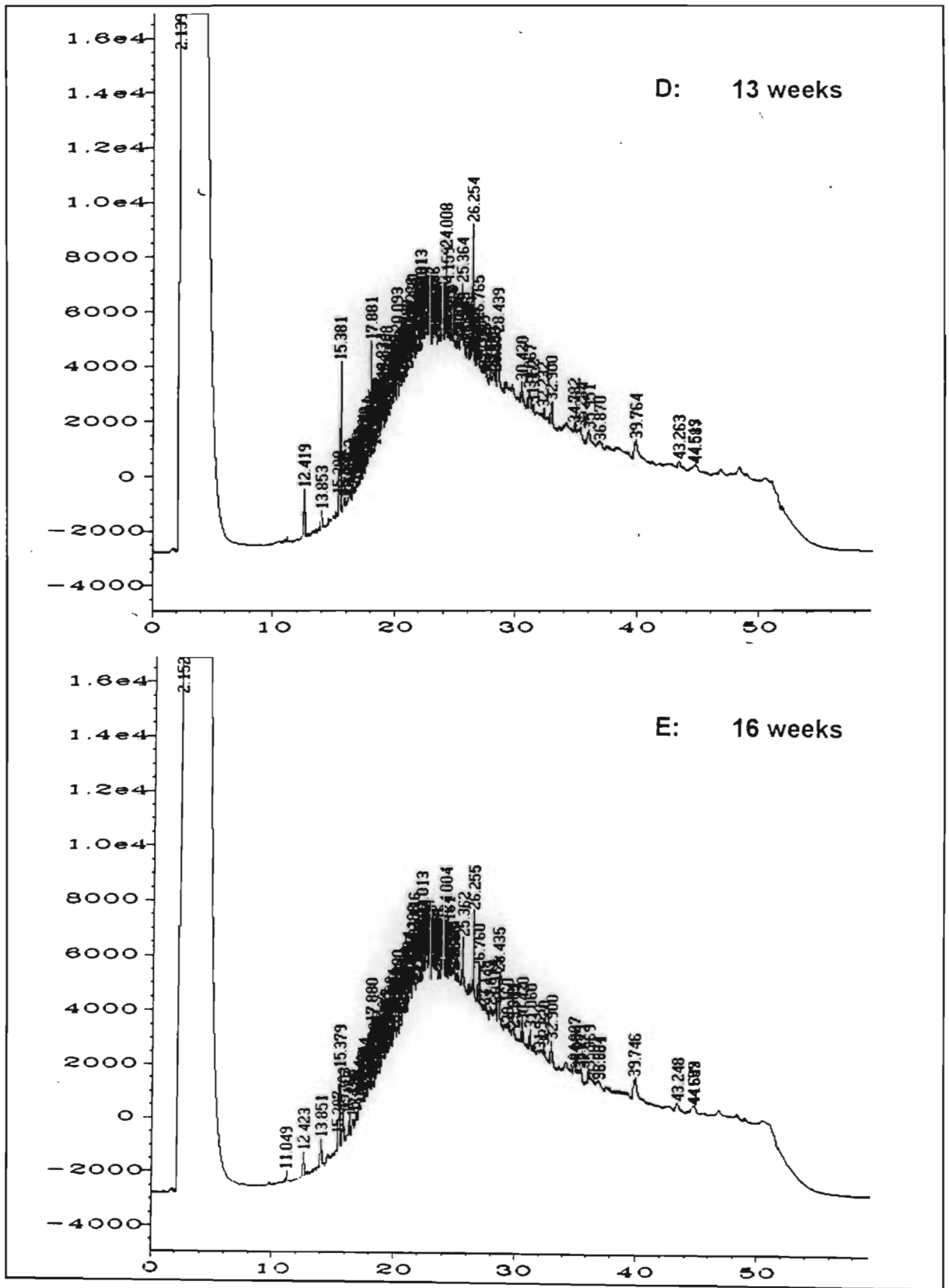


Figure 5.6 (continued): See previous legend.



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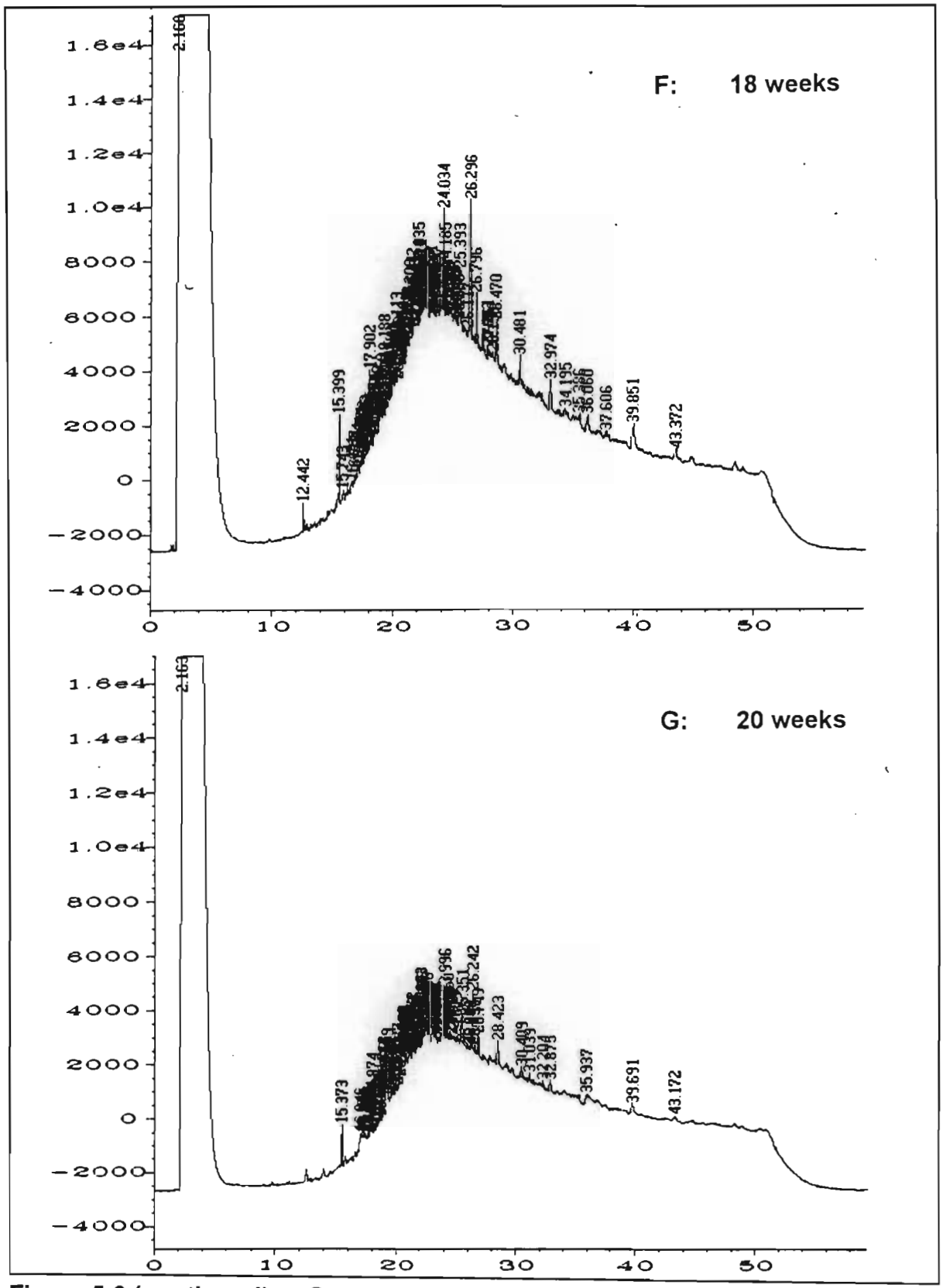

Figure 5.6 (continued): See previous legend.

the results obtained were inconclusive with respect to which marker gave the most accurate Biodegradation Index (Figure 5.5). It should be noted that the reference times to determine the Biodegradation Index were not fixed. The Indexes will change with different conditions and hydrocarbon mixtures and thus further experimentation is required.

Although no firm conclusion could be made on the validity of the Biodegradation Index, the data obtained suggested that the approach could be further developed, thus eliminating some of the problems associated with other analytical methods. An Index which has been validated has an advantage compared with other analytical techniques since it is unaffected by different contaminant concentrations which are often found in soil systems. However, much more work needs to be done, not only under various conditions but also with different hydrocarbon mixtures. In particular, the reference elution time is a point of contention since it was found that a variation between Index values resulted when the calculations were based on different reference retention times (Figure 5.5). This is considered to be the major stumbling block in the development of the Biodegradation Index. Future work should also focus on analysis made in conjunction with GC/MS analysis. This would allow specific hydrocarbons to be identified and the relative concentrations quantified.

It is also important to note that the Biodegradation Index could, potentially, be affected by leaching. Due to the relatively high water solubilities of the lower boiling point hydrocarbons, water movement through the soil may result in the selective leaching of the lighter hydrocarbons. This would give a Biodegradation Index which would incorrectly depict degradation. Although this problem could be overcome by employing a soil washing control it is still a factor which must be kept in mind during future monitoring studies.

It is hoped that with future work, the Biodegradation Index and the underlying principles involved, could be applied to hydrocarbon bioremediation studies. In spite of the obvious problems associated with the development of this method, it is believed by the author that

the Biodegradation Index offers potential advantages over other analytical techniques.

CHAPTER 6

HEAVY METAL- AND HYDROCARBON-SOIL INTERACTIONS

6.1 INTRODUCTION

An important factor to consider in the treatment of contaminated soil is the role that sorption plays in contaminant mobility. Adsorption to soil affects the volatilization, diffusion, leaching and availability of chemicals for microbial or chemical degradation (Riser-Roberts, 1992). Compounds which adsorb strongly onto solids are retarded in their movement through soils and aquifers. Sorption may occur if the compound of interest has a low affinity for water or high affinity for the surrounding solid material. The main subsurface solids responsible for adsorption include solid organic matter, clay minerals and amorphous minerals (Pettyjohn and Hounslow, 1983).

Movement of contaminants such as heavy metals and polyaromatic hydrocarbons is of particular concern because of their potential environmental toxicities (Pettyjohn and Hounslow, 1983). Knowledge of contaminant migration patterns through the soil and into groundwater is thus important.

The hydrocarbon sludge examined in this study was not only recalcitrant, but also contained high concentrations of heavy metals (Table A.3). It was a concern, therefore, that the rate of hydrocarbon migration through the soil could have been greater than the rate at which the hydrocarbons were degraded and removed from the soil through microbial action. This would then have increased the potential for groundwater contamination.

Where heavy metals and hydrocarbons are present in soil, the situation is exacerbated as the heavy metals are not susceptible to microbial degradation. In such cases, adsorption and accumulation within the microbial biomass are the main mechanisms of limiting heavy metal movement (Gadd and Griffiths, 1978). However, microorganisms

may also influence the local soil conditions by decreasing both the pH and the redox potential (Baker and Herson, 1994). Under such conditions heavy metal mobility may be promoted.

In certain instances, metal ions may form complexes with a number of microbial metabolites or organic ligands (chelation). The organic ligands mask the positive charges of the metal ions and reduce the ability to adsorb to the negatively charged clay particles (Francis, 1985; Wildung and Garland, 1985). Solubilities of the metals are, thus, increased which may potentially lead to groundwater contamination.

The object of this part of the research programme was to assess the adsorption behaviour of hydrocarbons and heavy metals in both contaminated and uncontaminated soil. In addition, factors such as the soil buffering capacity and the effects of pH were investigated as controlling factors of pollutant migration.

6.2 EXPERIMENTAL

6.2.1 Heavy Metal Adsorption Studies

Hydrocarbon contaminated soil (2.1) was placed into individual 100m/ Erlenmeyer flasks in increasing increments of 2g up to a final soil mass of 20g. A 20 m/ volume of metal solution, containing Mn, Zn, and Ni in the ratio 500:220:78 $mg/^{-1}$, was added to each flask. The flasks were sealed with rubber bungs and incubated at 25°C in the dark for 24 hours in a New Brunswick shaker incubator (70 rpm). All of the treatments were prepared in duplicate. Subsequently, the supernatant metal concentrations were analysed by atomic absorption spectrophotometry (2.8.2, 2.9.3). A duplicate experiment was then made with uncontaminated soil.

6.2.2 Hydrocarbon Adsorption Studies

Uncontaminated soil (2.1) was placed in individual 100m/ Erlenmeyer flasks in 2g

increments up to a mass of 20g as described in Section 6.2.1. A 20ml volume of water soluble hydrocarbon fraction was added to each flask. This fraction was obtained by equilibrating 1kg of contaminated soil in 2 litres of distilled water in the dark for 7 days at 30°C. The flasks were sealed with rubber bungs and incubated at 25°C for 24 hours in a New Brunswick shaker incubator (60 rpm). All of the treatments were prepared in duplicate. The liquid fraction hydrocarbon concentration was measured with a UV Spectrophotometer (PYE Unicam SP6-550) at a wavelength of 277nm. Due to the presence of natural soil chemicals which could cause absorbance at 277nm, supernatant, which was obtained by adding water to uncontaminated soil (1kg uncontaminated soil soaked in 2 litres distilled water for 24 hours), was used as a blank for each soil increment sample.

6.2.3 Soil Buffering Capacity

A mass of 30g uncontaminated soil (2.1) and 110ml distilled water were placed in a 150ml Erlenmeyer flask. Acid (1M HCl) was added to the soil suspension in 0.2ml increments. The soil slurry was continuously stirred and monitored for changes in pH.

6.2.4 Metal and Hydrocarbon Mobility in Soil

Treatments to ascertain the mobility of hydrocarbons and heavy metals were carried out in 60cm columns constructed from PVC pipes with an internal diameter of 4.5cm. The bottom of each column was closed with a silicone rubber seal which was equipped with a leachate outlet tube. Glass wool was used to prevent clogging of, and soil loss through the outlet. Each column was packed with 40cm uncontaminated soil which was added with continuous agitation on a vortex shaker. A further 10cm mixture of contaminated soil and hydrocarbon sludge (9:1 w/w dry soil:sludge) was added above the 40cm uncontaminated soil for each treatment. The uncontaminated soil was of the same type as the contaminated soil and was obtained from the same source (2.1). The columns were incubated at 25°C. A second set of columns for each treatment was built with the column lengths extended to 120cm. For these treatments the uncontaminated

soil depth in each column was 100cm. The columns were subjected to the treatments detailed in Table 6.1. All of the treatments involved the application of 70ml liquid (equivalent to 50mm "rainfall") each week. Leachate samples were continuously collected and the pH values determined with pH paper strips. The Mn and Zn concentrations were also monitored (2.9.3).

Table 6.1: Description of treatments during the course of the study.

Treatment	Description
A: Tap Water	Untreated tap water (pH 6.8).
B: Acid	Tap water with a pH adjusted to 4.5 with 1N HCl immediately prior to application. After 12 weeks the pH was decreased to 2 and further decreased to 1 after 18 weeks.
C: Nutrients	Nutrient medium containing: 0.5g KH_2PO_4, 2g NH_4NO_3, 0.2g $MgSO_4.7H_2O$, and 0.5 ml trace element solution [50% mixture of trace element solutions A and B (2.4.1)] in 1 litre distilled water;
D: Nutrients and Lime (Nuts+L)	Treatment D was similar to that of treatment C but in this case the uncontaminated soil was supplemented with 5.6g $CaCO_3$ kg^{-1} soil prior to column packing. (This is equivalent to approximately 27 tonnes of $CaCO_3$ per hectare when incorporated to a depth of 40cm and was to ensure a high pH under all test conditions).

After 28 weeks of treatment the 60cm columns were destructively sampled, divided into 5cm sections and the soil dried in a desiccator at 35°C for 48 hours. Because of an initial column sectioning error, the Water treatment column was divided into 7 cm

sections and dried as previously described. The soil samples were analysed gravimetrically (2.7.1, 2.9.2) for hydrocarbon content (adjusted for presence of extractable natural soil organics), pH (2.9.5), and Mn, Zn, Ni, Cd, Ca and Mg (2.8.1, 2.9.3).

6.3 RESULTS AND DISCUSSION

6.3.1 Hydrocarbon/Heavy Metal/Soil Adsorption Relationships

The adsorption characteristics of heavy metals onto the uncontaminated soil are shown in Figures 6.1, 6.2 and 6.3. The soluble concentrations of Mn, Zn and Ni were found to remain relatively constant at low soil mass. However, the soluble concentrations of all three metals were found to decrease with increasing uncontaminated soil mass after the initial equilibrium. This inferred that uncontaminated soil had the potential to adsorb some proportion of the aqueous phase heavy metals. In contrast, the soluble metal concentrations did not decrease when incubated in the presence of contaminated soil (Figures 6.4, 6.5 and 6.6). The aqueous phase zinc concentrations were, in fact, found to increase with increased contaminated soil mass (Figure 6.5). A similar trend was also observed for Mn. This lack of adsorptive capacity of the contaminated soil may be explained by the presence of hydrocarbons on the soil surfaces. A coating of hydrophobic oil sludge may have resulted in a reduction of the natural adsorptive capacity of the soil by covering the adsorptive sites and/or acting as a chelating agent. The net result would thus have been the prevention of adsorption of the heavy metals onto soil surfaces.

From the analysis of the hydrocarbon sludge which coated the contaminated soil, it was found that a high concentration of heavy metals, which included Mn, Zn and Ni, was present (Table A.3). Non-chemical or thermodynamic mediated release of Mn and Zn present in the hydrocarbon sludge and which coated the soil, may have accounted for the apparent increases observed in the soluble concentrations of these metals. The continued application of heavy metal containing sludge to a soil site which has been

previously contaminated with the same sludge is thus of concern. However, provided that a sufficient amount of uncontaminated soil is present below the contaminated soil layer, heavy metal migration may be attenuated to some extent. It should be also stressed that the attenuation of hydrocarbon migration will also depend strongly on the properties of the underlying soils and sediments.

Water soluble hydrocarbons, which were present in the aqueous phase, were found to decrease only slightly with increasing uncontaminated soil weight (Figure 6.7). These adsorption characteristics indicated that the ability of the uncontaminated soil to retard the movement of the soluble hydrocarbon fraction through the soil profile was limited. This is significant particularly as water soluble hydrocarbons pose the greatest environmental health risk. However, because of their hydrophobic nature, heavier hydrocarbons would be more likely to be retained in the upper soil horizon.

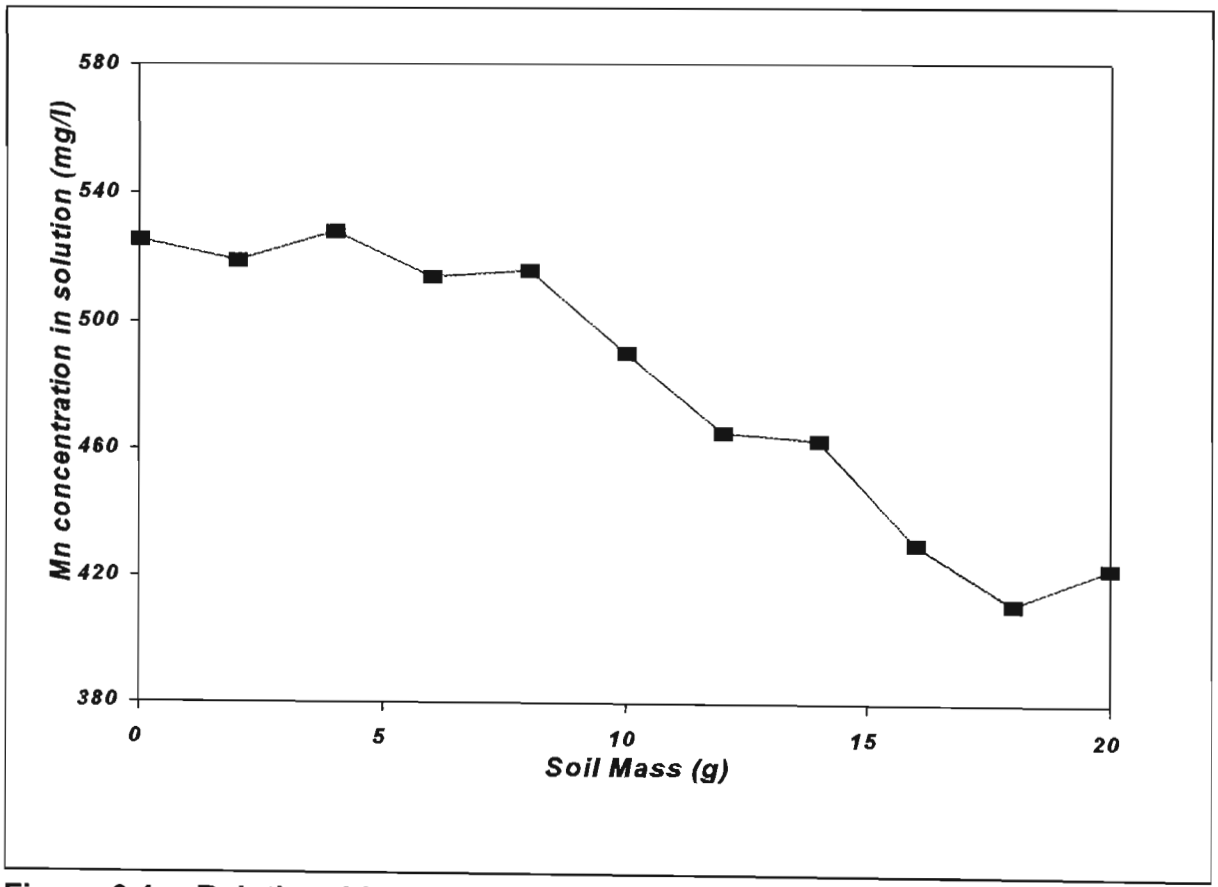

Figure 6.1: Relationship between aqueous Mn concentration and uncontaminated soil weight.

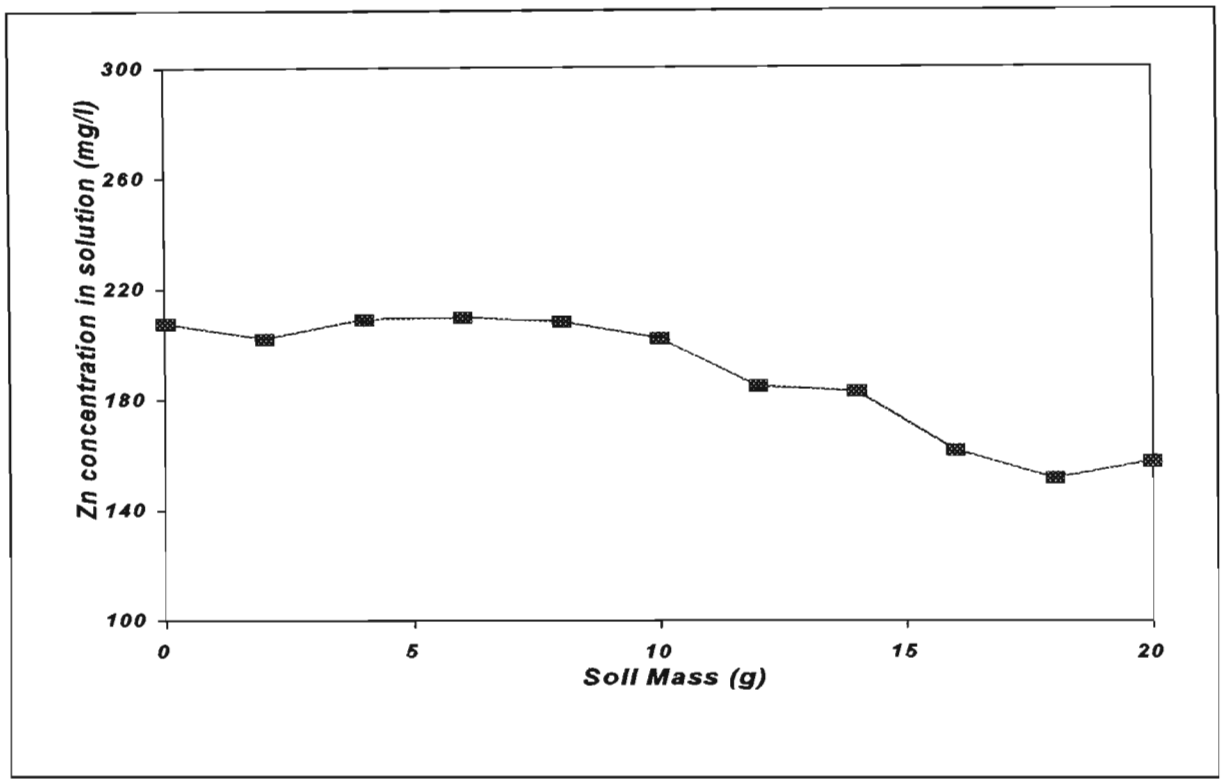

Figure 6.2: Relationship between aqueous Zn concentration and uncontaminated soil weight.

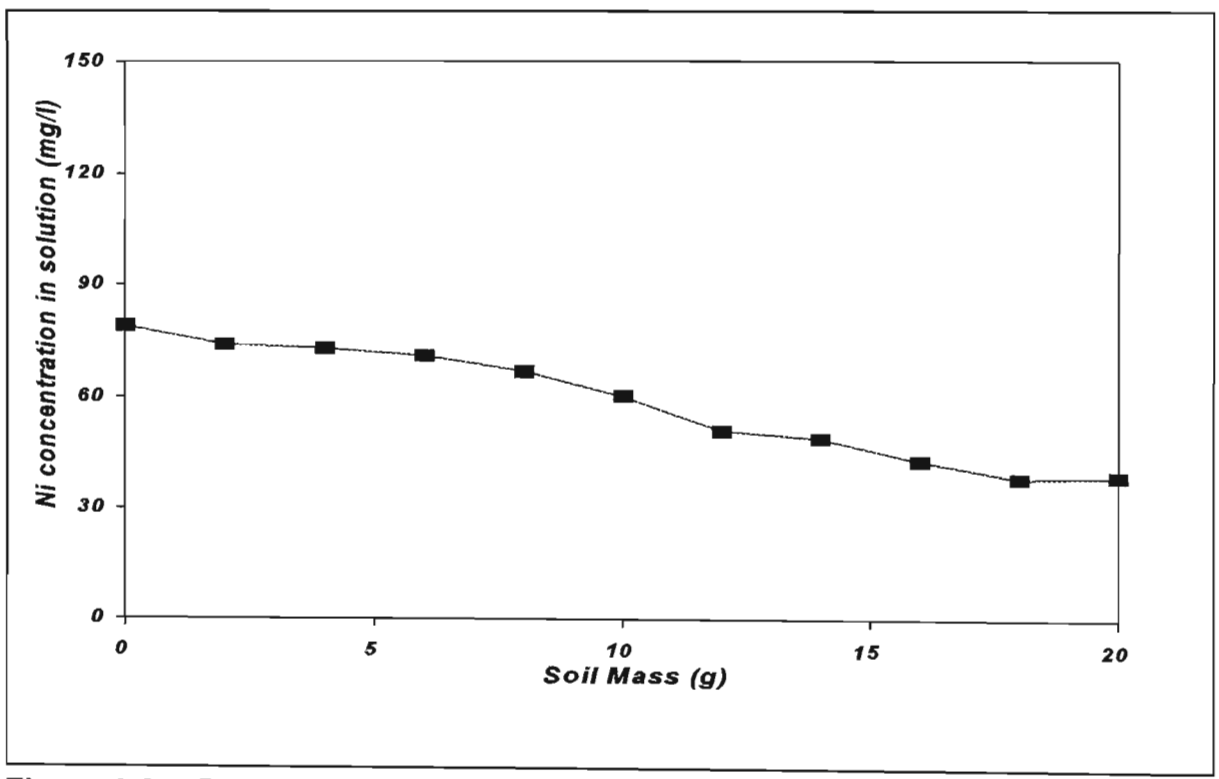

Figure 6.3: Relationship between aqueous Ni concentration and uncontaminated soil weight.

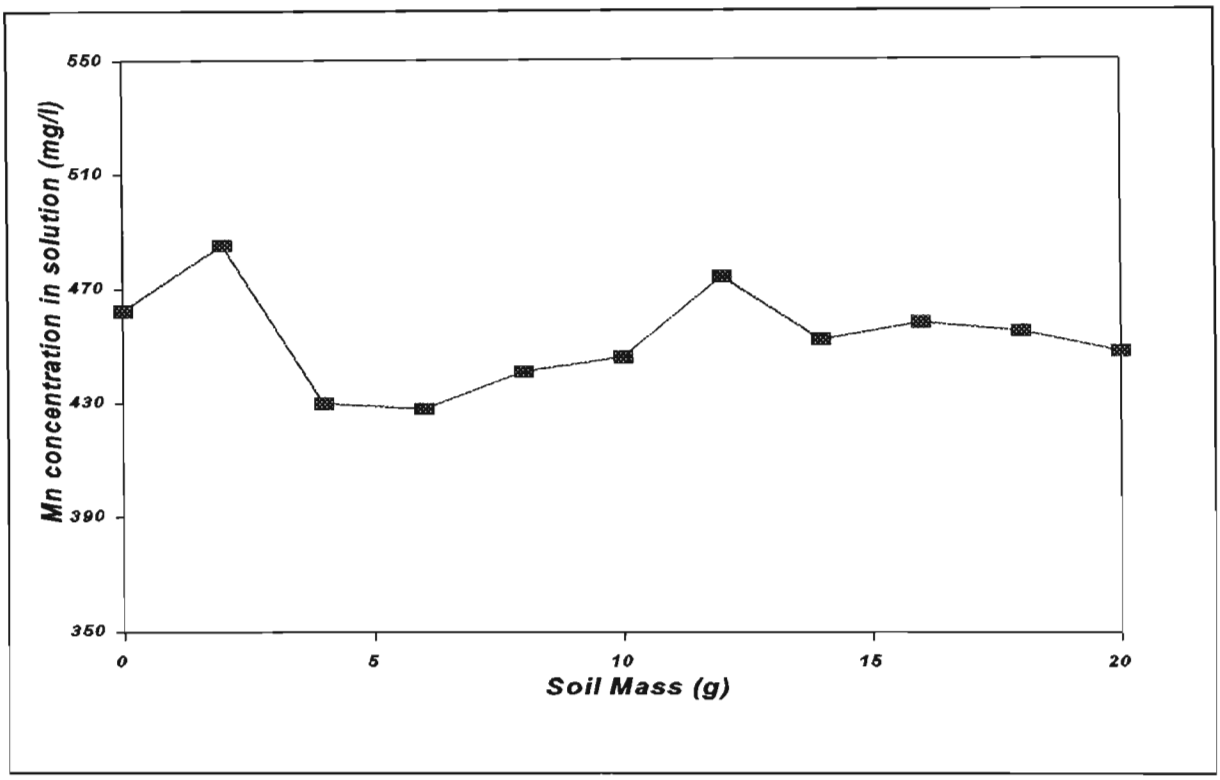

Figure 6.4: Relationship between aqueous Mn concentration and contaminated soil weight.

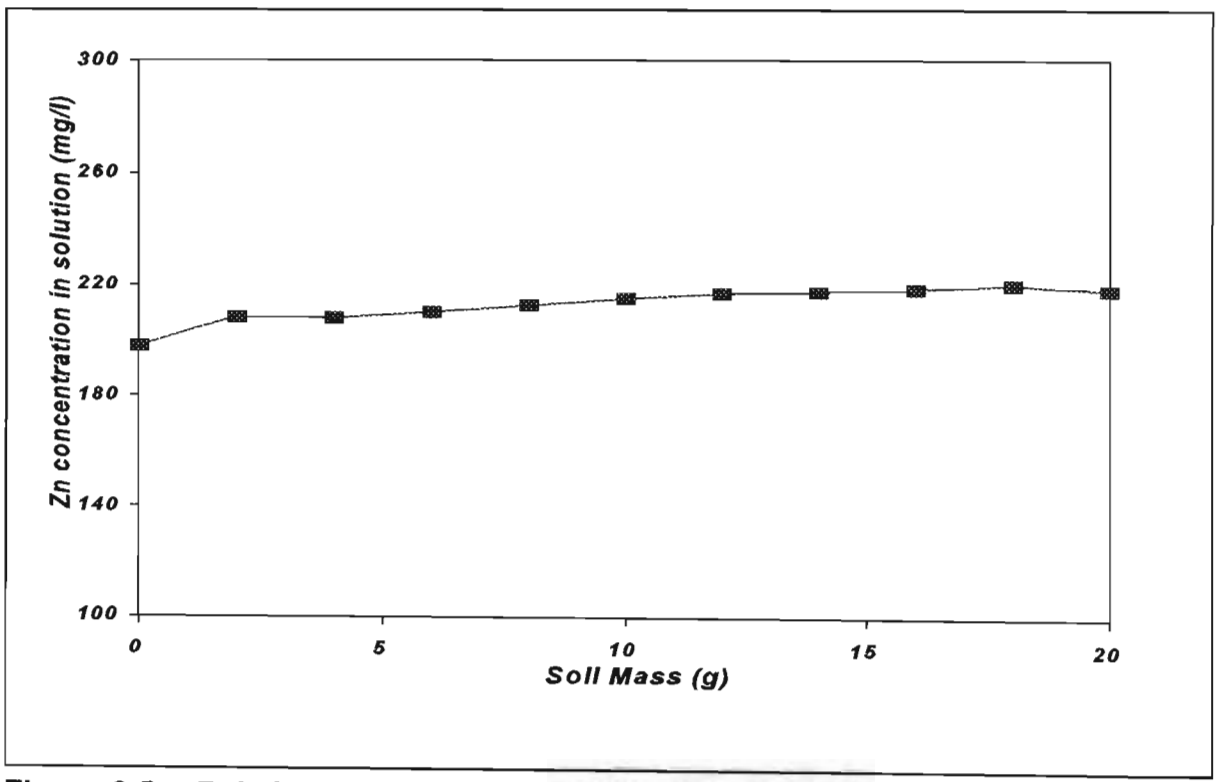

Figure 6.5: Relationship between aqueous Zn concentration and contaminated soil weight.

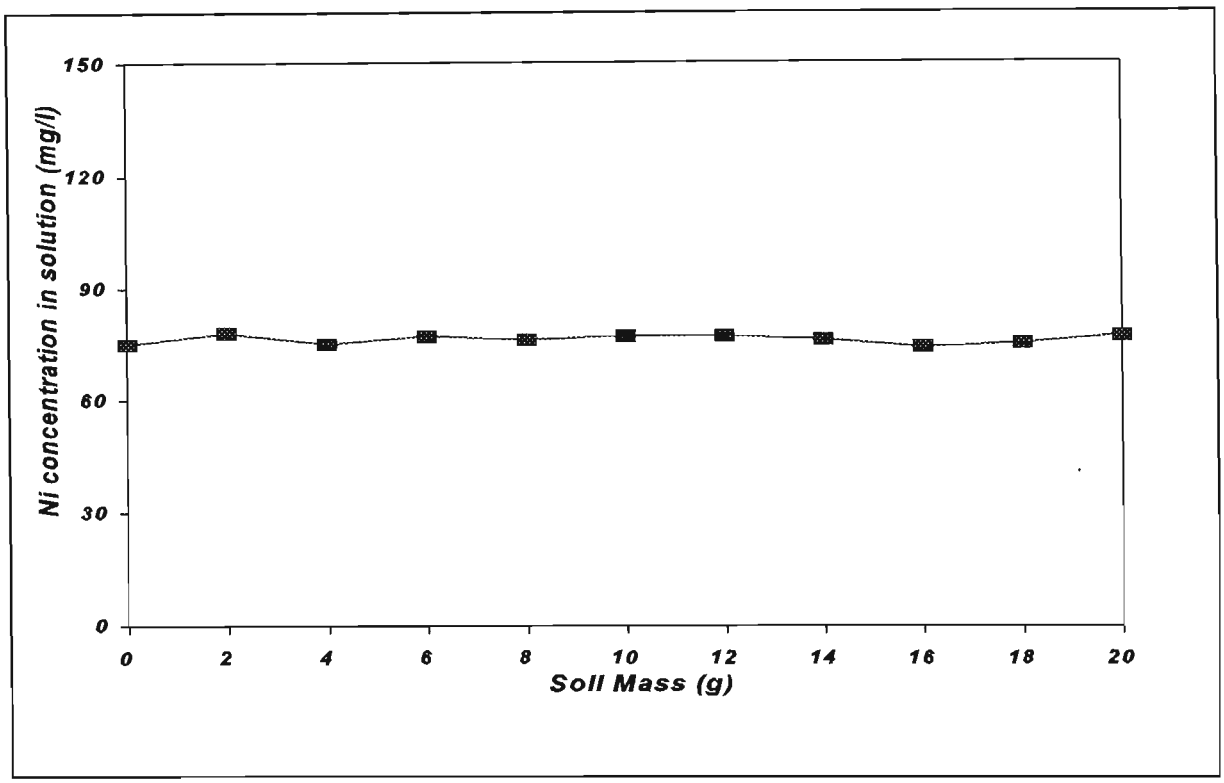

Figure 6.6: Relationship between aqueous Ni concentration and contaminated soil weight.

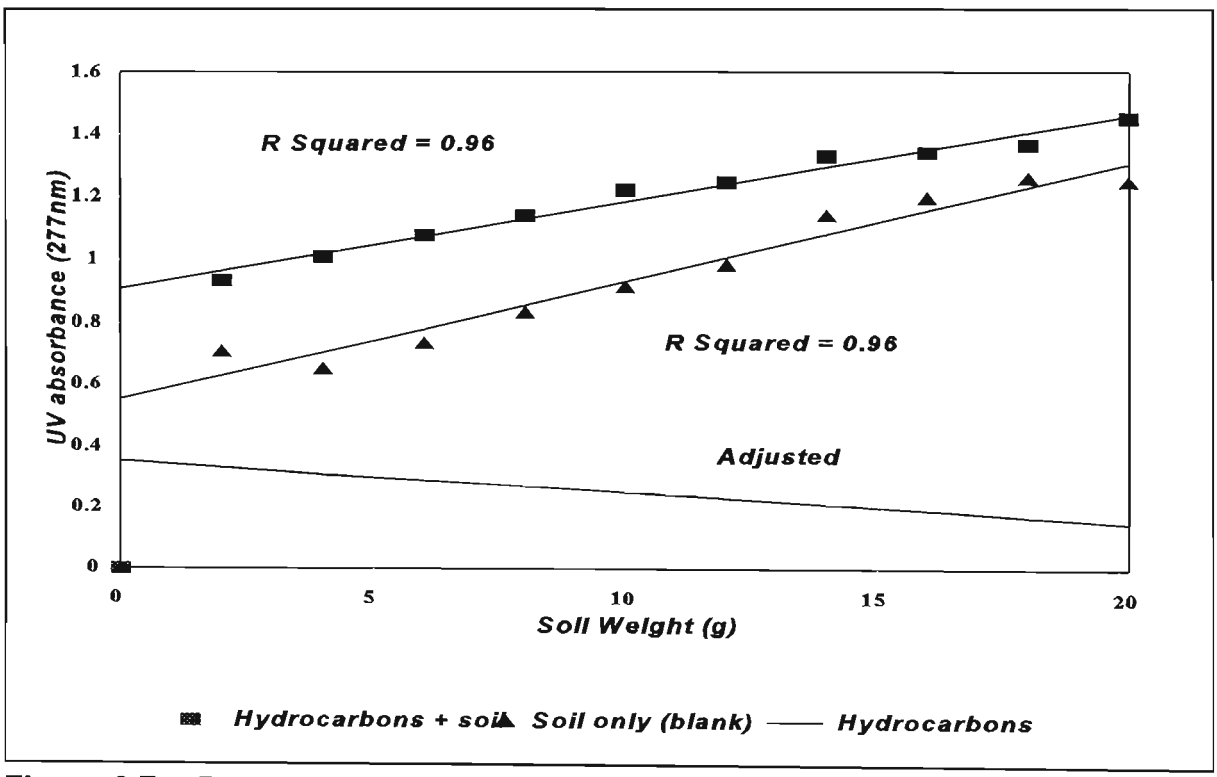

Figure 6.7: Relationship between water soluble hydrocarbon fraction and increasing uncontaminated soil weight after adjustment for background soil interference effects.

6.3.2 Soil Buffering Capacity

The soil's buffering capacity is shown in Figure 6.8. The best buffering appeared to occur at a pH of 4.7. The buffering capacity was exceeded after 3.2m/ HCl had been titrated. At this point, the total H^+ concentration of the soil was calculated to be 10.6 $cmol_c kg^{-1}$. This value is high when compared to the total cation exchange capacity (CEC) of the soil which was found to be 9.9 $cmol_c kg^{-1}$ (Table A.1). It should also be noted that the CEC determinations were made at a pH of 6.5 which was higher than the pH of 3.8 which was reached after addition of 3.2m/ of HCl. It could thus be assumed that the soil CEC at pH 3.8 was lower than at pH 6.5. The H^+ concentration of 10.6 $cmol_c kg^{-1}$ thus exceeded the total CEC of the soil. Under such circumstances, buffering should not have occurred and it may be concluded that the soil buffering capacity was not simply related to the CEC. This was later confirmed by the observation of excessively high Ca concentrations (Section 6.3.3, Table 6.2).

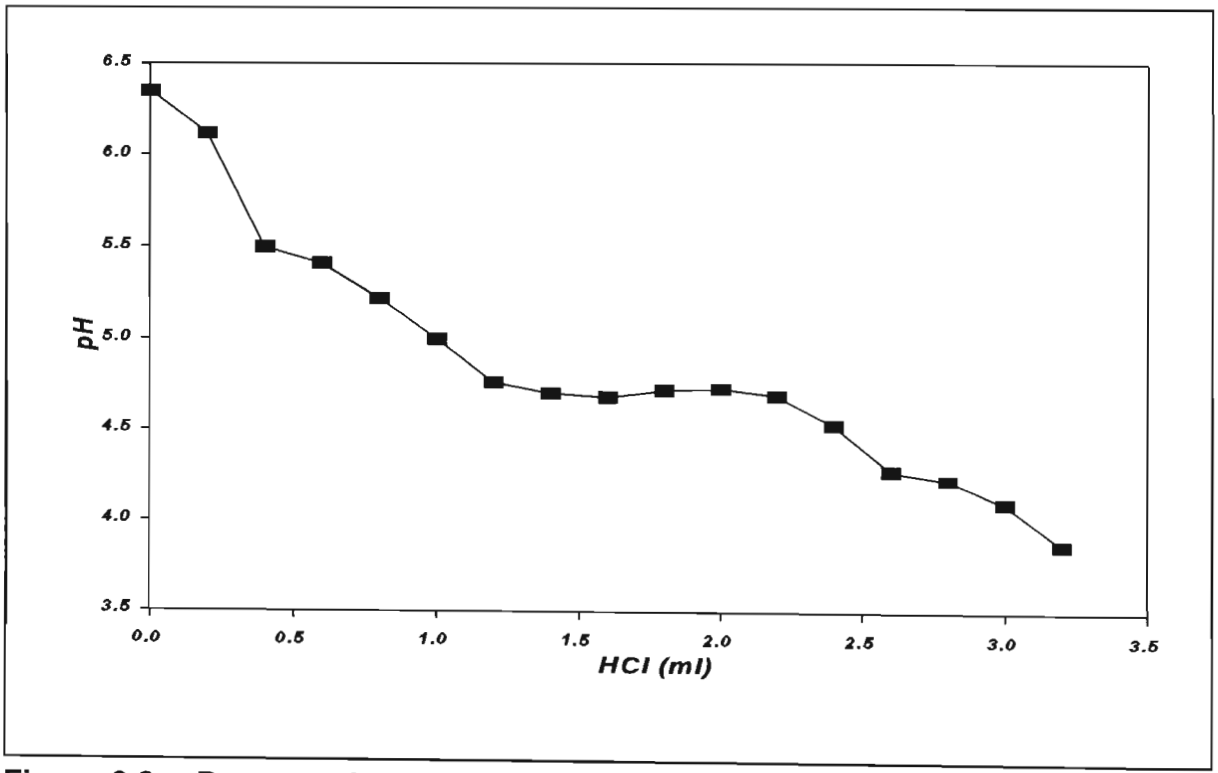

Figure 6.8: Decrease in soil slurry pH with addition of 1M HCl to the uncontaminated soil.

The soil buffering capacity is an important factor as metal mobility is directly related to increased acidity as a result of microbial hydrocarbon degradation. Consequently, the buffering capacity of the uncontaminated soil below the polluted layer should aid in the prevention of heavy metal migration.

6.3.3 Heavy Metal and Hydrocarbon Migration

In the initial design of the column experiments, allowance was made for the possibility that the heavy metal or hydrocarbon constituents would migrate further than 60cm. To account for this possibility 120cm columns were also built. However, after dismantling and analysing the soil of the 60cm columns, it was found that pollutant migration did not exceed the 60cm soil depth. Thus, it was only necessary to consider the soil analysis data of the 60cm soil columns.

For all treatments, an increase in soil pH was found to have occurred in the first 15-20cm of the soil profile (Figures 6.9, 6.10, 6.11, 6.12, 6.13A). The fact that the tap water control also had an initial low pH indicated that the subsequent pH increases were not directly due to the different treatments. The changes of pH with soil depth probably resulted from the buffering effects discussed in Section 5.3.2. This buffering capacity may have been further enhanced by the formation of $CaCO_3$ (Alloway, 1990) as a result of the high concentrations of Ca present in the soil (Table 6.2). The initial low soil pH values recorded in the Water (A), Nutrient © and Nutrients + Lime (D) treatments, could be attributed to microbial activity, while the hydrochloric acid was the most likely cause for the low pH in treatment (B). The rapid increases in pH observed in the first 15-25cm of each treatment were most likely as a result of the buffering capacity of the soil.

Table 6.2: Initial heavy metal concentrations and soil pH of uncontaminated soil before treatment.

pH(KCl)	6.78
Zn	66 mg kg^{-1}
Mn	12 mg kg^{-1}
Ca	2450 mg kg^{-1}
Mg	157 mg kg^{-1}
Cd	0.2 mg kg^{-1}
Ni	2.8 mg kg^{-1}

The biologically available heavy metal concentrations, determined after extraction by the Ambic method (2.8.1), and the relative pH values at discrete soil depths for the various treatments are shown in Figures 6.9, 6.10, 6.11 and 6.12. It was found that, in general, for all the treatments, heavy metal migration from the upper layer of contaminated soil and the hydrocarbon sludge into the uncompromised soil had occurred. Because of the effect that pH has on the relative mobility on heavy metals, the pH values are also shown in these figures.

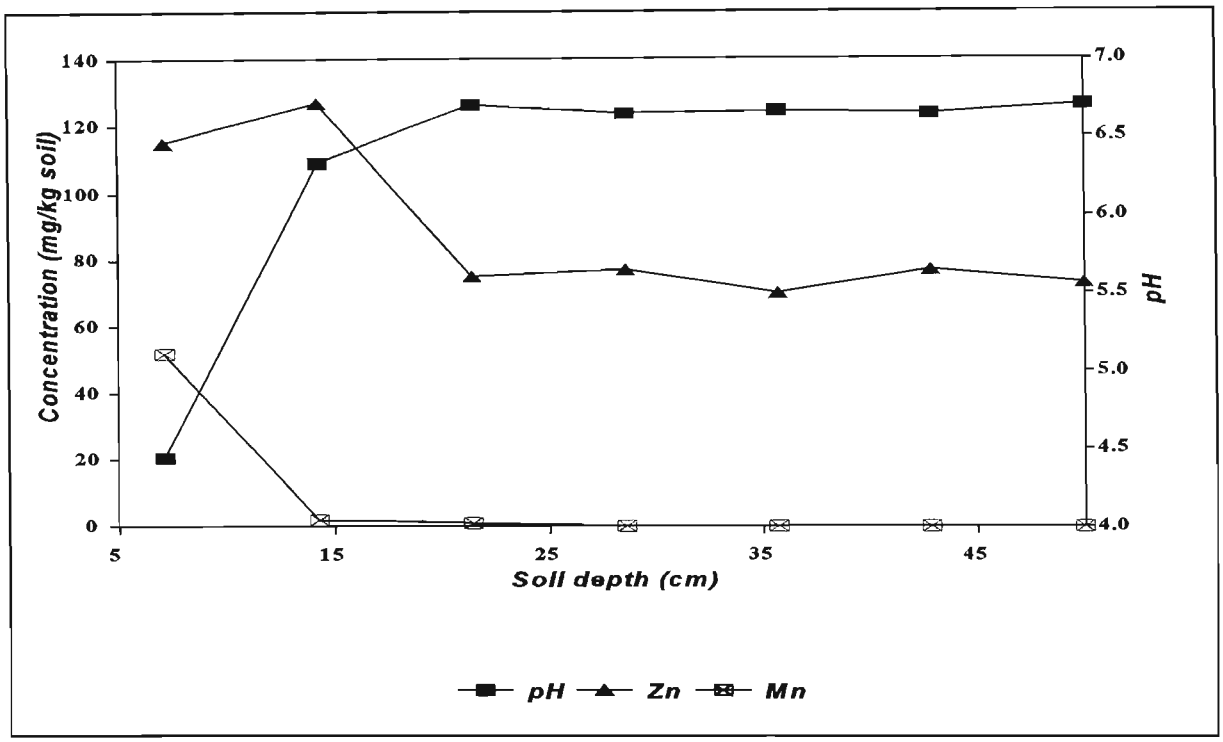

Figure 6.9A: Changes in pH, and Mn and Zn concentrations with soil depth for the Water treatment.

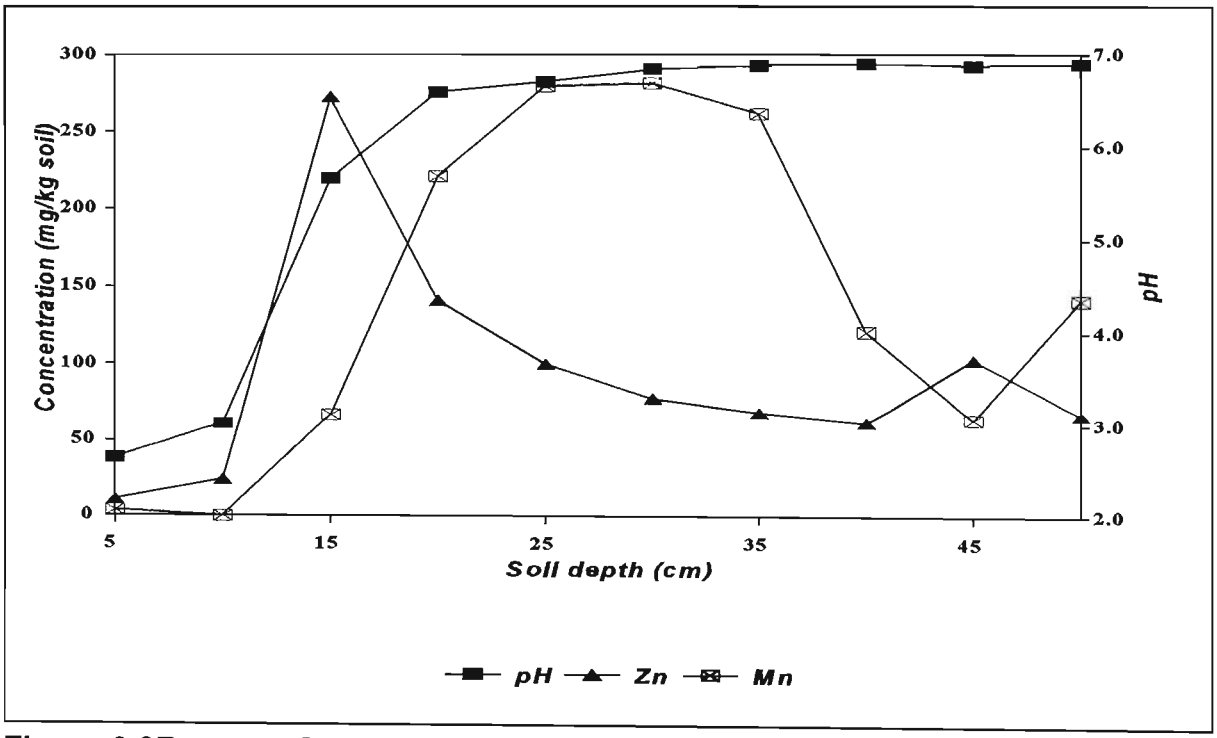

Figure 6.9B: Changes in pH, and Mn and Zn concentrations with soil depth for the Acid treatment.

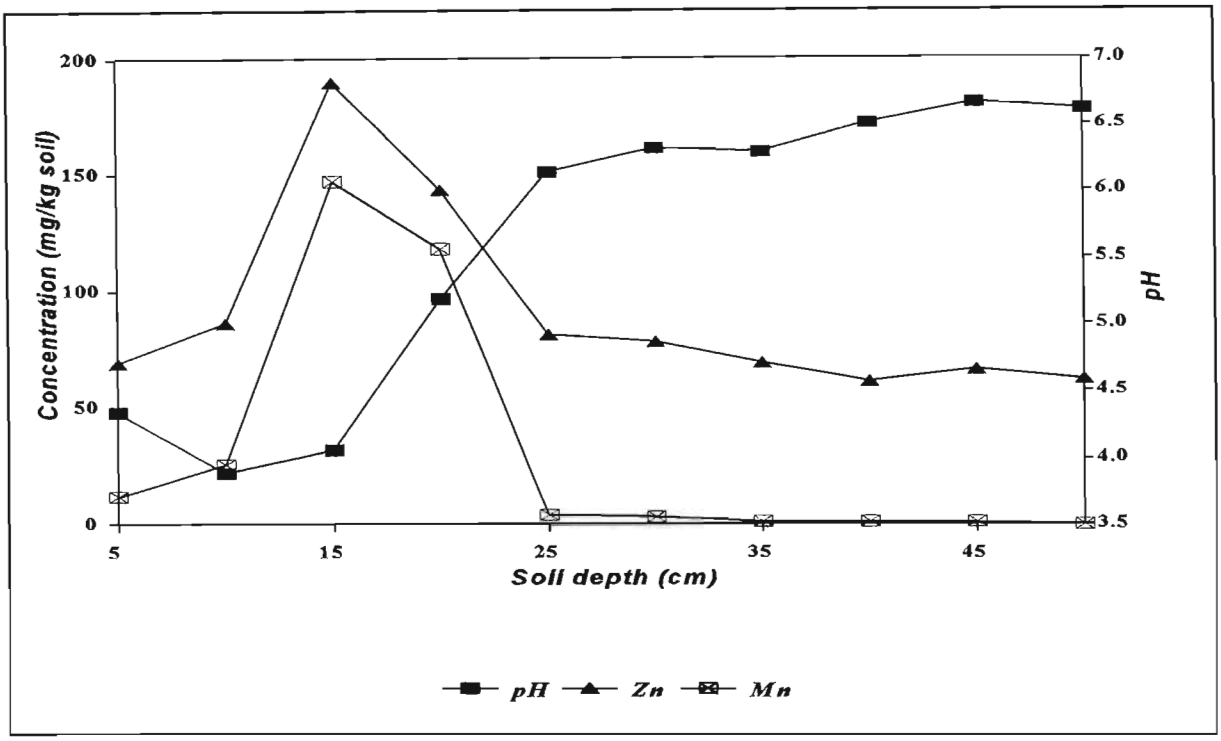

Figure 6.9C: Changes in pH, and Mn and Zn concentrations with soil depth for the Nutrients treatment.

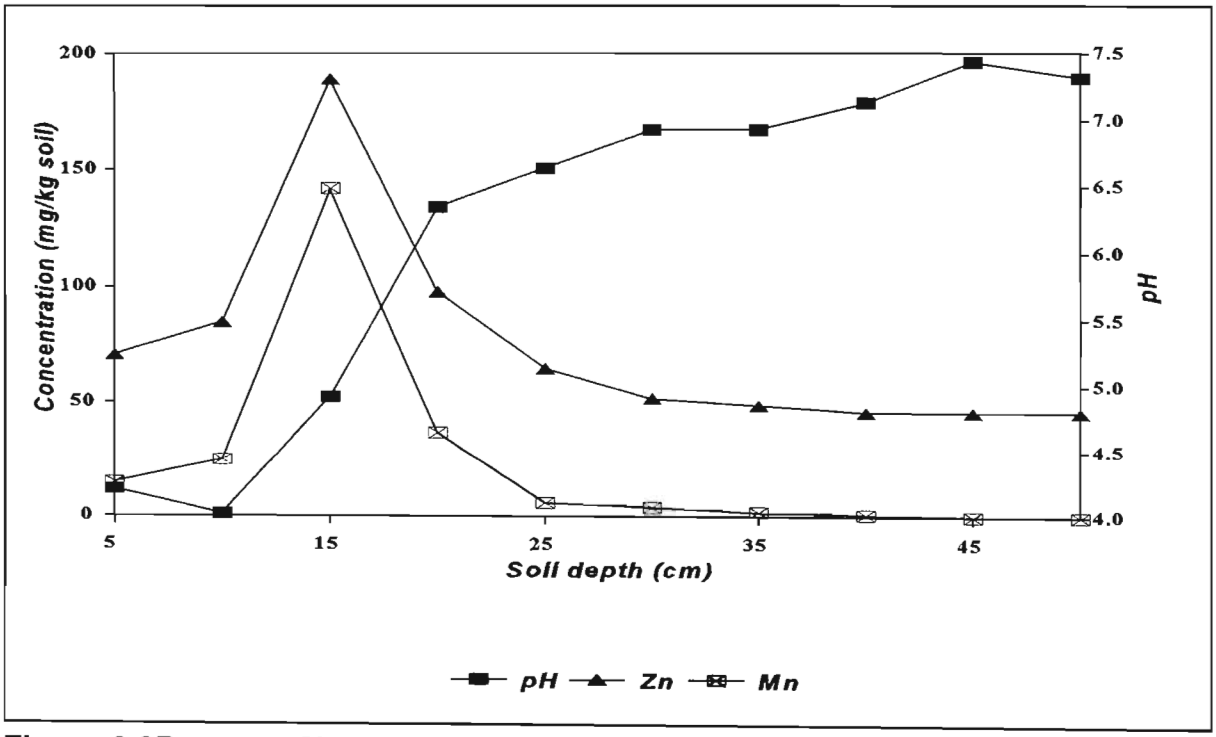

Figure 6.9D: Changes in pH, and Mn and Zn concentrations with soil depth for the Nutrients+Lime treatment.

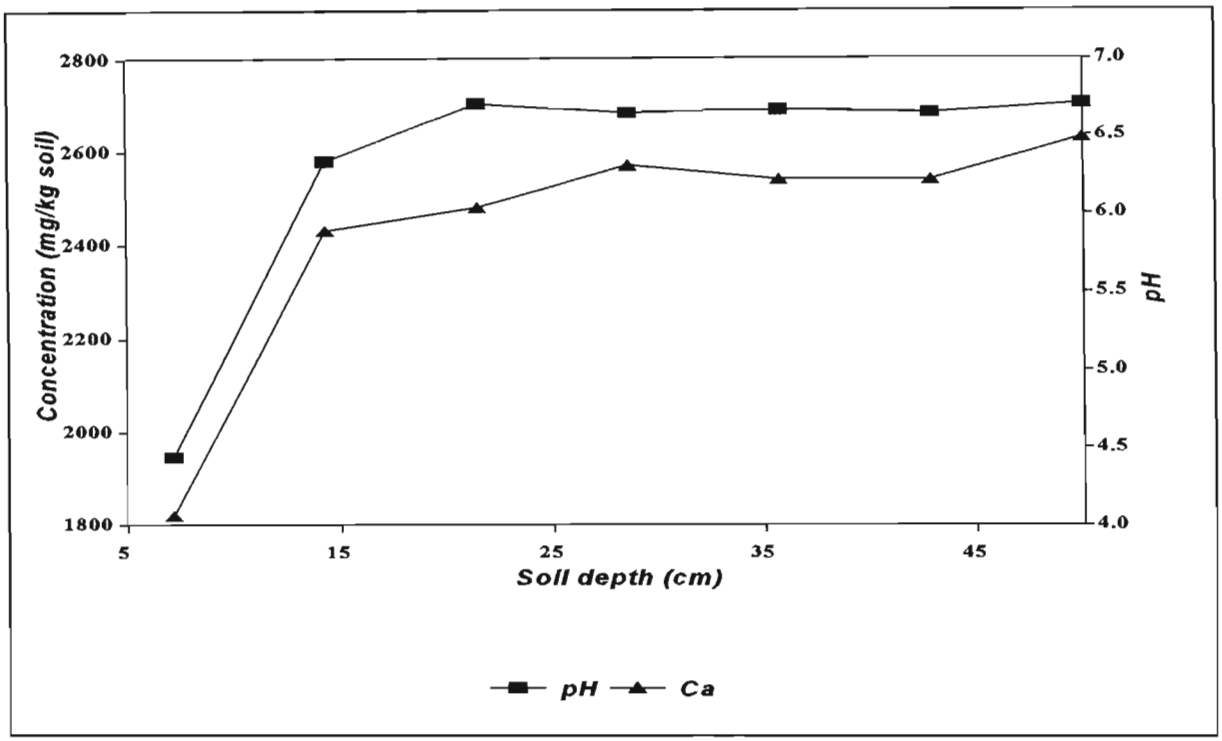

Figure 6.10A: Changes in pH and Ca concentrations with soil depth for the Water treatment.

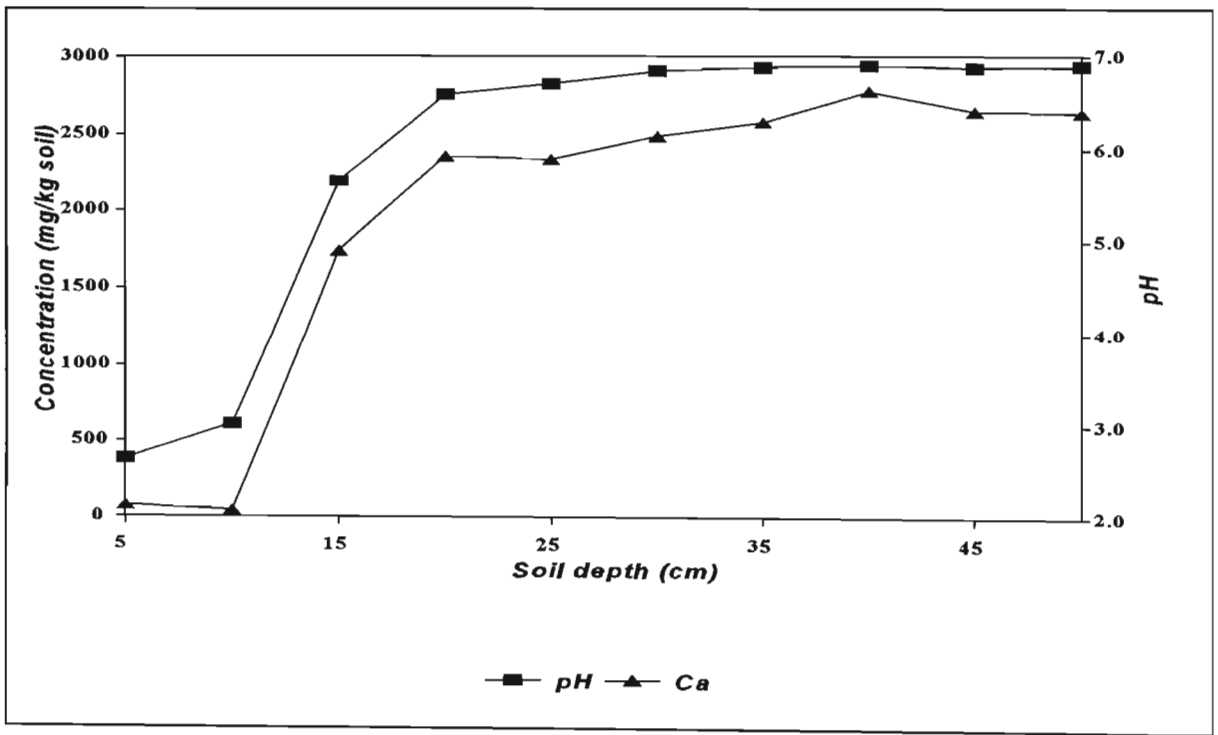

Figure 6.10B: Changes in pH and Ca concentrations with soil depth for the Acid treatment.

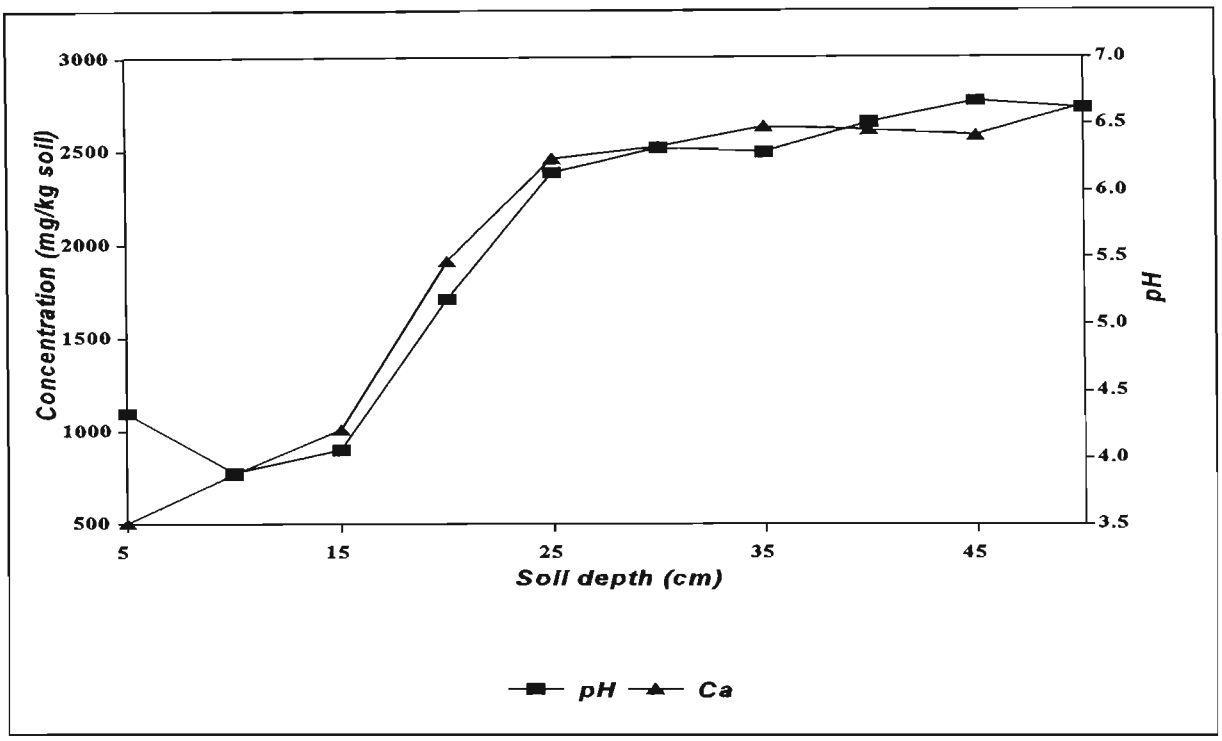

Figure 6.10C: Changes in pH and Ca concentrations with soil depth for the Nutrients treatment.

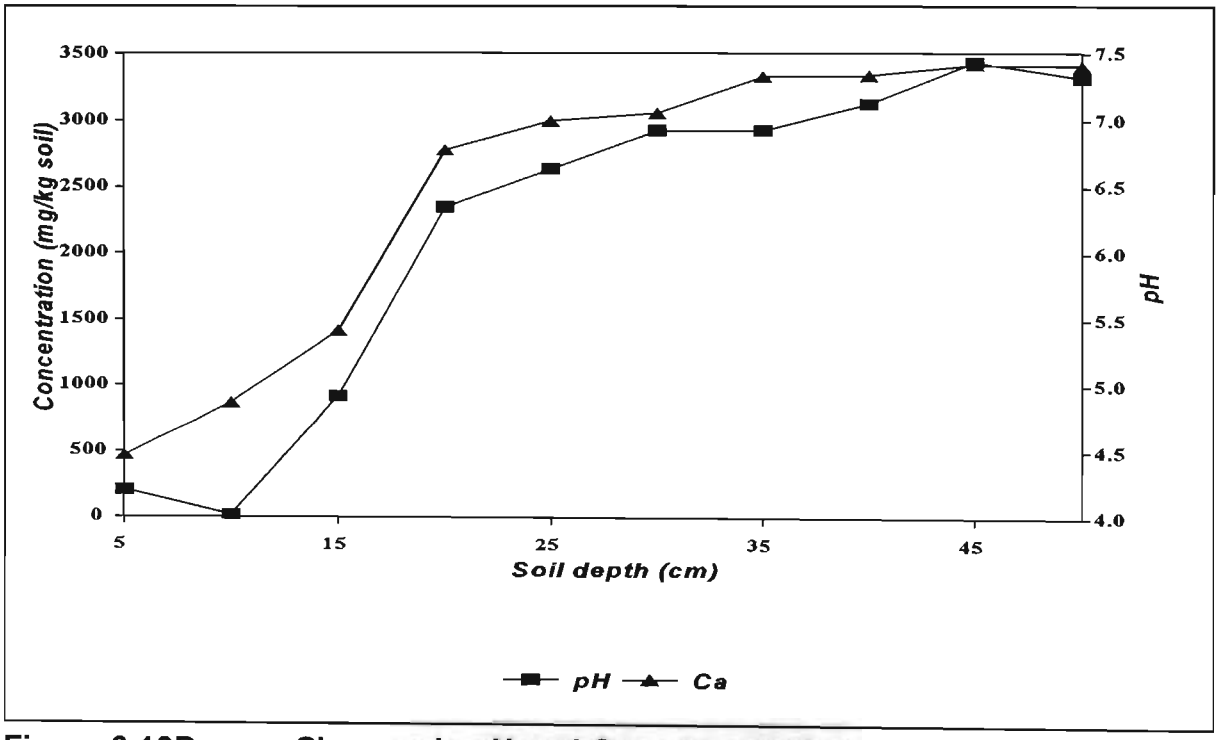

Figure 6.10D: Changes in pH and Ca concentrations with soil depth for the Nutrients+Lime treatment.

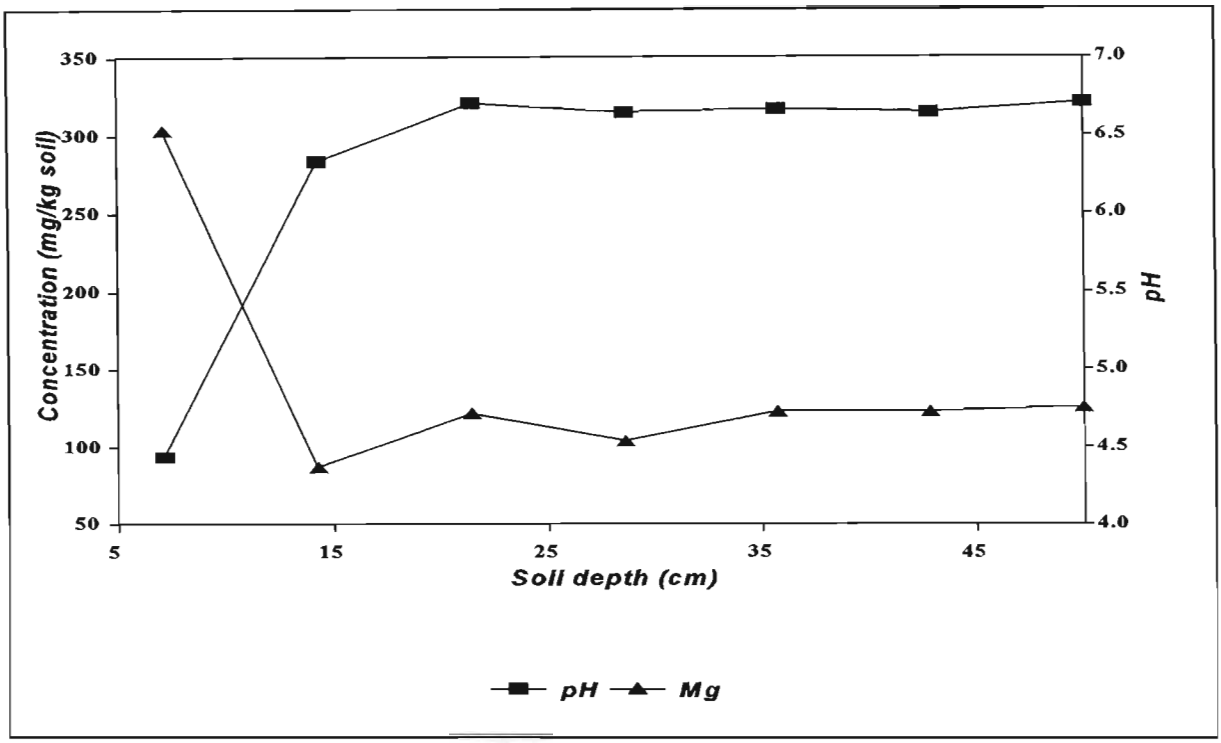

Figure 6.11A: Changes in pH and Mg concentrations with soil depth for the Water treatment.

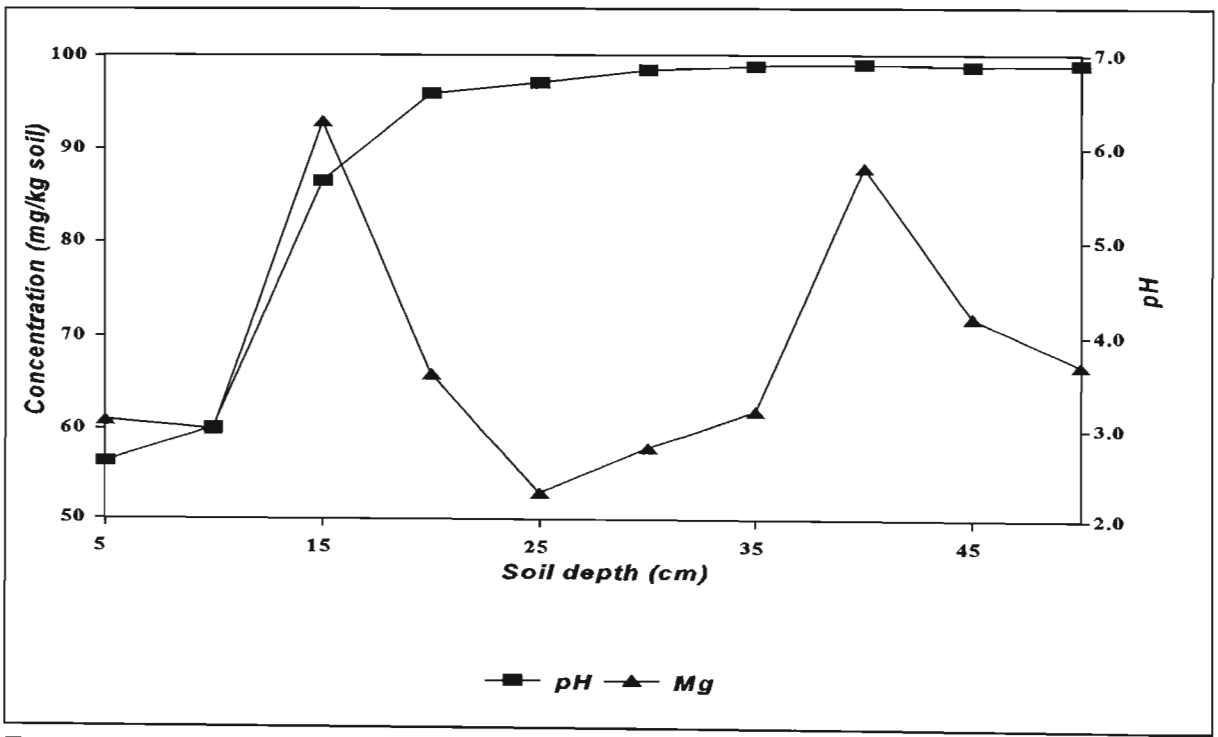

Figure 6.11B: Changes in pH and Mg concentrations with soil depth for the Acid treatment.

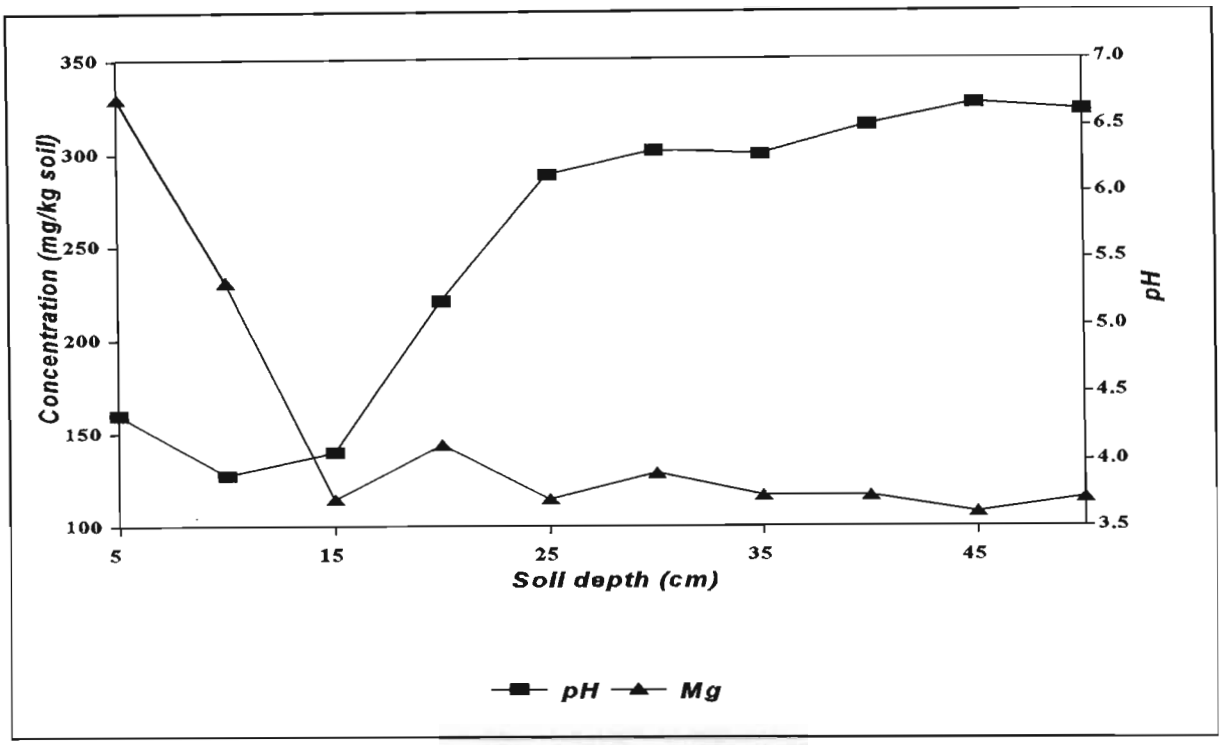

Figure 6.11C: Changes in pH and Mg concentrations with soil depth for the Nutrients treatment.

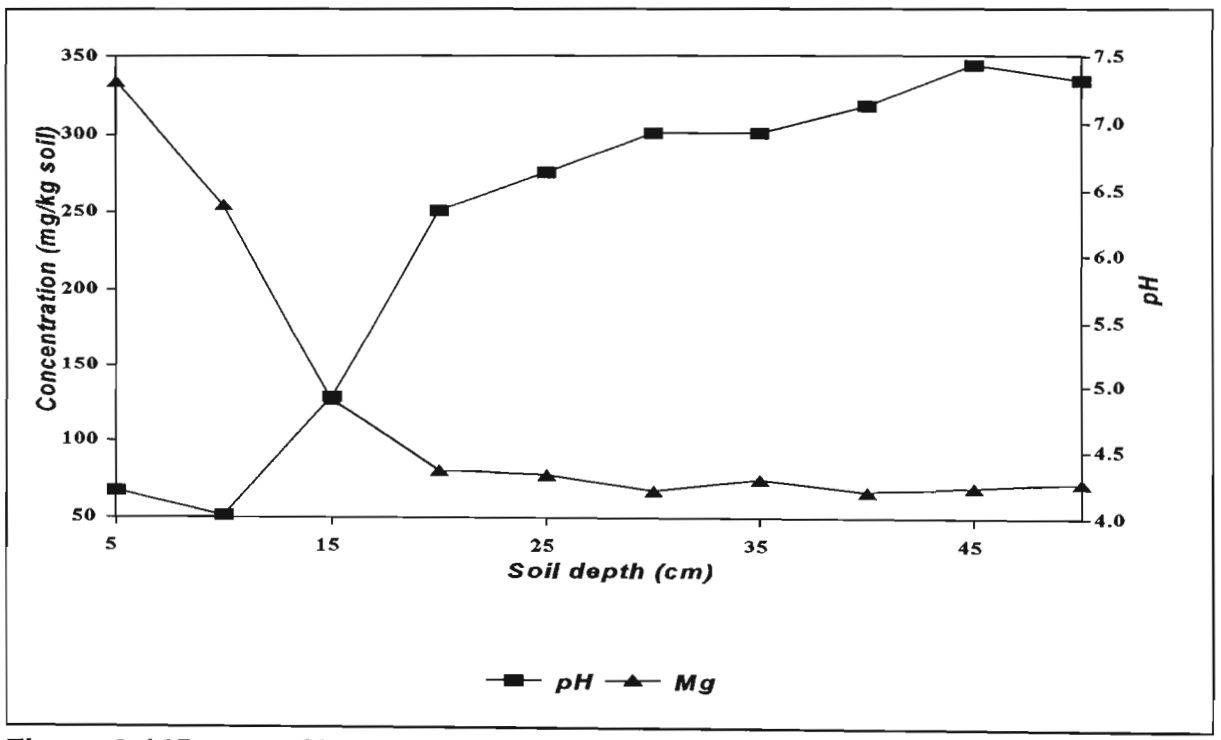

Figure 6.11D: Changes in pH and Mg concentrations with soil depth for the Nutrients+Lime treatment.

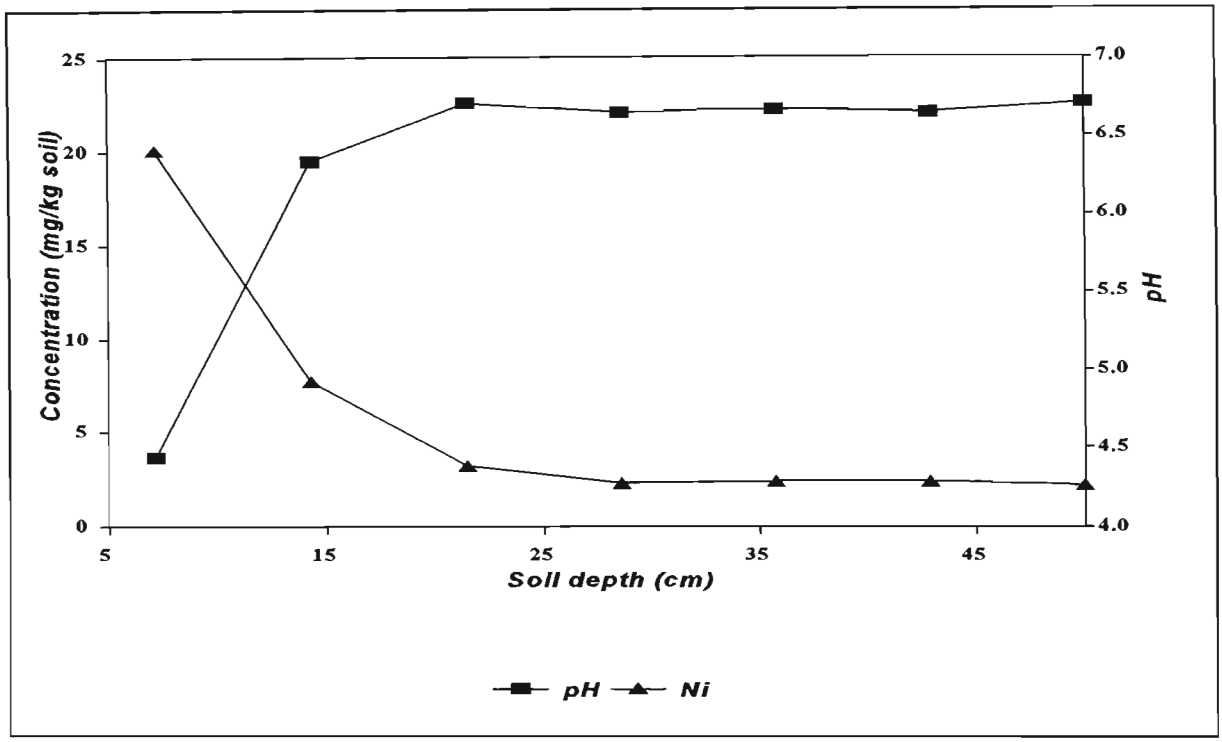

Figure 6.12A: Changes in pH and Ni concentrations with soil depth for the Water treatment.

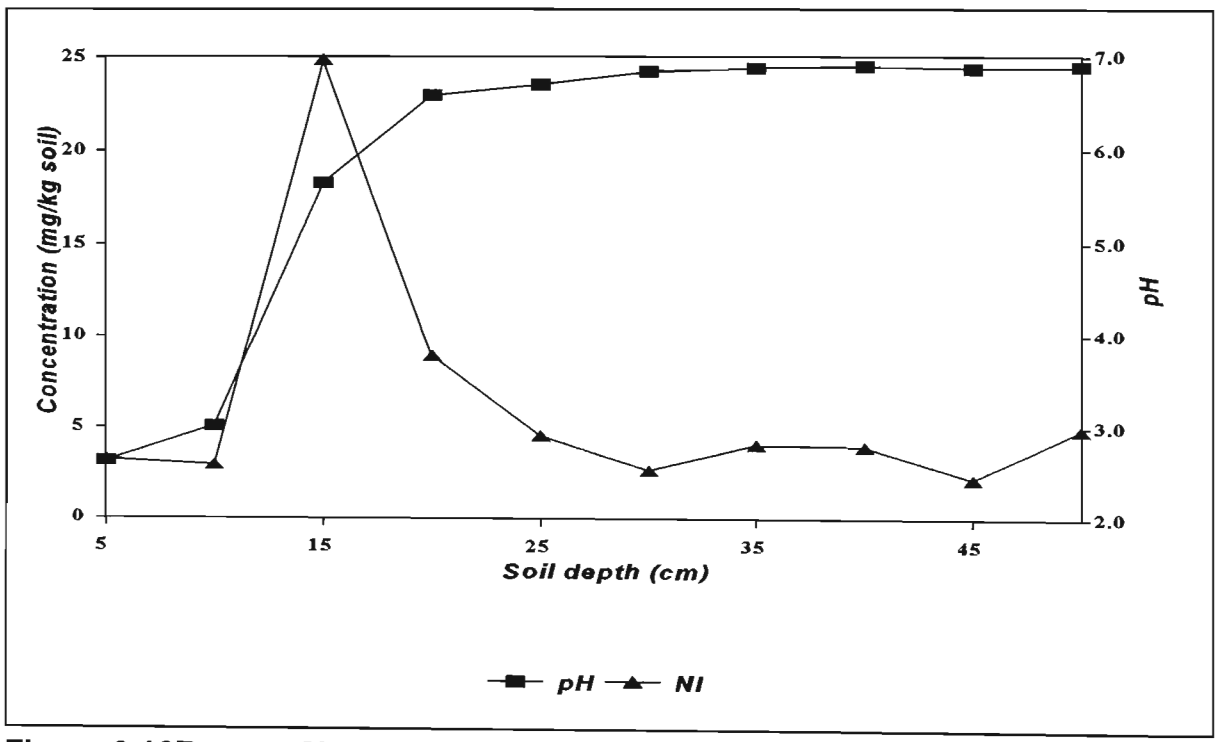

Figure 6.12B: Changes in pH and Ni concentrations with soil depth for the Acid treatment.

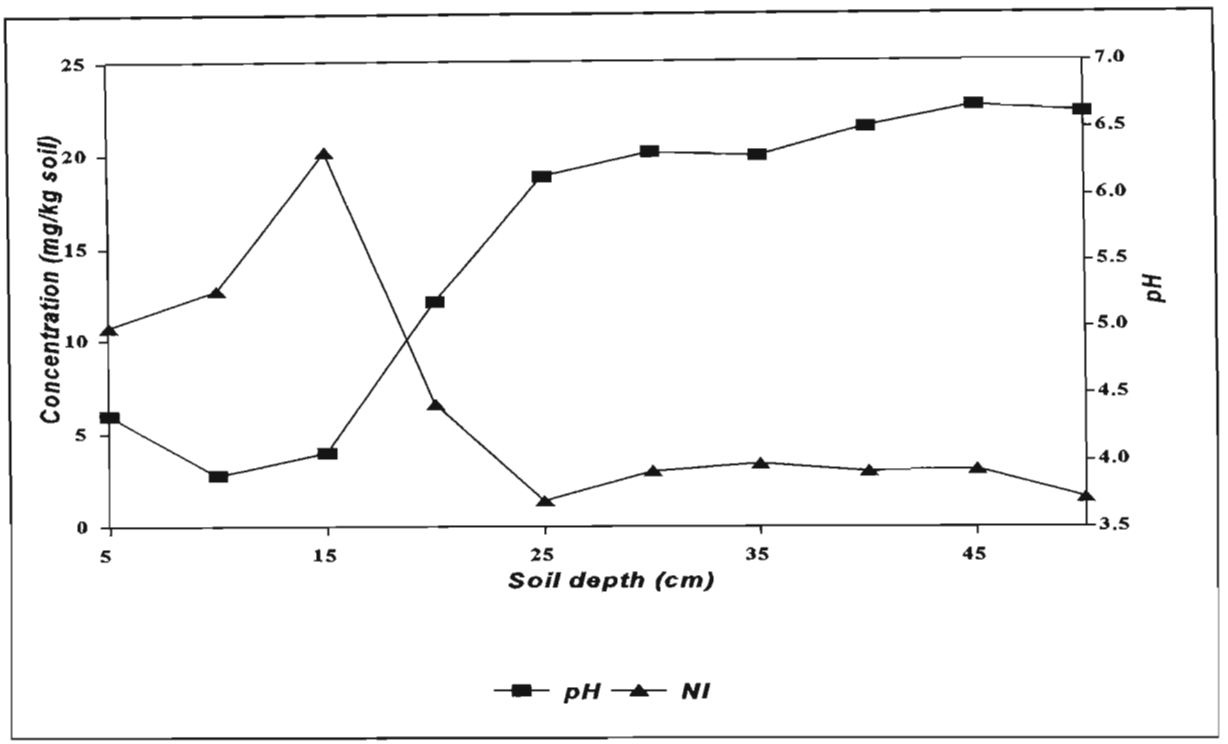

Figure 6.12C: Changes in pH and Ni concentrations with soil depth for the Nutrients treatment.

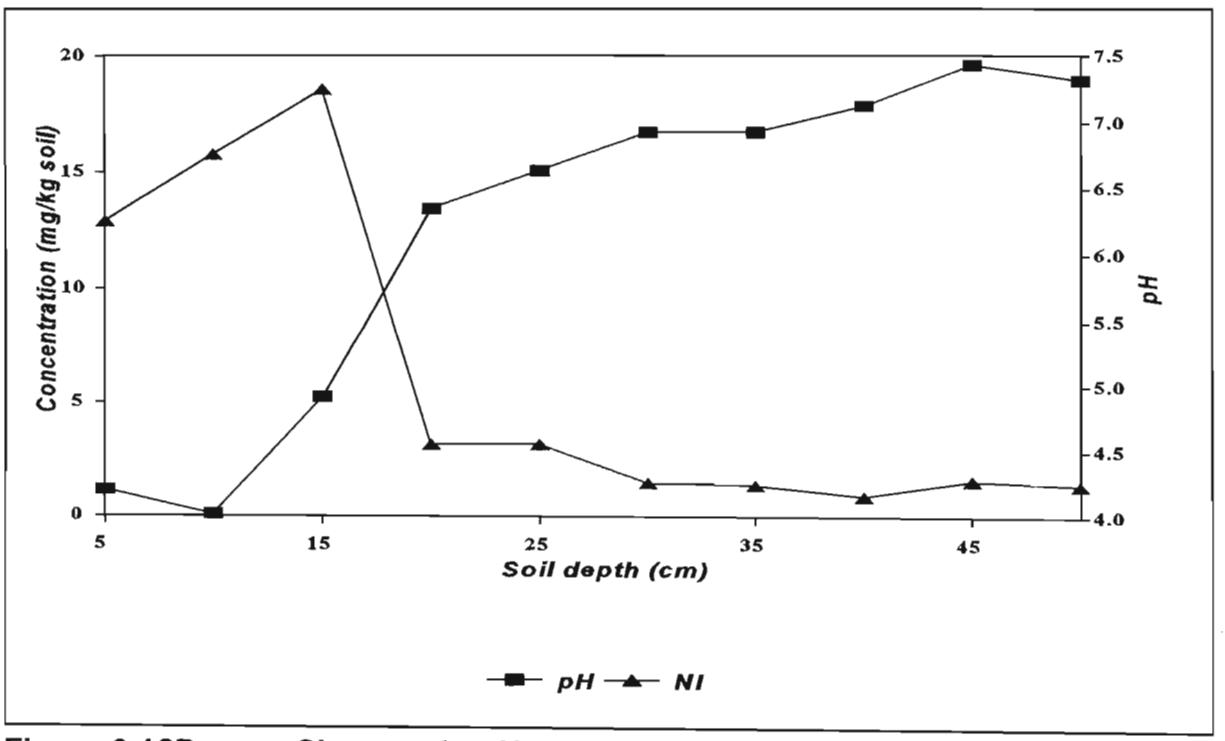

Figure 6.12D: Changes in pH and Ni concentrations with soil depth for the Nutrients+Lime treatment.

The mobility and distribution of Mn and Zn throughout the soil profiles appeared to be pH mediated (Figure 6.9). The pH, as recorded for the acid treatment (Figure 6.13A), was significantly lower (1 - 1.5 units) than for the other treatments in the first 5-15cm depth of soil. As a result, Zn and Mn mobility from the same depth of soil should have increased, thus leading to the presence of elevated concentrations of metals in the soil zone succeeding the acidic region (Figure 6.9A). The mobility of Mn appeared to be influenced by the acidic conditions to a greater extent than the other treatments (Figures 6.9A, 6.9B, 6.9C, 6.9D, 6.13C) with the metal widely distributed through the soil to a depth of 35cm. Although the soil pH was buffered to normal levels within the first 20cm soil depth, the significantly lower pH in the contaminated soil zone would have accounted for an increased migration from this zone. The zinc concentrations of the Acid treatment (6.9B) in the 15-20cm soil zone were relatively high in comparison to the other treatments (Figures 6.9A, 6.9C, 6.9D, 6.13B). However, in contrast to the Mn, Zn migration into the uncontaminated soil was relatively limited. This suggested that reduced mobility through soil buffering was more effective in limiting Zn movement.

It was also noted that treatment with Nutrients, and Nutrients and Lime, had marked effects on metal migration (Figures 6.13B, 6.13C, 6.9 A-D). The manganese and zinc concentrations, as well as the depths of migration for these treatments were observed to be greater than the Water treatment but lower than the Acid treatment. These differences were attributed to the various pH effects induced by the different treatments (Figure 6.13A). The nutrient treatments did not produce low pH conditions in the 15-20cm zone as compared to the Acid treatment. However, because fermentative microbial catabolism was probably stimulated by the addition of nutrients, the two nutrient treatments mediated conditions which were more acidic than the water treatment.

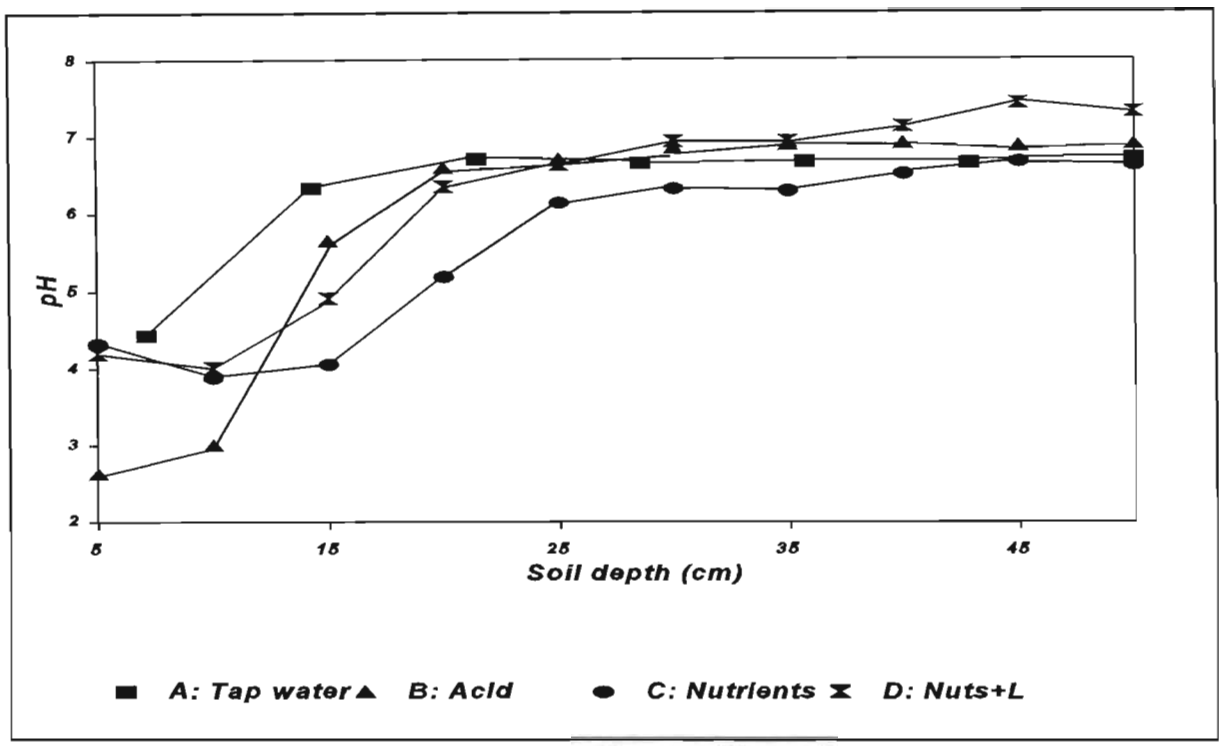

Figure 6.13A: Changes in pH values with soil depth for the different treatments.

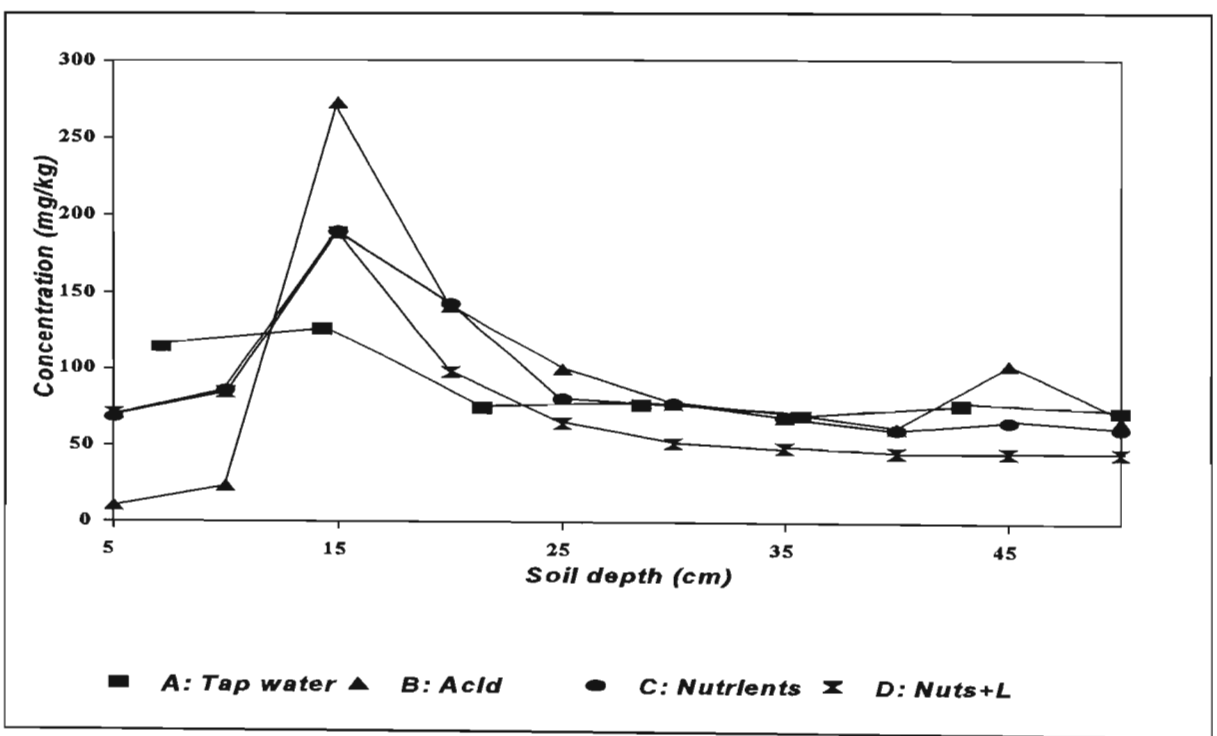

Figure 6.13B: Changes in Zn concentrations with soil depth for the different treatments.

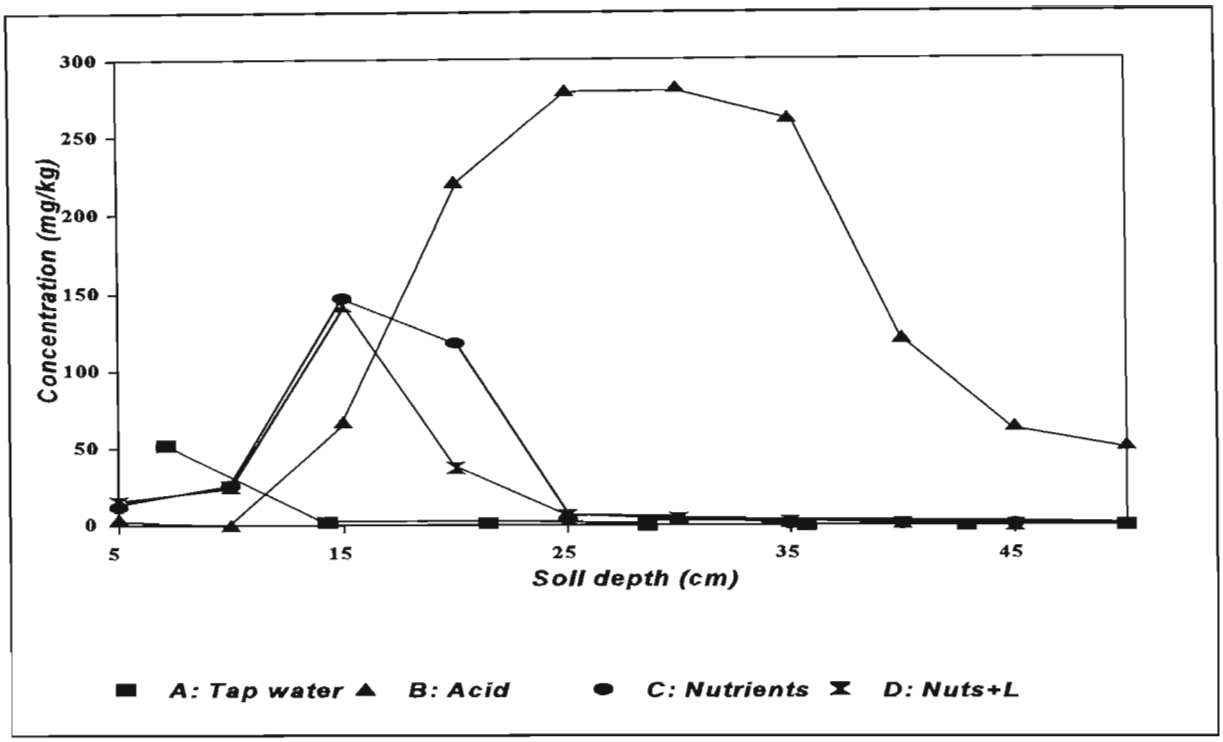

Figure 6.13C: Changes in Mn concentrations with soil depth for the different treatments.

A close relationship between the soil pH, Ca concentration and soil depth was observed. An increase in soil pH was noted along with an increase in Ca concentration. From Figure 6.10 (A-D), it can be seen that extremely high concentrations (2500mgkg^{-1}-3500mgkg^{-1}) of Ca were present which suggested that Ca had been previously added to the soil and was not of natural origin. This was later confirmed when the concentration of Ca was compared to the concentration of Mg (Figure 6.11 A-D). Generally, the ratio of Ca to Mg tends to be 2:1 in natural soils (C. A. du Plessis, 1995 personal communication). This was, however, clearly not the case for this particular soil.

The Ca distribution after the various treatments was found to be similar for the Acid, Nutrients, and Nutrients and Lime treatments. The Ca concentration following water treatment was significantly higher in the first 15cm depth of soil than for the other three treatments and was probably due to immobilization because of the relatively high pH in this zone (Figures 6.10A 6.13A and 6.13D). The calcium concentrations in the 20-50cm zones were similar for the Water, Acid and Nutrient treatments (Figure 6.13D).

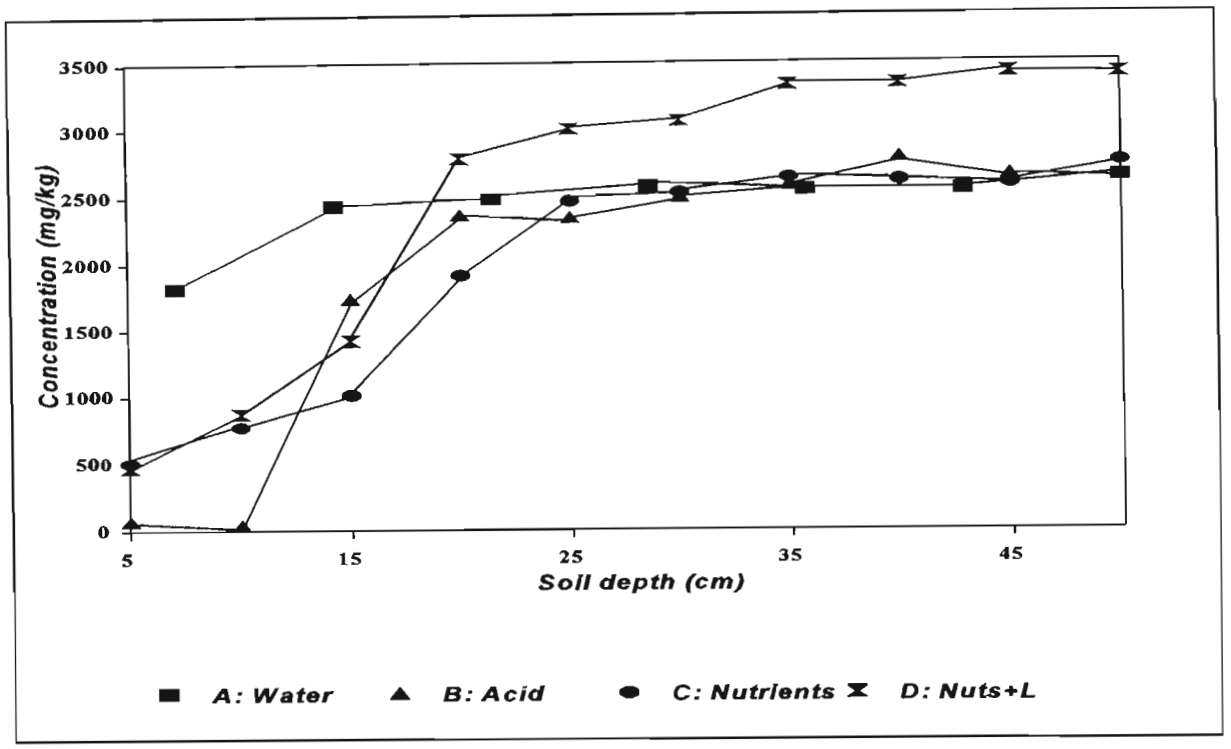

Figure 6.13D: Changes in Ca concentrations with soil depth for the different treatments.

However, the Ca concentrations for the Nutrients plus Lime treatment were found to be greater in the 20-50cm zone and these were attributed to the lime ($CaCO_3$) addition.

Magnesium was observed to have an inverse distribution to that of calcium for the Water, Nutrients, and Nutrients and Lime treatments (Figures 6.11 A-D and 6.13E). The Mg concentrations in the upper layer (0-15cm) of the contaminated soil were found to be high and limited movement from the contaminated soil and hydrocarbon sludge layer was observed. The Acid treatment resulted in a maximum concentration of Mg at a soil depth of 15cm. The greater depth distribution of Mg in this column could be attributed to the low pH of the acidic treatment. It must be noted that the general concentrations of Mg throughout this column were generally less than those found in the other columns (Figure 6.13E).

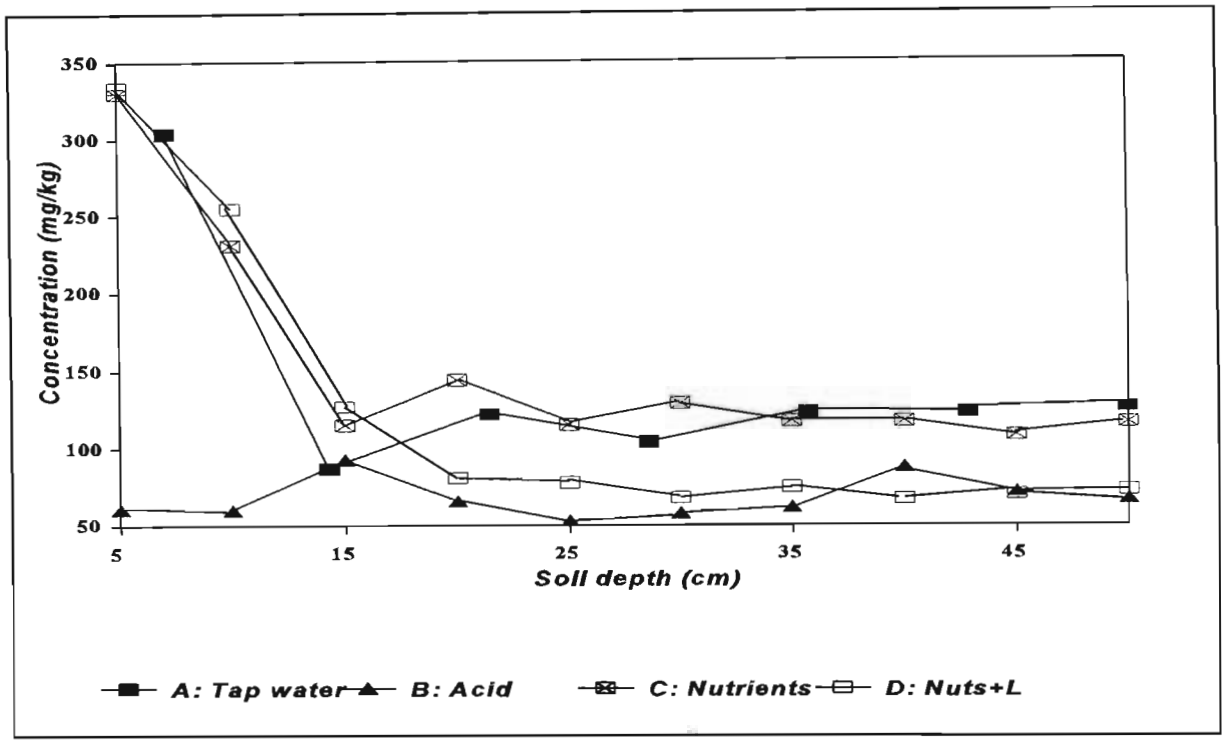

Figure 6.13E: Changes in Mg concentrations with soil depth for the different treatments.

Nickel distribution through the soil in relation to pH is shown in Figure 6.12 A-D. The Water treatment did not tend to influence migration of nickel from the upper contaminated zone (5-10cm) to any great extent. In contrast, the Acid treatment was found to increase the nickel migration with the highest concentration observed at 15cm. This again suggested that the application of acidic water had resulted in the migration of nickel from the hydrocarbon sludge. The Nutrients and Nutrients plus Lime treatments (Figure 6.13F) also produced a nickel peak at an intermediate soil depth (15cm), but with lower nickel concentrations than was observed with the Acid treatment. This was again probably due to the intermediate acid conditions.

For all treatments, the heavy metals were distributed throughout the soil profiles below the concentration maxima. It should, however, be noted that the heavy metal concentrations at these depths generally did not deviate excessively from the concentrations observed in the uncontaminated soil before treatment (Table 6.2). The heavy metals which were detected in the deeper soil zones were thus present before

sludge application onto the uncontaminated soil.

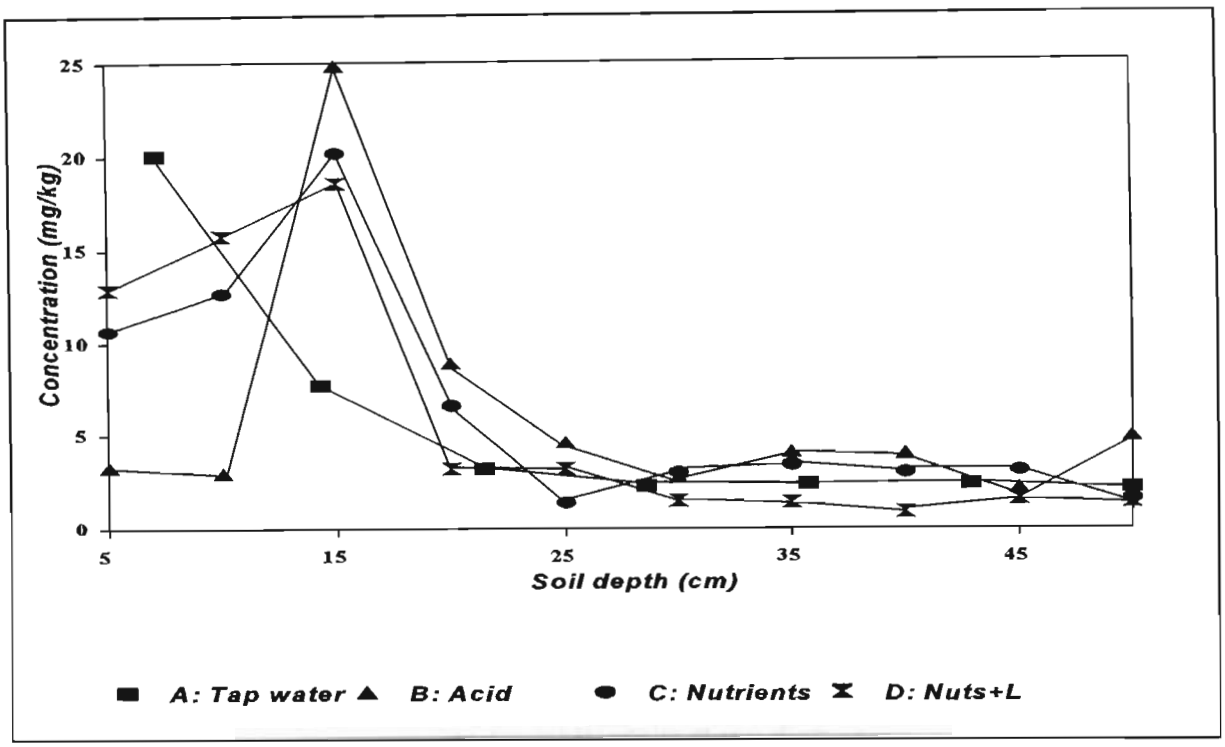

Figure 6.13F: Changes in Ni concentrations with soil depth for the different treatments.

Figure 6.14 (A-D) shows leachate data for the 60cm and 120cm columns. The metal concentrations recorded during the experimental time period showed a number of fluctuations with relatively high peaks for all the metals observed after 24 weeks. These could have resulted from the movement of low concentration metal fronts which were present in the uncontaminated soil below the layer of contaminated soil and sludge (Table 6.2). The metal concentrations were, however, relatively low and were not thought to be of any particular significance.

The results obtained showed that the metals (excluding Ca as this was present in high concentrations prior to the sludge application) were localised between 10 and 25cm. Although the Acid treatment resulted in a wider heavy metal distribution throughout the soil profile, this was still limited to the 20-35cm zone. Manganese,

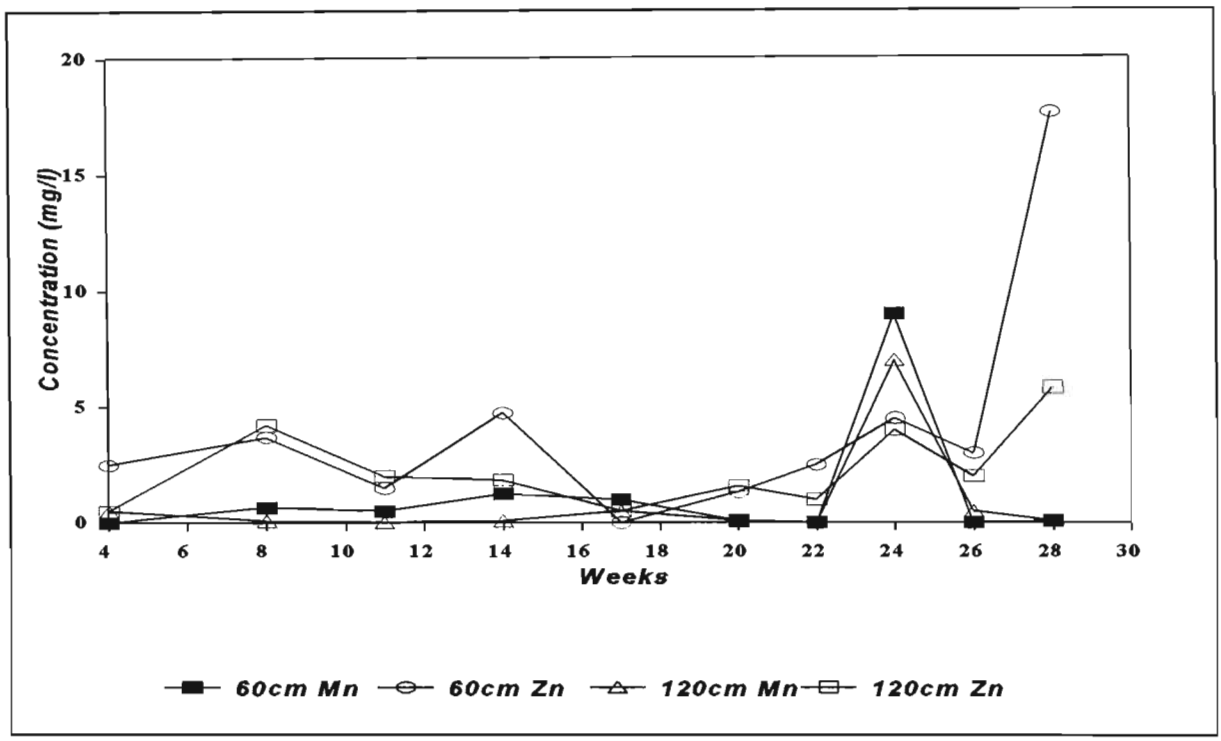

Figure 6.14A: Changes in heavy metal leachate concentrations for the Water treated columns during the 30-week study period.

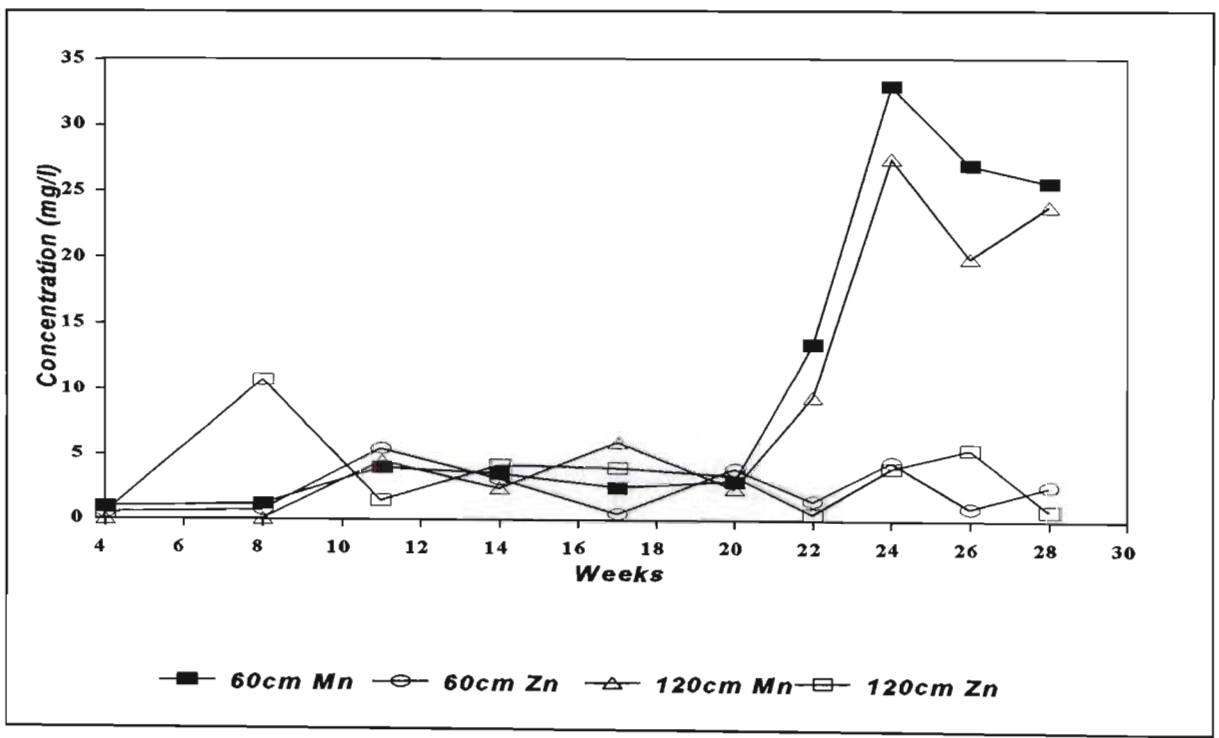

Figure 6.14B: Changes in heavy metal leachate concentrations for the Acid treated columns during the 30-week study period.

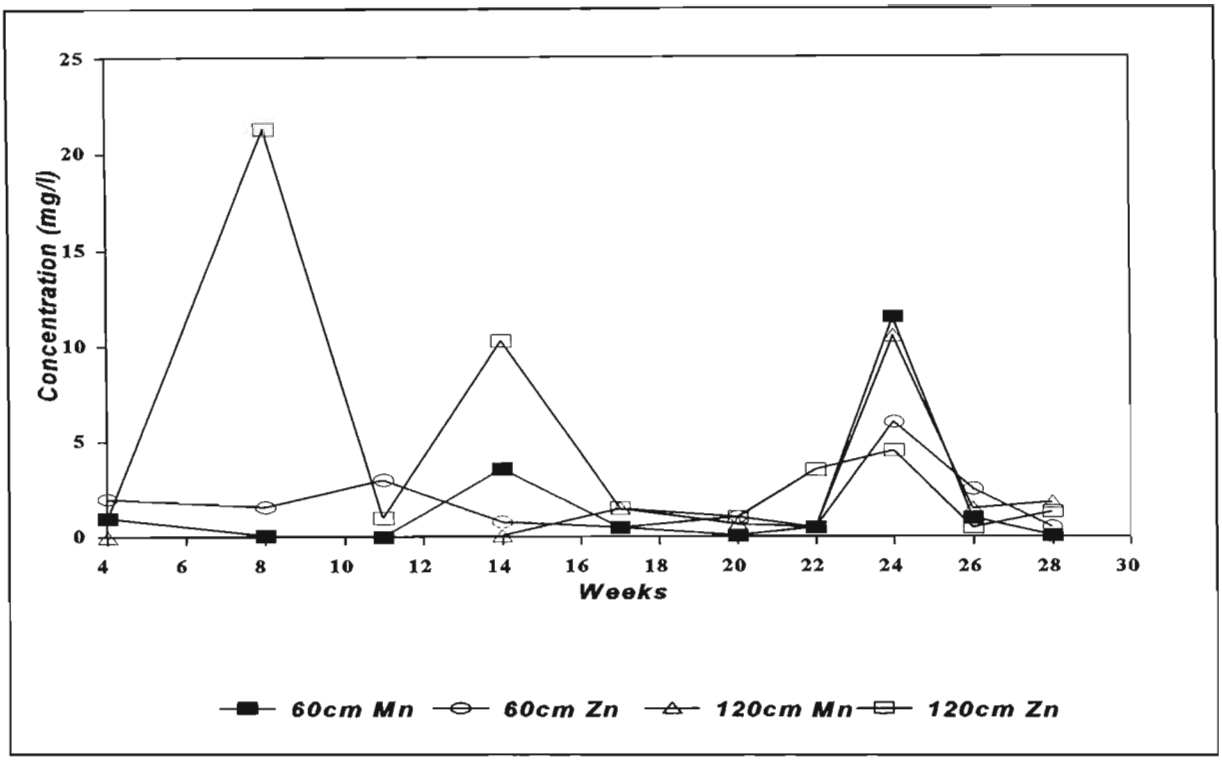

Figure 6.14C: Changes in heavy metal leachate concentrations for the Nutrient treated columns during the 30-week study period.

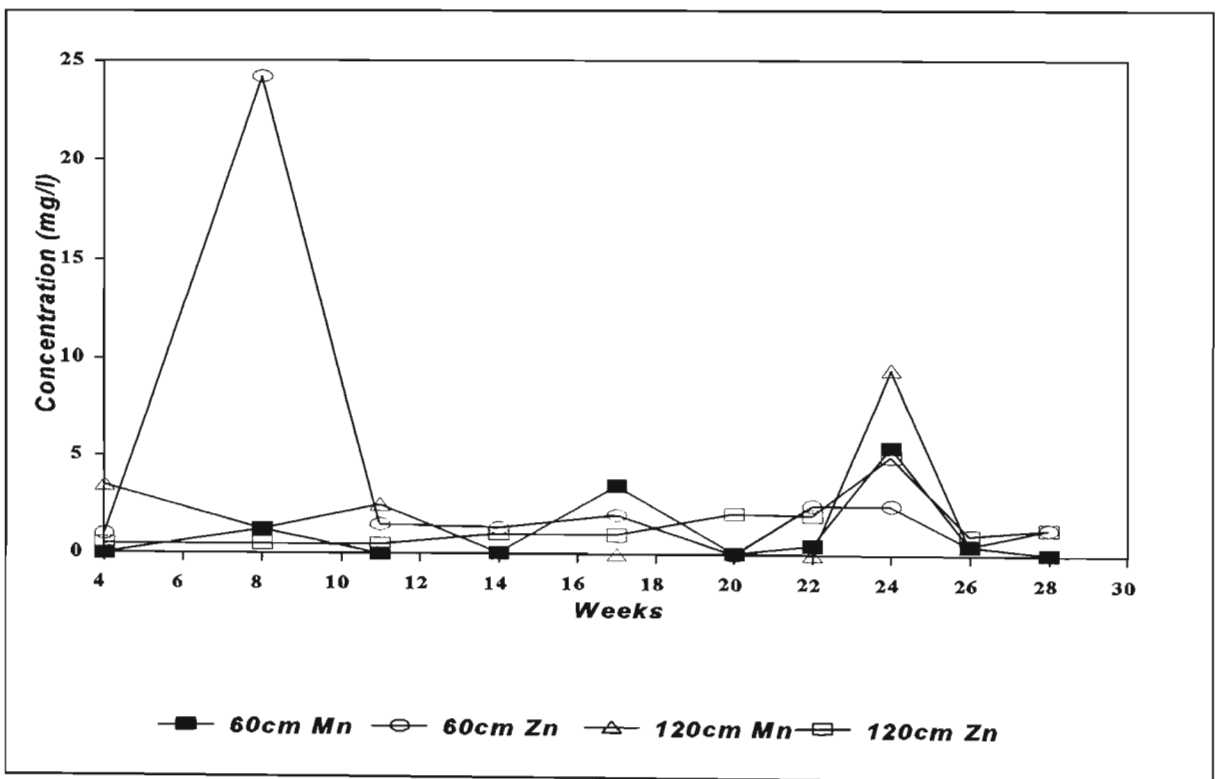

Figure 6.14D: Changes in heavy metal leachate concentrations for the Nutrient+Lime treated columns during the 30-week study period.

however, proved to be the exception. Relatively high concentrations of this metal were found in the 40-45cm zone and, with continued treatment, would be expected to migrate, at a very slow rate, to greater depths.

It can thus be concluded that heavy metal migration down the soil profile, even under acidic conditions was retarded due to the adsorptive nature of the soil. This phenomenon was also aided by the buffering capacity of the soil which was a function of the high Ca concentration in the uncontaminated soil. However, it should be noted that the presence of heavy metals in the soil leachate was limited. Thus, metal migration down the soil profile did not necessarily infer that high concentrations of heavy metals would compromise the groundwater quality.

An important factor to consider is that the treatments simulated a site rainfall pattern equivalent to 1500mm or 5 years condensed into 30 weeks. The migration patterns of heavy metals examined here would thus represent a worst case scenario. For the purposes of short-term planning, the potential for groundwater contamination is, as a result, minimal since such saturation of the soil is unlikely to occur over a short time period. It is also unlikely that the soil pH would decrease to below 4 at which point increased concentrations of heavy metals would be expected in the soil solution. However, with a protracted time span, the migration of heavy metals, although slow, could eventually lead to the presence of certain metals such as Mn at greater soil depths and, subsequently, in the groundwater.

The concentrations of hydrocarbons throughout the soil profile after 30 weeks for the various treatments is shown in Figure 6.15. The hydrocarbons did not appear to migrate further than the first 10cm of soil during the experimental period. Only the Acid treatment provided conditions for the limited movement of hydrocarbons into the first 10cm of soil. Hydrocarbon migration was relatively limited and was not of any major concern as the water soluble fraction tends to be labile. Despite nutrient application, gravimetric analysis did not show appreciable loss of hydrocarbons due to biodegradation in the first 5cm of soil. A puzzling aspect is the lack of hydrocarbons

detected in the 5-15cm layer. Since hydrocarbon sludge and soil were mixed into the upper 10cm of soil, a higher proportion of hydrocarbon would be expected than was actually detected (Figure 6.15). It can only be surmised that hydrocarbon in the 5-10cm layer has to some extent been attenuated. The exact measurement and slicing of 5cm soil sections was also achieved with some difficulty. During the slicing process it is also possible that a proportion of the 10-15cm slices may have become incorporated into soil from the upper layer, ultimately resulting in greater hydrocarbon dilution. If, however, it can be accepted that degradation would be expected to be greatest in the aerobic 0-5cm zone, it would appear that the hydrocarbon molecules are recalcitrant. This finding correlated with the results presented in Chapter 4 which indicated that this particular hydrocarbon sludge was amenable to very slow rates of biodegradation. Although this is of concern, the relative immobility of the hydrocarbon fraction suggested reduced risk of soil and water contamination.

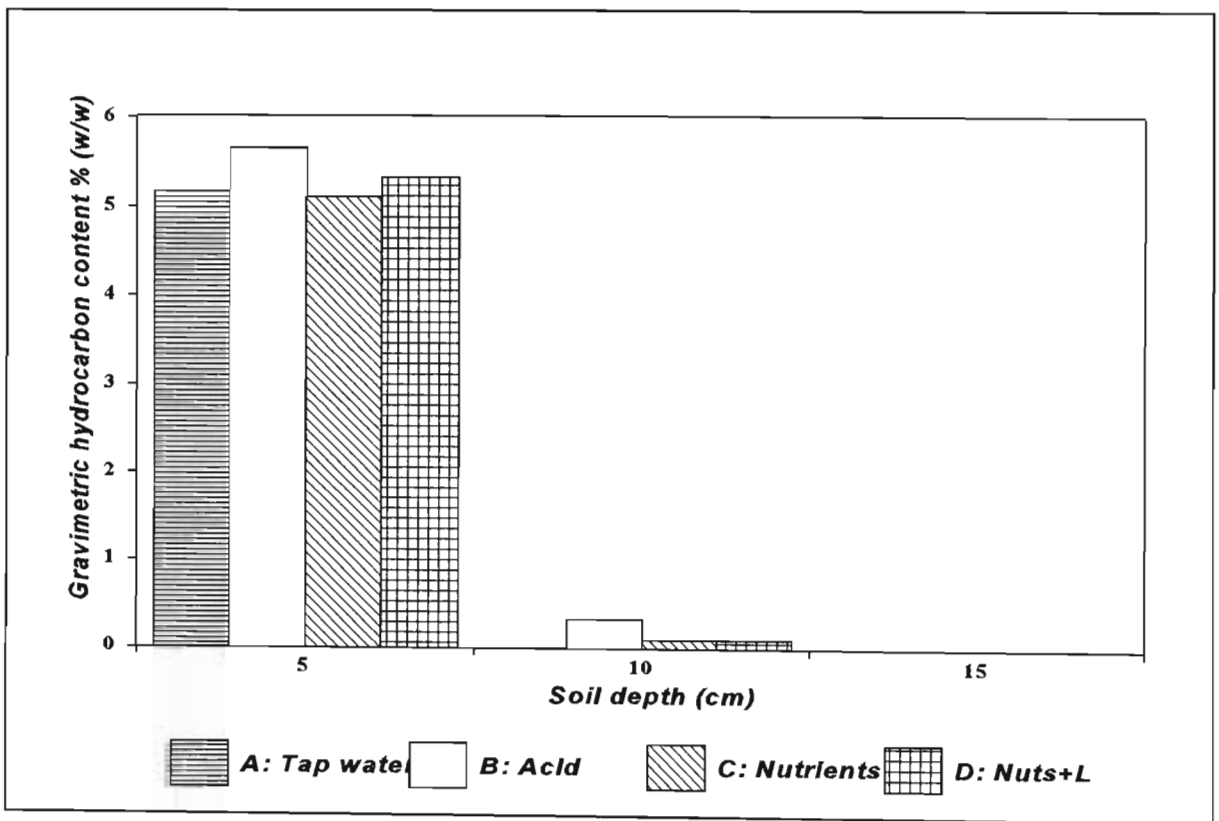

Figure 6.15: Gravimetric hydrocarbon content with increasing soil depth for the different treatments after the 30-week study period.

CHAPTER 7

RAPID BIOREMEDIATION ASSAYS WITH IODONITROPHENYLTETRAZOLIUM CHLORIDE

7.1 INTRODUCTION

The selection of appropriate analytical protocols and procedures is critical for evaluating bioremediation efficacy (Block *et al.*, 1990) and thus provide reliable and complete hydrocarbon quantification. Several methods have been developed for hydrocarbon quantification which include infrared (EPA method 418.1) and capillary gas chromatography methods (Morgan and Watkinson, 1989). Both these approaches require appropriate preparation of the sample, equipment and machinery which may be time consuming. Although relatively reliable quantification of hydrocarbon contamination results, the methods are unsuitable for the rapid evaluation of the bioremediation processes *in situ*. In addition, the capital outlay required to purchase and maintain the analytical equipment required is substantial. It is, thus, often desirable to monitor the state of bioremediation progress without the associated sample preparation time and cost involved. A quick and simple method to indicate the state of such aspects would thus be advantageous.

The soil examined in this study was contaminated with a complex petroleum hydrocarbon and heavy metal mixture. If it is accepted that the hydrocarbons are the major source of organic carbon present in the soil (A.1) and available for microbial metabolism, then microbial activity should be indicative of bioremediation progress.

A relatively cheap and simple method for measuring microbial activity is with tetrazolium salts. These provide an assay which reflects electron transport system (ETS) activity and, hence, the activity of a microbial population within a soil system (Trevors *et al.*, 1982). Electron transport system activity is a consequence of metabolic processes and is closely linked with many of the biochemical pathways involved in

microbial respiration. The ability to measure ETS activity should, thus, provide a means of assessing the microbial status of various soils (Trevors, 1982).

An estimation of microbial activity can be made by measuring the rate of reduction of tetrazolium salts (Casida, 1977). Iodonitrophenyltetrazolium chloride (INT) is a tetrazolium salt which is a colourless, water soluble redox indicator, which upon reduction is converted to a water insoluble, red formazan derivative in viable cells (Oren, 1987). Tetrazolium reduction measurement should thus give a general estimation of the enzymes involved in microbial energy metabolism in a particular matrix as a function of electron transport system activity (Oren, 1987). The correlation between ETS and metabolism was confirmed by Maki and Remsen (1981) who reported that reduction of INT to formazan by ETS was a function of respiration.

Fluorescein diacetate (FDA) is another chemical which has been used in assays as an indicator of microbial activity (Söderstrom, 1977; Brunius, 1980; Lundgren, 1981). Fluorescein diacetate is hydrolysed by a number of different enzymes such as proteases, lipases and esterases (Guilbault and Kramer, 1964; Rotman and Papermaster, 1966). The product of this conversion is fluorescein which can be quantified by fluorometry or spectrophotometry. If the production of fluorescein is a function of microbial activity then, as with INT, FDA could be used to estimate activity in hydrocarbon polluted soil and, hence, the progress of remediation.

The aim of this section of the programme was, therefore, to establish wether indicators of microbial respiration could be used as a rapid method for monitoring bioremediation progress in hydrocarbon contaminated soils.

7.2 EXPERIMENTAL

7.2.1 INT Assay Methodology Development

Literature describing previous assays, carried out under different conditions, indicated

that a wide range of procedures were followed. For this reason it was important to develop a protocol specific for hydrocarbon contaminated soil. Several factors were, thus, investigated to identify and achieve optimum analytical conditions.

The general procedure adopted for the method development was to incubate 1g soil (2.1) samples with 0.5m/ 0.4% (w/v) INT in 15cm x 1.5cm test tubes, in the dark at 25°C. The extraction procedure dictated that formazan should be removed by dissolution in methanol. To establish which volume of methanol would provide the best extraction, the following procedure was carried out: Soil (1g), known to have an active microbial population (obtained from soil surrounding a healthy rhizosphere), was incubated with INT as described above for 24 hours. Extraction of formazan with 6m/, 8m/ and 10m/ volumes of methanol was then carried out. This was achieved by adding methanol to the soil, mixing and sonicating for 15 minutes. The soil/methanol mixtures was then filtered through Whatman no.1 filter paper into test tubes. Finally, the quantity of formazan produced was estimated at A_{490}nm with a Milton Roy 801 spectrophotometer. Sterilized soil (autoclaved at 121°C for 15 minutes) was used as the control.

The incubation time required for optimal formazan development was also tested by monitoring the optical density changes at different time intervals. This was achieved by incubating 1g hydrocarbon contaminated soil samples with INT as described above. After incubation, 8m/ methanol were added to each, mixed and sonicated for 15 minutes. Extraction and analysis of formazan were carried out as described above. Sterilized soil (autoclaved at 121°C for 15 minutes) was used as the control.

To investigate the possibility of oil interference effects, 1g hydrocarbon contaminated soil samples were assayed with INT and compared to a control sample. The controls consisted of 1g contaminated soil samples which had been treated in the same way as the experimental hydrocarbon samples, with the exception that 0.5m/ water was used instead of INT. The methanol extracts were then compared over a range of light wavelengths.

7.2.2 Soil Biological Activity Assays

To establish wether differences between microbially active soils and relatively inactive soils could be distinguished, several soil types were tested by the INT assay. The soil types tested were hydrocarbon contaminated soil (SAS), pot-house soil (obtained from a greenhouse) soil (N) and soil (FC treatment, Chapter 4) which had been bioremediated for 120 days (OIL). The soil assays were carried out as described in 2.10

Soil microbial counts were made with various types of solid media. For organisms selectively utilizing hydrocarbons as their carbon source OIL agar was used. This was made by preparing an emulsion of 4% (v/v) hydrocarbon sludge with a nutrient medium which contained (l^{-1} distilled water): 0.5 g KH_2PO_4, 1.5g K_2HPO_4 ,1g NH_4NO_3 , 0.2g $MgSO_4.7H_2O$, and 20g agar. For general counts, Martin's Rose Bengal Agar (10g glucose, 5g peptone, 1g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 0.033g Rose Bengal, 20g agar and 0.03g streptomycin dissolved and diluted to a final volume of 1 litre) and Soil Extract Agar (1g glucose, 0.5g K_2HPO_4, 100m/ soil extract and 20g agar in a final volume of 1 litre, and with the pH adjusted to between 6.8 and 7.0).

Several bioremediation box trials were also monitored over a period of 12 weeks. This was done to establish whether the progress of various bioremediation treatments could be monitored by the INT assay. Contaminated soil (1kg) was placed in soil boxes (16x21x8cm) and subjected to several treatments as shown in Table 7.1. The soil boxes were incubated at ambient temperature (approximately 25°C). The treatments were assayed weekly by the INT method as described in 2.10.

7.2.3 FDA Assays

Fluorescein diacetate (2mgml^{-1}) was prepared in acetone to make a 2 litre stock solution. Aliquots (0.2m/) of the stock solution were added to 10m/ sterile 60mM phosphate buffer which was contained in test tubes (15cm x 1.5cm). The inoculum was

prepared by incubating

Table 7.1: Various soil box treatments carried out over a 12-week treatment period.

Treatment	Description
1: Control	The box contained contaminated soil which was untreated.
2: Nutrients	As for Treatment 1 but 70 m/ nutrient medium (2.4.1, medium 1) were added every week.
3: Water	As for Treatment 2 but water was added instead of nutrients.
4: Ash	An ash/contaminated soil (50% w/w dry weight) mixture was used and supplemented as for Treatment 2.
5: Pine-bark	A pine-bark/contaminated soil (30% w/w dry weight) mixture was used and supplemented as for Treatment 2.

1g contaminated soil samples in sterilized (autoclaved at 121°C for 15 minutes) nutrient broth ($16g\ell^{-1}$) overnight at 25°C and in the dark. A dilution series (10^3 to 10^6) of the inoculum was then prepared in sterilized water. 2m/ samples of the different dilutions were then added to the test tubes. After shaking on a rotary shaker at 24°C for 120 minutes, the reactions were halted by filtering the mixtures through Whatman no.1 filter papers and placing the supernatants on ice. The amount of FDA hydrolysed was measured at A_{490} with a Milton-Roy 801 spectrophotometer.

7.3 RESULTS AND DISCUSSION

7.3.1 INT Methodology Development

It was anticipated that the use of methanol as a solvent would lead to problems with spectrophotometric analysis. The solubilization of lighter fraction hydrocarbons in methanol was expected to interfere with the readings of the formazan. The optical density results of both the methanol soluble hydrocarbon fraction and the formazan are shown in Figure 7.1. These results indicated that for every absorbance obtained for formazan, methanol soluble hydrocarbons contributed a significant proportion of the apparent reading. Although interference due to hydrocarbons was observed to be negligible at 523nm, the sensitivity of formazan detection was so reduced that detection at this wavelength proved to be impractical. For this reason, a wavelength of 490nm was used to detect formazan. Furthermore, background readings of methanol extracted hydrocarbons were used to correct the soil activity readings.

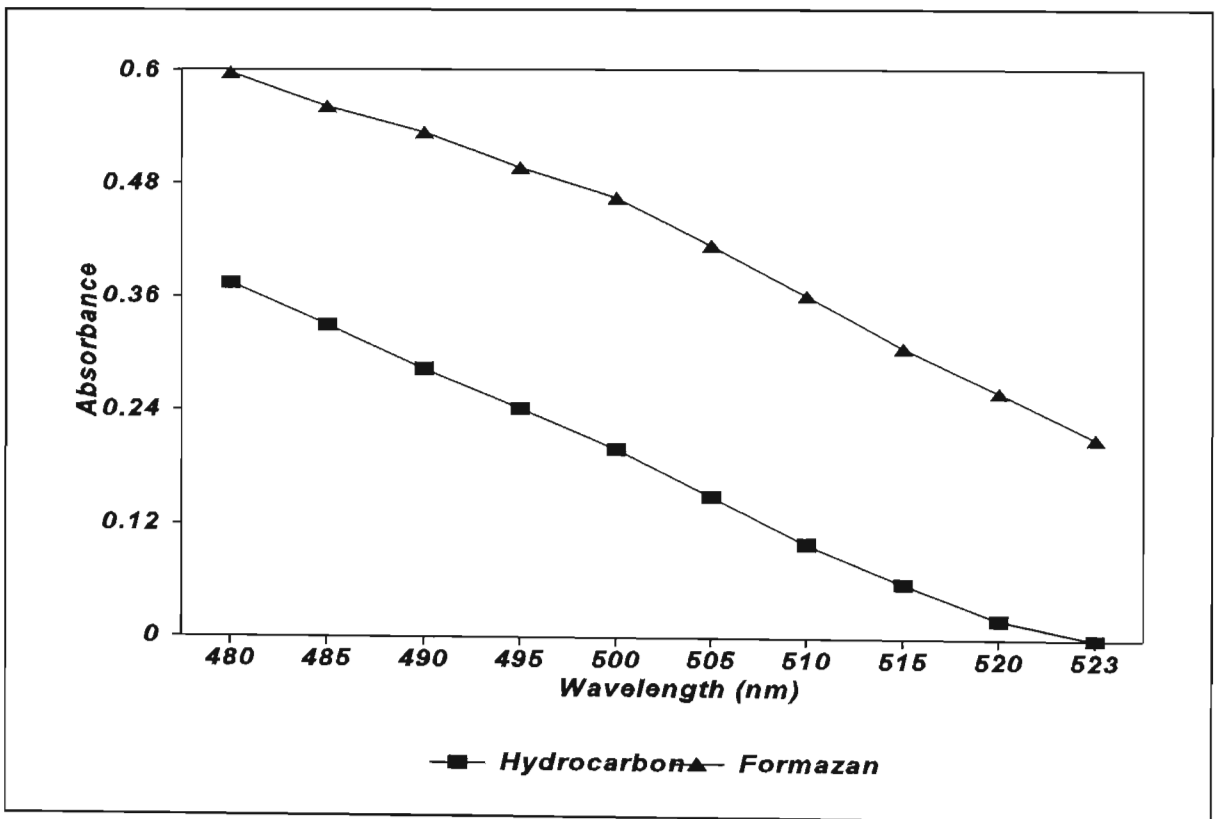

Figure 7.1: Optical absorbance readings obtained between the wavelengths of 480nm and 523nm for methanol soluble hydrocarbons and formazan.

Incubation time was an important factor to consider since formazan development is dependent on the INT contact time with the microorganisms. Where conditions such as

high concentrations of hydrocarbons and heavy metals are present, microbial activity may be suppressed. Incubation time was, in fact, found to influence formazan development, with a stronger colour reaction observed after increased incubation time intervals (Figure 7.2). For the hydrocarbon contaminated soil under investigation, where the toxicity effected an initial low level of microbial activity, the minimal incubation time was found to be 96 hours (4 days). This time interval was, subsequently, adopted for other experimental procedures.

The volume of methanol used by other workers has varied. The volume is, however, critical as excessive methanol introduces a dilution effect. As a result, various volumes of methanol were tested to determine their dilution effects. The extraction volumes of methanol did not appear to significantly alter the concentration and hence spectrophotometric readings of extractable formazan (Figure 7.3). Subsequently, 8ml of methanol were used for the extraction procedures and adopted for further assays.

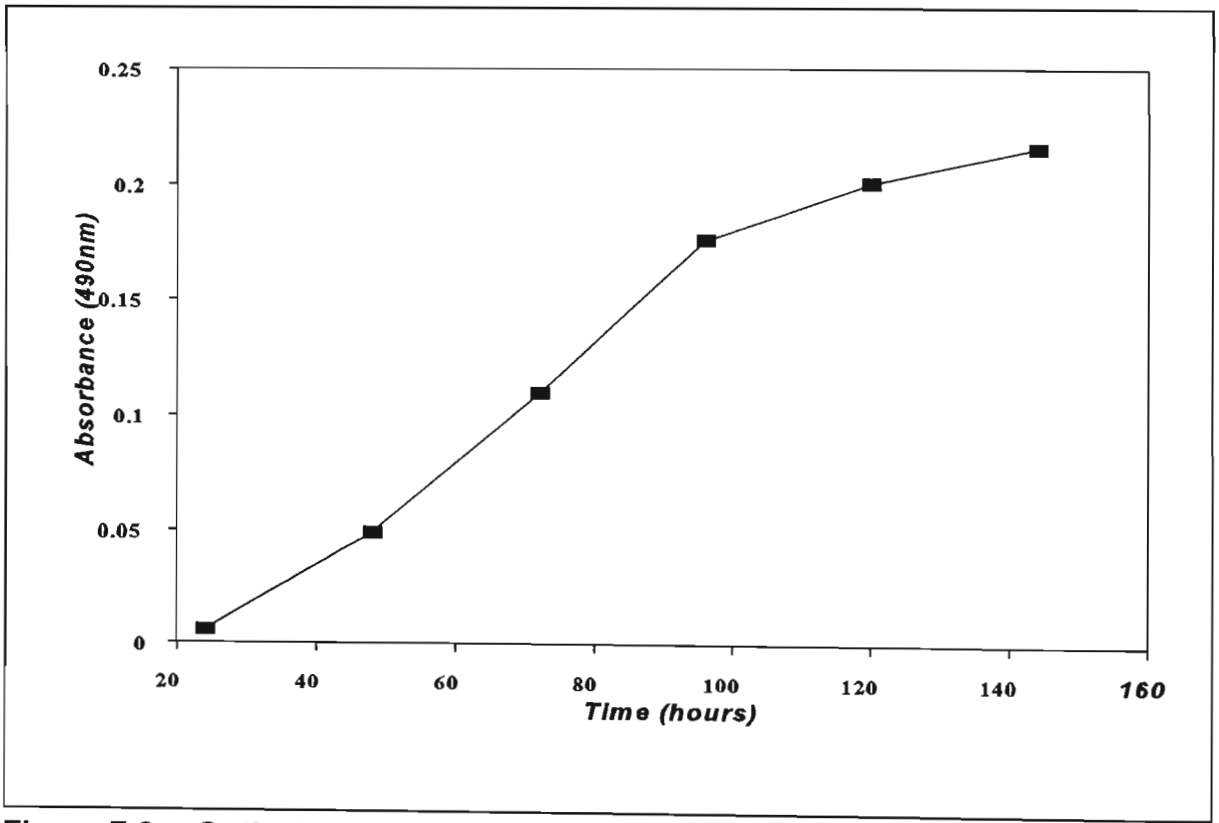

Figure 7.2: Optical absorbance (A_{490}) readings of formazan development with incubation time.

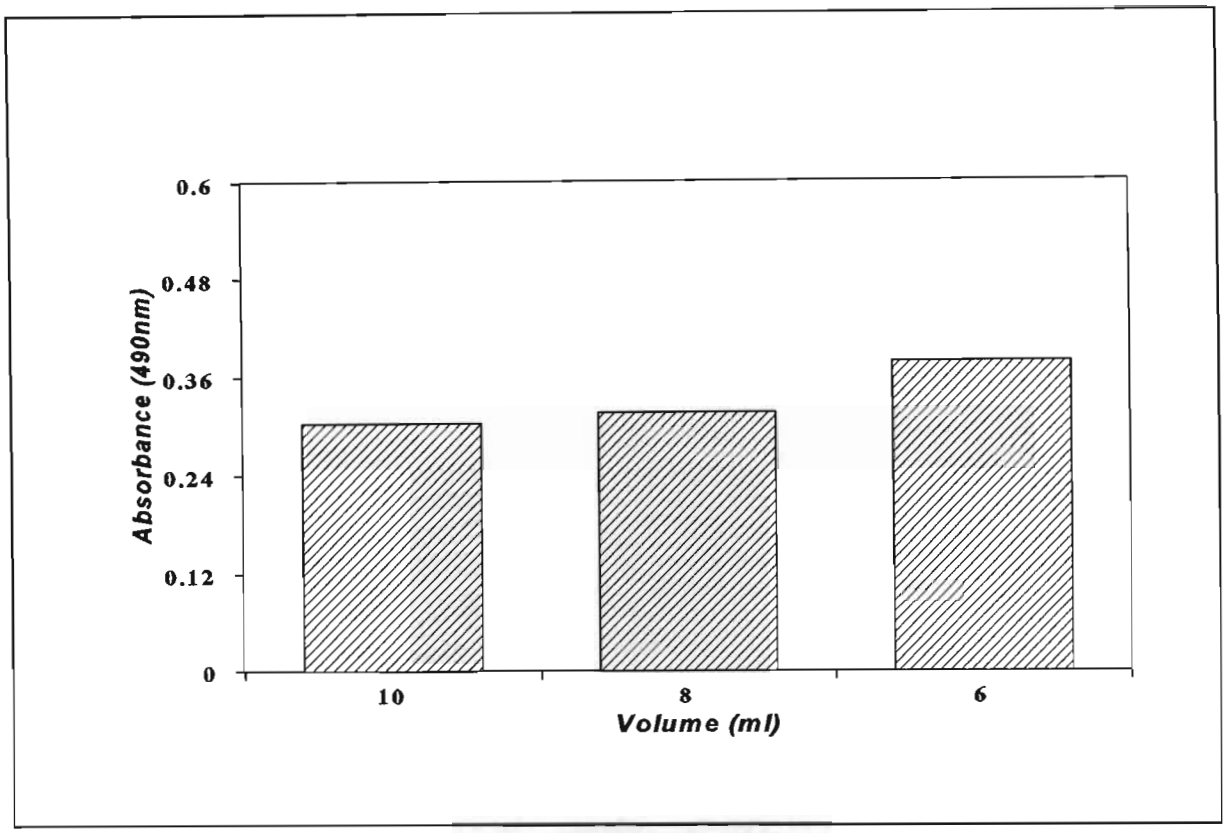

Figure 7.3: The effect of various methanol extraction volumes on the optical absorbance values obtained for formazan.

7.3.2 FDA Assays

Fluorescein diacetate was also tested as a measure of microbial activity in hydrocarbon contaminated soils. Unfortunately, the initial results (not shown) indicated that absorbance interference by acetone extractable hydrocarbons exceeded the detectable absorbance of fluorescein produced from an actively metabolising microbial population. Based on this indicator, FDA was not considered a suitable assay for the detection of microbial activity in hydrocarbon contaminated soils.

7.3.3 Soil Biological Activity Assays

The INT assay recorded different microbial activities for the different soils tested (Figure 7.4). Normal pothouse soil (N) had the highest activity, followed by the contaminated soil (SAS) and the bioremediated soil (OIL). Plate counts of colony forming units made on the same soils (Figure 7.4), indicated that high microbial numbers did not necessarily correlate with high electron transport chain activity. Microbial numbers for OIL and SAS were higher than that for N, but microbial activity, as determined by the INT assay, gave the opposite trend. These results suggested that

the INT assay could be used to distinguish between soils with an active microbial population (pothouse soil) and soil with low microbial activity caused by contaminant toxicity (OIL). Such a distinction would be difficult to determine with standard plate count methods since microbial survivors are selected mainly on the basis of recovery rates and not biological activity.

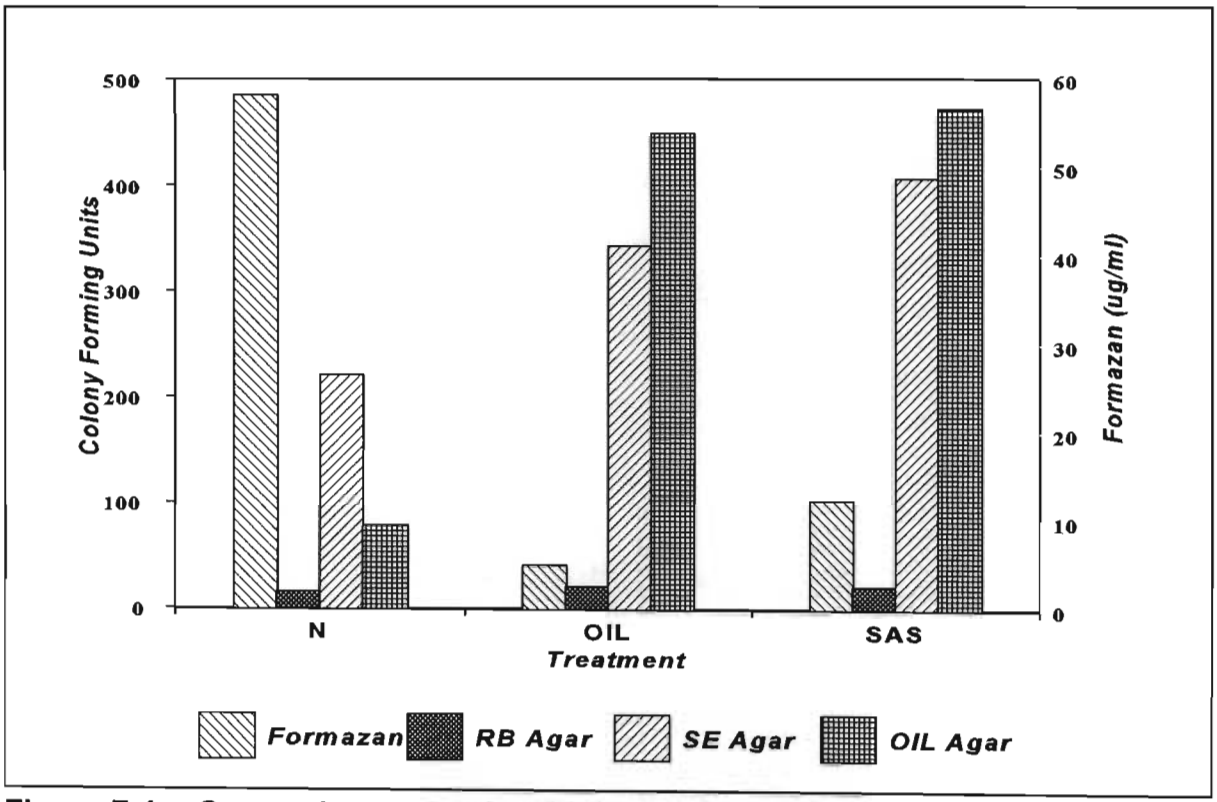

Figure 7.4: Comparison of microbial activity, as determined by formazan development (INT assay), and counts of colony forming units on various solid media for normal (N), contaminated untreated (SAS) and bioremediated soil (OIL).

Progress monitoring of the various bioremediation treatments, with the INT assay, is shown in Figure 7.5. Only formazan concentrations up to 65ugml^{-1} are shown for ease of results representation. The full formazan development profile for the Ash treatment is shown in Figure 7.6. The Ash treatment showed the highest microbial activity for the total treatment time followed by the Nutrients and Pine-bark treatments. This suggested that improved soil aeration, as provided by the bulking effect of ash, resulted

in enhanced microbial activity. Similar increases in hydrocarbon degradation rates with

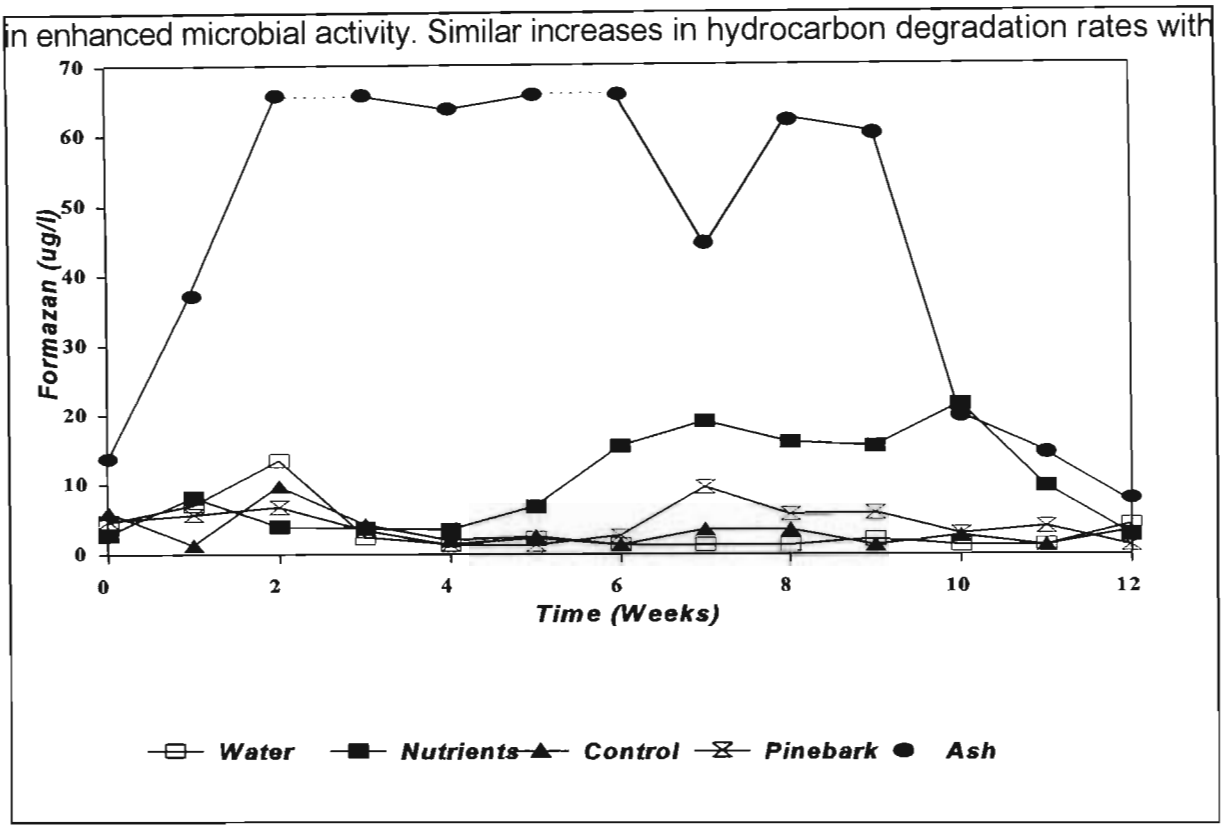

Figure 7.5: Microbial activity, as determined by the INT assay, after water, pine-bark, nutrients and ash treatments.

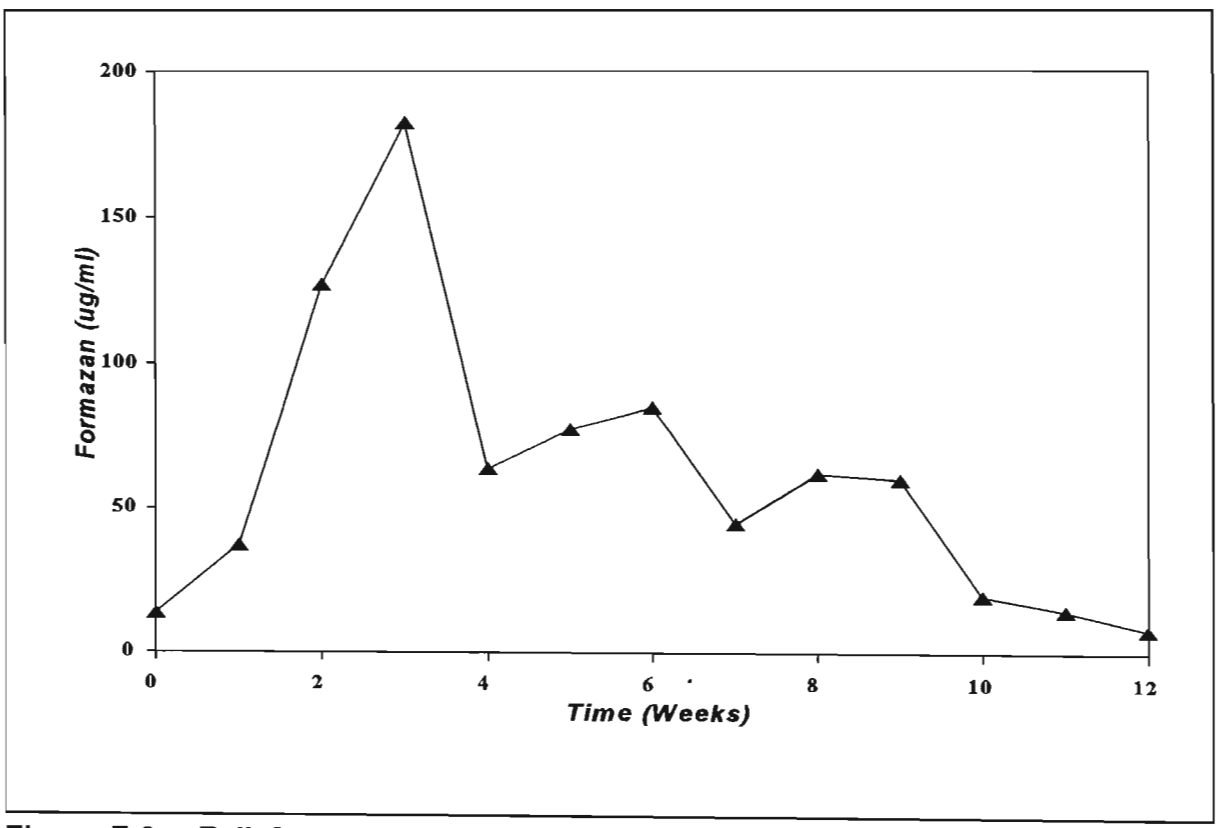

Figure 7.6: Full formazan development profile, as determined by the INT assay, after the Ash treatment.

improved oxygen availability have been reported by other authors (Trevors *et al.*, 1982). Microbial activity for the Ash treatment was exceptionally high (Figures 7.5 and 7.6) and occurred between 2 and 10 weeks. In the case of the Nutrients and Pine-bark treatments, increases in activity which could be attributed to hydrocarbon catabolism were only observed between weeks 6 and 10. This discrepancy could be explained in terms of rapid aerobic metabolism of hydrocarbons in the Ash treatment which resulted in high microbial activity soon after treatment application. Similarly, the benefits of nutrient supplementations can be seen where microbial activity was considerably enhanced compared with the effects of water addition. Pine-bark did not appear to increase the microbial activity above the level that was obtained with nutrients alone. This suggested that pine-bark is less effective in increasing soil porosity than the ash.

Microbial activity, as monitored by the INT method, for the different treatments showed a positive correlation with the results presented in Chapter 3. The greatest hydrocarbon attenuation resulted from the bulking (Ash) and nutrient (FC) treatments. It can thus be inferred that high microbial activity was indicative of hydrocarbon catabolism and that monitoring such activity may provide a marker for assessing the progress of bioremediation.

Increases in microbial activity, as indicated by formazan production, following electron donor addition have been observed by other authors (Trevors *et al.*, 1982; Thom *et al.*, 1993). It is, thus, reasonable to assume that a similar effect could be expected following the addition of hydrocarbons to soil. The use of INT offers the advantage of detecting electron transport activity in not only a wide range of microorganisms (Thom *et al.*, 1993) but also under anaerobic conditions (Trevors *et al.*, 1982).

The difficulties of working with hydrocarbon contaminated soils were, however, well illustrated in this study. Clearly, assays, particularly for the determination of microbial activity, are complicated by hydrocarbons which interfere with spectrophotometric absorbance readings. Interference effects also require that the use of conditions best suited for obtaining the greatest formazan (colour) development are advantageous to

accurately gauge microbial activity. It is felt that with the correct methodology development to eliminate interference effects, INT assays could be used as relatively cheap (approximately 25 cents per sample) and rapid assessments of bioremediation progress in a range of soils.

In general, the use of INT as an indicator of microbial activity in a hydrocarbon contaminated soil was found to be satisfactory although several factors must be considered. Wide variations in readings were observed and, hence, the INT assay can only be used as a rough guide of microbial activity in general. Interference from hydrocarbons may vary greatly due to the heterogeneous distribution of hydrocarbons through the soil. This may result in non-linear hydrocarbon dissolution in methanol and, thus, it is recommended that several background readings are made. It is also important to recognize that this method may be site specific and different conditions may require adjustments in the assay protocol.

In conclusion, the assay should prove useful in preliminary treatment investigations i.e. to ascertain wether the soil under investigation has sufficient microbial numbers to facilitate bioremediation. The progress of any subsequent treatment may also be followed by monitoring the microbial activity.

CHAPTER 8

GENERAL SUMMARY AND CONCLUSIONS

8.1 Contaminated Site

From studies conducted under various treatment conditions, it was clear that biodegradation of hydrocarbons occurred in the soil but that this was limited to a small fraction of the total hydrocarbons present. The high concentrations of heavy metals present in the sludge and soil did not appear to adversely affect microbial metabolism and catabolism of the hydrocarbons. This observation was confirmed by oxygen consumption experiments and light and electron microscopy studies. The apparent heavy metal resistance of the microorganisms may be ascribed to low solubilities and immobility of the heavy metals because of the relatively high soil pH and adsorptive capacity. It was also significant that microbial activity, as determined by the tetrazolium salts assay was low for the contaminated soil at the start of treatment. This may have resulted from the excessive and toxic concentrations of the hydrocarbons present.

Although slow rates of hydrocarbon biodegradation are obviously not desirable, the risks of groundwater contamination are not substantially high. Increased biodegradation rates would, obviously, be more desirable but hydrocarbon mobility is also an important consideration for environmental risk assessment. It was found that hydrocarbon mobility in the soil was negligible under accelerated simulated rainfall and low pH conditions and, thus, poses little risk for ground water contamination over the short term. However, with continued hydrocarbon sludge application, the situation may become exacerbated as the increased concentration of hydrocarbon contamination may drastically reduce microbial activity. This would in effect result in soil which is non- treatable by biological means and may require alternative treatment remedies. The costs associated with such treatment is high. Although little legislation exists for penalties concerning polluted soil, it is expected that this situation will change and the appropriate steps should be taken to prevent soil pollution in the meanwhile. It is, however, important to point out that the

uncontaminated soil's capacity for adsorption of metals was greatly reduced by the hydrocarbon application. Hydrocarbon coated soil particles were found to have lowered the soil metal adsorption capacity. From a practical point of view it would, therefore, be important to have sufficient uncontaminated soil underlying the contaminated zone. In relation to this, an additional precaution would be to reduce hydrocarbon loadings onto the site. Further steps should also be taken to prevent a drop in pH which would result in mobilisation of heavy metals. However, an approach such as liming should only be considered after continuous site monitoring as the soil pH is unlikely to drop below 4 at which point the metals could be expected to mobilize into the soil water.

If it can be assumed that the uncontaminated soil used in this study is similar to that which underlies the hydrocarbon contaminated soil (the soil was supplied by the contract holder and could not be verified by the researcher) a few calculations can be made. The soil can most likely (and safely) be allowed to adsorb approximately 10% of its maximum adsorption capacity for Mn, Zn and Ni. This would give a maximum application[1] of 6237kg Mn, 3000kg Zn and 2214kg Ni per acre (assuming a 1m soil depth) which should, preferably, not be exceeded.

For removal of the majority of hydrocarbons present in the soil, bioremediation may prove to be an effective treatment over an extended time period. This could be best achieved by placing emphasis on aeration of the upper soil zone to provide rapid aerobic degradation. Work conducted under anaerobic conditions suggested that anaerobic degradation processes may become more significant with an extended treatment time. Thus, in the deeper soil zones where oxygen is unlikely to penetrate, denitrifying conditions should be promoted possibly with the addition of nitrate in the form of fertilizer. Before the application of nitrate is advocated, however, it is important to recognize that nitrate is, in itself, an important environmental contaminant,

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[1] All calculations are simply for estimation purposes and should not be considered to be absolute since several soil and environmental factors which could potentially effect these values could not be incorporated in the calculations.

particularly when applied in the soluble form.

Provided that the appropriate bioremediation steps resulted in the biodegradation of the hydrocarbon sludge, the problem of heavy metal contamination will still not be resolved. It may be necessary to ensure that the metals are immobilized within a specific soil zone. The most acceptable method of achieving this would be by maintaining suitably alkaline pH conditions through the application of lime. However, should national soil legislation change to penalize the presence of metals in soil then an alternative remedy would have to be found. Recent work in the field of phytoremediation could provide a solution.

Phytoremediation is the use of plants for environmental cleanup, including heavy metals, which can be divided into three areas:
Phyto-extraction, in which metal accumulating plants concentrate heavy metals into harvestable parts of the roots and above ground shoots;
Rhizo-filtration, in which plant roots absorb, precipitate and concentrate toxic metals from polluted effluents; and
Phyto-stabilization, where mobility of heavy metals is reduced due to alleviation of soil erosion and decrease in soil leaching potential (Salt *et al.*, 1995).

Recent surveys indicate that the *Brassicae* (mustard) plant family demonstrates the greatest potential for phytoremediation, displaying high levels of tolerance, rapid growth rate, high biomass yield and the ability to accumulate high concentrations of heavy metals. In relation to treatments such as excavation and removal to landfills, chemical fixation in the soil, soil leaching, and phytoremediation may provide a cost effective alternative (Salt *et al.*, 1995). Phytoremediation would thus be ideal for use in conjunction with microorganism based bioremediation in a hydrocarbon, heavy metal contaminated site. Vegetation removal could be performed after a suitable treatment time and subjected to chemical treatments such as precipitation, flocculation, ion exchange, reverse osmosis and microfiltration with the aim of recovering any metals of interest. The protracted time period required for total metal removal would be offset by

financial gains when compared to expensive soil excavation followed by off-site chemical treatment or disposal. It is, thus, recommended that phytoremediation should be examined for use as a potential site treatment strategy. It should, however, be noted that phytoremediation may be limited due to hydrocarbon toxicity. It is suggested that initial studies be concentrated around developing plant resistance to high hydrocarbon soil concentrations.

8.2 Alternative Treatment Options

Of chief concern is the continuous application of hydrocarbon and heavy metal sludge onto soil which is already contaminated. This would in effect increase the concentration of these contaminants to a level which may be inhibitory to microbial catabolism. A relatively simple solution could be achieved by preventing or modifying sludge loading rates onto the site currently contaminated.

To accommodate excess sludge new disposal sites should be selected and disposal on these areas rotated to prevent excessive contaminant loads. Uncontaminated soil which would be present on the new sites would also aid in limiting heavy metal migration to deeper soil areas. However, due to the recalcitrant nature of the hydrocarbons, the utilization of new sites may still not be sufficient to accommodate loading rates over the long term. It may thus be necessary to pretreat the sludge before soil application to aid in hydrocarbon breakdown and heavy metal removal. The costs of pretreatment through the use of bioreactors is a significant factor to consider and this aspect should be investigated for its financial viability. Other disposal and treatment options such as incineration and landfill discarding should also be considered but are not without their own financial and legal constraints. The selection of new sites should also be carefully evaluated before disposal implementation. Work conducted on the contaminated soil in this thesis has shown that heavy metals migrate at slow rates under leaching conditions. It is thus essential that liner installation be considered at any prospective site. A related issue is depth to groundwater. No data was supplied concerning details of site aquifers, groundwater and soil morphology. Information of this

nature is critical in assessing remedial options. It must be stressed that the selection of new sites must be approached with caution. Disposal onto such sites, even with stringent waste management procedures, will ultimately lead to contamination of more soil. Ultimately, the answer may be found in waste minimisation at source. Until such options are fully evaluated, it is recommended that the following bioremediation practices should be maintained:

1. Monitor soil pH and maintain between 6-8. Lime if necessary (soil laboratory to be consulted).

2. Addition of fertilizer to maintain C:N:P ratio at 500:10:1.

3. Soil aeration by tilling on a weekly basis. The installation of a comprehensive aeration system in the form of blowers or hydrogen peroxide injection wells (with caution) should also be considered.

4. Maintain soil moisture at between 20% and 50% field capacity of the soil.

5. Regular monitoring of the soil and groundwater to establish effectiveness of treatment strategies.

The type of fertilizer which is selected should be fully evaluated before use. Fertilizers such as ammonium sulphate, ammonium nitrate and ammonium phosphate generate more acidity than fertilizers such as urea and anhydrous ammonia (Brady, 1984). However, in general, all ammonium or ammonium-forming fertilizers are acid producing during the nitrification process. Thus, to prevent exacerbating heavy metal migration due to increased acidity, liming may be necessary. Alternatively, nitrate fertilizers may be considered as an option since these are not subjected to nitrification and hence would delay the onset of decreased soil pH.

In spite of the laboratory studies which have been made, a number of questions remain

unanswered. It is unclear as to what extended *in situ* treatment would have to offer in terms of hydrocarbon biodegradation and heavy metal migration. Treatment under field conditions may improve hydrocarbon biodegradation efficiency without providing an answer for dealing with the heavy metals. To fully gauge the outcome of such treatment it is thus essential that full scale studies should be carried out on-site and *in situ*. These studies should be further supplemented by investigating the feasibility of using alternative treatment technologies either individually or as part of a treatment train.

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APPENDIX

Table A.1: Hydrocarbon contaminated and uncontaminated soil chemical analysis (Cedara).

Analysis	Uncontaminated soil	Contaminated soil
Sample density ($g\ cm^{-3}$)	1.2	0.99
P ($mg\ l^{-1}$)	230	3
K ($mg\ l^{-1}$)	168	39
Ca ($mg\ l^{-1}$)	1645	383
Mg ($mg\ l^{-1}$)	143	79
Acidity (Al + H) ($cmol_c\ l^{-1}$)	0.06	0.05
Total cations ($cmol_c\ l^{-1}$)	9.87	2.70
Acid saturation %	1	2
pH (KCl)	6.28	4.95
NIRS organic carbon %	0.34 (min)-2.9 (max)	5.0
Mn ($mg\ l^{-1}$)	68	49
Zn ($mg\ l^{-1}$)	5	41

Table A2: Hydrocarbon contaminated and uncontaminated soil texture analysis (Cedara).

Size fraction %	Uncontaminated soil	Contaminated soil
Clay (<0.002mm)	9	21
Fine Silt (0.02-0.002mm)	5	9
Sand & Coarse Silt (2.0-0.002mm)	86	71

Table A.3: Metal composition of hydrocarbon sludge (SASTECH).

Metal	Concentration ($mgkg^{-1}$)
Al	676
Ni	14
Si	70
Mn	27
Fe	2284
Cr	4
Mg	317
Na	152
Zn	109
V	41
Ca	1077
Cu	7
Pb	11
K	35
Ba	12
Co	1

B	1.5
Hg	0.027
Se	0.020

Table A.4: Semi quantitative analysis (ICP MS) of NH_4-EDTA extractable soil metal concentrations ($mgkg^{-1}$) from the A horizon of the contaminated site (SASTECH).

Metal	Concentration ($mgkg^{-1}$)
Cr	0.3
Cu	7.1
Ni	48.7
Cd	0
Zn	136.7
Mn	311.4
Pb	6.8
V	59.4
La	12.5
Sr	15.3
Co	10.3