BIOREMEDIATION OF OIL-CONTAMINATED SOIL : A SOUTH AFRICAN CASE STUDY

Zoë Marie Lees (B.Sc. Hons., N.H. Dip.)

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Faculty of Science, University of Natal, Pietermaritzburg

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" The contaminated land genie is out of its bottle, and try as they might no one will be able to shove it back in."

(*Julian Rose, New Scientist, August 1993*)

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PHYTOREMEDIATION : SOIL RECLAMATION USING SOYBEAN PLANTS IN CONJUNCTION WITH NUTRIENTS AND COMPOSTED PINEBARK

DECLARATION

The experimental work described in this document was conducted in the Department of Microbiology and Plant Pathology and the Department of Agronomy, University of Natal, Pietermaritzburg, under the supervision of Professor E. Senior and Professor J.C. Hughes. These studies, except where the work of others is acknowledged, are the result of my own investigations.

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ABSTRACT

In 1990, an oil recycling plant situated in Hammarsdale, South Africa, was decommissioned and a decision was taken by management to rehabilitate the site in preparation for resale. The heavily impacted area covered over two hecatares and oil contamination penetrated soil to depths in excess of three metres, making excavation and removal of the soil very expensive. The options for remediation of the site were limited. No facility for incineration of contaminated soil exists in South Africa, and landfilling was not permitted. The emphasis in developing a remediation strategy, therefore, focussed upon the possibility of *in situ* remediation with minimal excavation of soil. This study, the first of its kind in South Africa, was subsequently initiated to assess the feasibility of this approach, the results of which would underpin a full-scale cleanup programme.

The development of such a strategy involved four key stages of work : (1) a comprehensive site investigation to evaluate and fully understand the particular problems at the site; (2) treatability studies to determine the potential for biological treatment of the contaminated soil and the optimisation of such treatments, particularly in terms of time and cost; (3) the testing of some of the more effective treatments on a pilot-scale; and (4) recommendations for full-scale bioremediation of the contaminated site. Various conditions unique to South Africa had to be considered at each stage viz. the lack of funds and remediation experience, which created numerous problems and emphasised the requirement for a simple, "low-tech" approach.

Site investigations revealed that *in situ* remediation may be possible due to the high permeability of the sandy soils and low concentrations of heavy metals. Laboratory experiments also showed that a mixed association of indigenous microorganisms was present which, once stimulated by nutrient

supplementation at C:N:P ratios of between 10:1:1 and 20:1:1, was capable of degrading total petroleum hydrocarbons at an average rate of 11% week^{-1}. Further experimentation, aimed at reducing the cost of remediation and improving the soil quality, focussed on the efficacy of oil solubilisers, a soil ameliorant (composted pine-bark), indigenous fungi and higher plants in the remedial process.

Three commercial surfactants (Arkopal N-050, N-060 and E2491) and one natural solubiliser (soybean lecithin) were tested for their biotoxicity, solubilisation and biodegradability at various concentrations (0.01 - 1.0%). Formulation E2491 was able to support a microbial population and was selected as the preferred commercial surfactant if soil washing was to be recommended; however, lecithin was considered to be more useful *in situ* because of its localised solubilising effect, biological origin and nutritional contribution.

The use of fungi was of particular interest in addressing the persistent organic compounds, such as the heavy fractions of oil, for which bacterial remediation methods have been slow or ineffective. While it was not possible, however, to demonstrate in the laboratory that the indigenous fungi contributed significantly towards the degradation of the contaminating oil, the basic trends revealed that the fungal component of the indigenous microbial population was readily stimulated by the addition of nutrient supplements. The bulking-up process was also a success and additional exploratory work was proposed in the form of a larger scale composting design.

Finally, the potential for using higher plants and 20% (v/v) composted pine-bark (in addition to nutrients) to increase the microbial degradation of the contamination was investigated in both greenhouse and field plot studies. Greenhouse investigations employed soybeans which were postulated to

have soil quality and cost benefits. However, although the soybeans were found to significantly enhance the remedial process, the complex soil-contaminant-plant interactions gave rise to strange nutritional effects and, in some cases, severe stunting. In contrast, the field studies employed grasses that had previously established on the site and which ultimately demonstrated a better tolerance for the contaminated conditions. Scanning electron microscopy revealed that there were considerable differences between the root tips of soybean plants which had been grown in contaminated soil and those which had been grown in uncontaminated soil. It was concluded that toxicity symptoms, which are readily observed in the root, could be used as an early indicator for determining the suitability of vegetation for remediation purposes. In both instances, despite the differences, the addition of composted pine-bark and nutrients (nitrogen and phosphorus) resulted in total petroleum hydrocarbon reductions of >85%, illustrating the benefits of plant establishment and oxygen availability.

The need to link results from laboratory or pilot-scale experiments to achieve reliable predictions of field-scale behaviour was an essential component of this research. The results of the field study provided evidence, similar to that found in the pot trial, of the accelerated disappearance of organic compounds in the rhizosphere. All experiments incorporated parallel measurements of hydrocarbon residues, microbial activity and pH changes in the contaminated soil, the results of which strongly supported the argument that biodegradation was the dominant component of the remediation process. Thus, after consideration of the significant interactions which dominated the study (time-contaminant-nutrient; time-contaminant-pine-bark; and time-contaminant-pine-bark-plant), it was clear that, aside from these limiting factors, little should preclude the *in situ* bioremediation of the impacted soil.

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GLOSSARY OF TERMS AND ACRONYMS USED IN BIOREMEDIATION

Abiotic reactions :.Occurring without the involvement of microorganisms. Included are inorganic, organic, photolytic, surface-catalysed, sorptive, and transport processes.

Aerobic respiration : The process whereby microorganisms use oxygen as an electron acceptor to generate energy.

Air sparging : Injection of air into groundwater to remove volatile chemicals and deliver oxgen, which promotes microbial growth.

Air stripping : Above ground process used to remove volatile contaminants from water. It involves exposing the water surface to a large volume of air, usually by flowing water through a tower in one direction and air through the tower in the opposite direction.

Aliphatic hydrocarbon : A compound composed of carbon and hydrogen joined in a linear chain. Petroleum products are composed primarily of aliphatic hydrocarbons.

Anaerobic respiration : The process whereby microorganisms use a chemical other than oxygen as an electron acceptor. Common substitutes for oxygen are nitrate, sulphate and iron.

Aquifer : An underground geological formation that stores groundwater.

Aromatic hydrocarbon : A chemical formed from benzene rings, originally called "aromatic" because of benzene's distinctive aroma. Solvents, many types of pesticides and polychlorinated biphenyls (PCBs) are composed of aromatic hydrocarbons.

Bioaugmentation : The addition of non-native microorganisms to a site.

Biocurtain : A large quantity of organisms grown underground specifically to stop contaminant migration by creating localised clogging.

Biodegradation : Biologically mediated conversion of one compound (usually complex) to another (simpler compound).

Bioremediation : A managed or spontaneous process in which biological, especially microbiological, agents catalyse/act on pollutant compounds, thereby remedying or eliminating environmental contamination (biorestoration is a synonym).

Biotransformation : Microbially catalysed transformation of a chemical to some other product.

Bioventing : Circulation of air through the subsurface to remove volatile contaminants and provide oxygen, which stimulates microorganisms to degrade remaining contaminants.

BTEX : Acronym used for benzene, toluene, ethylbenzene and xylenes, which are compounds present in petroleum and other petroleum products, coal tar and various organic product formulations.

Co-Metabolism : A reaction in which microorganisms transform a contaminant even though the contaminant cannot serve as an energy source for the organisms. To degrade the contaminant, the organisms require the presence of other compounds (primary substrates) that can support their growth. Microbial growth does not result and co-metabolic reactions are not expected to accelerate. However, other microorganisms may be able to mineralise products of co-metabolism.

Desorption : Opposite of adsorption; the dissolution of chemicals from solid surfaces.

Diauxy : Selective biodegradation of some organic compounds over others, which sometimes occurs when the compounds are present in mixtures.

Electron acceptor : The compound (e.g. oxygen) which receives electrons (and, therefore, is reduced) in the energy-producing oxidation-reduction reactions that are essential for the growth of microorganisms and bioremediation.

Electron donor : The compound that donates electrons (and, therefore, is oxidised). In bioremediation the organic contaminant often serves as an electron donor.

Engineered bioremediation : A type of remediation that increases the growth and degradative activity of microorganisms by using engineered systems that supply nutrients, electron acceptors and/or other growth-stimulating materials.

***Ex Situ* :** Latin term referring to the removal of a substance from its natural or original position. Directly translated, these words mean "not in its original place" (the reverse of *in situ*).

Fermentation : The process whereby microorganisms use an organic compound as both electron donor and electron acceptor, converting the compound to fermentation products such as organic acids, alcohols, hydrogen, and carbon dioxide.

Fixation : Process whereby microorganisms obtain carbon for building of new cells from inorganic carbon, usually carbon dioxide.

Free Product Recovery : Removal of residual pools of contaminants, such as petroleum floating on the water table, from the subsurface.

Gas Chromatograph : Instrument used to identify and quantify chemicals in a sample, by separating compounds according to their boiling points and polarities.

Hydraulic conductivity : A measure of the rate at which water moves through a unit area of the subsurface under a hydraulic gradient ie the amount of groundwater that moves through a unit section of the subsurface in a given time.

Hydraulic gradient : Change in head (i.e. water pressure) per unit distance in a given direction, typically in the principal flow direction.

Hydrophobic compound : A water-repellent compound, such as oil, that has low solubility in water and tends to form a separate phase.

***In situ* :** In Latin, "in situ" means "in its original place".

Infiltration gallery : Engineered system which is used to deliver materials that stimulate microorganisms in the subsurface. Infiltration galleries typically consist of buried perforated pipes through which water containing the appropriate stimulating materials is pumped.

Intrinsic bioremediation : A type of bioremediation that manages the innate capabilities of naturally occurring microorganisms without taking any engineering steps to enhance the process.

Intrinsic permeability : A measure of the relative ease with which a liquid will pass through a porous medium (eg. the subsurface). Intrinsic permeability depends on the shape and size of the openings through which the liquid moves.

Kinetics : Refers to the rate at which a reaction occurs.

Landfarming : An above ground process used to stimulate microorganisms to degrade contaminants in soil. The process involves spreading the soil out, adding nutrients and tilling.

Metabolism : The chemical reactions in living cells that convert substrate sources to energy and new cell mass.

Micelle : An aggregate of molecules, such as surfactant molecules, that form a small region of nonaqueous phase within an otherwise aqueous matrix.

Microbe : The abbreviated term for microorganism.

Microorganism : An organism of microscopic size that is capable of growth and reproduction through biodegradation of "food sources", which can include hazardous contaminants.

Mineralisation : Conversion of an organic molecule to its inorganic constituents (e.g., CO_2). Mineralisation occurs when an organic compound is altered by central catabolic and anabolic cellular mechanisms. The responsible organism(s), typically, benefit from mineralisation reactions - thus, microbial growth is expected and a portion of the carbon in the original organic molecule is usually incorporated into biomass.

NAPL : 'Non Aqueous Phase Liquid'. A liquid solution that does not mix easily with water. Many common groundwater contaminants, including chlorinated solvents and many petroleum products, enter the subsurface as NAPL solutions.

Oxidise : The transfer of electrons away from a compound such as an organic contaminant. The coupling of oxidation to reduction usually supplies energy

that microorganisms use for growth and reproduction. Often, but not always, oxidation results in the addition of an oxygen atom and/or the loss of an hydrogen atom.

Phytoremediation : The use of green plants to remove, contain, or render harmless, environmental contaminants.

Plume : A zone of dissolved contaminants. A plume usually originates from the contaminant zone and extends for some distance in the direction of groundwater flow.

Primary substrates : The electron donor and electron acceptor that are essential to ensure the growth of microorganisms. These compounds can be viewed as analogous to the food and oxygen that are required for human growth and reproduction.

Pump and treat system : Most commonly used type of system for cleaning up contaminated groundwater. Pump and treat systems consist of a series of wells used to pump contaminated water to the surface and a surface treatment facility used to clean the extracted groundwater.

Rate-limiting material : Material whose concentration limits the rate at which a particular process can occur.

Reduce : The transfer of electrons to a compound, such as oxygen, that occurs when another compound is oxidised.

Reductive dehalogenation : A variation on biodegradation in which microbially catalysed reactions cause the replacement of a halogen atom on an organic compound with a hydrogen atom. The reactions result in the net addition of two electrons to the organic compound.

Sorption : Collection of a substance on the surface of a solid by physical or chemical attraction.

Solubility : The measure of the potential for the constituent to become dissolved in water. Solubility affects mobility, leachability, availability for biodegradation and the ultimate fate of the compound.

Surfactant : Soap or a similar substance that has a hydrophobic and a hydrophilic end. Surfactants can bond to oil and other immiscible compounds to aid their transport in water.

Toxicity : Toxic conditions can inhibit or not allow organics to be biodegraded.

Unsaturated zone : Soil above the water table, where pores are partially or largely filled with air (Vadose zone).

Vapour pressure (volatility) : The measure of the potential for the compound to evaporate readily at standard temperature and pressure. The higher the vapour pressure, the more volatile the compound. Volatility impacts on the level of off-gas control required for biological liquid/solids treatment and other bioremediation processes.

Viscosity : The resistance of a fluid to continuous deformation when subjected to a shear stress. High viscosity constituents tend to fill the soil pore spaces and reduce the effectiveness of technologies which require the flow of air and water.

CHAPTER ONE

BIOREMEDIATION : A PRACTICAL SOLUTION TO LAND POLLUTION

1.1 Introduction

The production and subsequent storage or transportation of hazardous materials are integral parts of the economy. Consequently, there is an enormous variety of synthetic organic chemicals (xenobiotics) which enter the environment by way of : leakages or spillages from pipes and tanks; deposition of airborne emissions; storage and disposal of raw materials or unwanted wastes and residues (for example, sludge lagoons, mixed landfills, slag areas, etc.); use of contaminated fill material; application of sewage or industrial sludge to land; and spraying of pesticides. In most instances, these problems involve the contamination of soils and/or groundwater and may also involve the contamination of sediments either on-site or in nearby water bodies (i.e. drainways, rivers, lakes). Furthermore, point- and non-point discharges of pollutants result in the contamination of sediments in natural surface water bodies. Although the majority of polluted sites have been identified in the United States and the European continent, few (if any) such records exist for most Third World countries where numerous uncontrolled waste disposal sites represent a critical problem. In addition, there are untold numbers of operating industries, as well as commercial, residential and agricultural lands, with on-site contamination problems. Amelioration is thus a daunting and often expensive challenge. Although many cleanup strategies may be considered, *in situ* bioremediation represents, arguably, the most cost-effective and ecologically friendly solution.

Bioremediation is the use of natural, enhanced or genetically engineered organisms to improve environmental quality by exploiting their ability to treat hazardous (including toxic) or merely offensive compounds at contaminated sites. This natural process has been used for decades to treat

wastes such as municipal sewage and effluents from industrial processes such as oil refining and chemical manufacture. It is also emerging as an extremely attractive alternative technology for the economic treatment of a wide range of environmental contaminants. Natural biological processes involved in bioremediation can be selectively enhanced and focused on the rapid removal of noxious or hazardous chemicals from soil, water and, even, the atmosphere. Bioremediation can mineralize waste products and hazardous chemicals into water, carbon dioxide, biomass or other innocuous products and thus negate the need to move the contaminants from one site or medium to another. In many cases, this may also be accomplished with considerably less environmental and worker exposure to hazardous substances.

The range of compounds treatable by bioremediation is immense. In addition to municipal waste and process waters, microorganisms can degrade pesticides, industrial chemicals, jet fuel, gasoline and many components of crude oil. Even compounds that, until recently, were not regarded as biodegradable, such as chlorinated solvents, polychlorinated biphenyls (PCBs), chlorofluorocarbons and other stable synthetic organics, are being biodegraded in the laboratory by selected strains or associations of microorganisms. It now appears that, given the right conditions, most organic compounds, both natural and man-made, can be catabolized by microorganisms, either through direct utilization or by co-oxidation. Bioremediation may also be used either directly or indirectly to treat inorganic wastes through, for example, the transformation of nitrogen- or sulphur-containing compounds, or the mobilization/ immobilization of metals.

A significant advantage of bioremediation is that it can be very cost-effective. Adding fertilizer, other nutrients, plants, and even selected microorganisms to contaminated soil/water, or otherwise manipulating the microbial environment, can often be much less expensive than alternative processes such as incineration, the use of adsorbents and catalytic destruction. Bioremediation also offers inexpensive treatment options for materials such as PCBs and other highly toxic contaminants which present a daunting liability to responsible industries. In

addition, we now have the tools of genetic engineering to manipulate the genetic material that encodes degradative enzymes and pathways and so can create vastly superior microorganisms which may have future use in bioremediation.

There are a number of types of bioremediation treatments. These include modifications of techniques used historically, in conventional waste treatment, as well as innovative methods. Approaches include: enhancing indigenous microbial activity by adding specially formulated fertilizers to soils or sediments contaminated with, for example, oil or other carbon-rich wastes; landfarming and composting techniques for degradation of refinery wastes and military explosive materials; the degrading of chlorinated compounds in soils by the intervention of microbial enzymes; treating PCBs in soils and sediments with microorganisms; phytoremediation; and degradation of recalcitrant compounds from lagoon sludges and contaminated soils in slurry reactors or other biological processing systems. Groundwater and surface water contamination can be treated by encouraging the growth of indigenous microorganisms to degrade the waste *in situ* by the addition of oxygen or by an alternative 'pump-and-treat' technology. Treatment of toxic or noxious substances (as vapours) is showing promise with bioreactors, containing solid supports, for microorganisms which degrade airborne contaminants as they pass through.

All of these technologies have their applications, either alone or in combination. In choosing an appropriate biotechnology for remediation of a specific hazardous waste problem, it is useful to make a selection based on reliable information. It should be the goal of the waste technologist to base (and record) every decision on a sound evaluation of the alternatives by an unbiased assessment of their applicability from both a technical and nontechnical perspective. Successful bioremediation techniques must address both the heterogeneous nature of the many contaminated waste sites and the complexity of using living organisms. Not all sites can be treated effectively. Bioremediation is not a panacea. At the same time, strong evidence is emerging that the right application of biotreatment, combined with a thorough knowledge of the limitations of this technology, can be highly effective in remediating contaminated sites.

Treatment technologies are selected by considering three factors: (1) cost; (2) time to completion; and (3) familiarity (King, Long and Sheldon, 1992). When choosing an appropriate strategy, selection should be made for the approach that:

(a) most effectively degrades the target compound(s);

(b) best addresses remediation of the matrix or phase in which the contamination is present (dissolved, adsorbed or both);

(c) has proven to be the most effective in transporting nutrients and oxygen (or other electron acceptors) to the microorganisms at the point of contamination;

(d) will make use of as many existing site conditions, utilities and/or labour as possible;

(e) has generated positive case histories for this type of matrix and contaminant;

(f) is least disruptive to the facility;

(g) can be most readily permitted by the authorities involved, or is deemed acceptable by those regulators;

(h) can be most rapidly deployed and initiated;

(i) best suits the site layout or is most mobile for use at other site locations;

(j) will close the site (i.e. reach minimum concentrations);

(k) will allow salvage of the capital equipment once the project is completed;

(l) will offer the most flexibility in system operations;

(m) will most readily complement other technologies to be utilized at the site;

(n) will not require excessive treatment of off-gas, sludges or other residuals; and

(o) will best reduce or eliminate client liability (King *et al.*, 1992).

1.2 Microbial Nutrition and Environmental Requirements

It has been said that every biochemically-synthesised organic compound is biodegradable and that somewhere there is a class of microorganism which possesses the metabolic and enzymatic potential to degrade it (Chakrabarty, 1982). This availability of a "work force" for bioremediation makes most sites suitable for treatment. All sites contain microorganisms but the challenge is to harness their metabolic potentials. The key factors are the environmental parameters at a given site and the nutritional factors, which will facilitate the biodegradation of target

contaminants and so produce the desired remedial end point.

The mineralization, or complete degradation, of an organic molecule in soil/water is mostly a consequence of microbial activity. Few abiotic mechanisms in nature totally convert organic compounds of any kind to inorganic products and mineralization sequences characterize the microbial metabolism of several classes of synthetic compounds. As they convert the organic substrate to inorganic products, they make use of much of the carbon in the substrate and convert it to cell constituents. At the same time, in most cases, energy is released and the populations increase in number and biomass as they assimilate some of the carbon and acquire energy for biosynthesis. Detoxification is a common outcome of mineralization except when one of the products itself is of environmental concern (Alexander, 1981).

Together with the above requirement for carbon, there are at least eleven essential nutrients that must be present in the soil in correct proportions and forms to sustain microbial growth. These are nitrogen, phosphorus, potassium, sulphur, iron, calcium, manganese, zinc, copper, cobalt and molybdenum. A typical bacterial cell is (w/w): 50% carbon, 14% nitrogen, 3% phosphorus, 2% potassium, 1% sulphur, 0.2% iron and 0.5% each of calcium, magnesium and chloride (Alexander, 1977). It is not usually necessary to supplement soil/water with the latter nine elements as they are normally present in sufficient concentrations (Clarke, 1979) and are rarely limiting factors. In contrast, nitrogen and phosphorus are of concern since they may become limiting and, thus, supplementation is usually necessary. As a general rule, nitrogen must be present in the form of nitrate (Swindell, Aelion and Pfaender, 1988a) although some species can utilize ammonia or nitrogen gas, while orthophosphate must be available as the source of phosphorus.

Microorganisms also require moisture for their growth and metabolism. Elemental uptake is by absorption and transport of solubilised molecules across cell membranes. Thus, the amount of water present in the treatment matrix is critical in making the target molecules available to the microorganisms. Optimal

water content for aerobic bioremediation of soils is usually 10 to 20% by mass (Hinchee and Arthur, 1991). Over the range of 30 % to 90 % of the water-holding capacity of the soil, the moisture content has little effect on biodegradation rates. Overwatering will, however, saturate the soil and promote anoxic conditions which, in most cases, are undesirable.

During aerobic biodegradation, carbon atom oxidation releases electrons which are passed directly to molecular oxygen. When free oxygen is not available many microorganisms can oxidize carbon through anaerobic pathways, utilizing nitrate or sulphate as a terminal electron acceptor. In general, aerobic degradation proceeds at a faster rate than anaerobic degradation and, as a result, most remediation technologies have focused on an aerobic solution.

Together with nutritional requirements, microbial activities are influenced by environmental conditions. Although it may be said that microorganisms are found in most extreme situations, the majority prefer a pH of between 6 and 8. Similarly, they metabolize at temperatures between 0° and 80°C (Higgins and Burns, 1978). Bioremediation rarely takes place at temperatures beyond these limits although it must be realized that temperature profoundly affects the rates of degradation and substrate availability (Dragun, 1988) and must, therefore, be seriously considered.

A familiarity of the basic responses of microorganisms to their physical and chemical environments, and their nutritional needs, is fundamental to the application of bioremediation.

An exhaustive discussion of the many biodegradative pathways known to exist for organic compounds can be found elsewhere (Stewart and Kallio, 1959; Treccani, 1964; Trudgill, 1978; van der Linden, 1978; Cerniglia, 1984; Perry, 1984; Singer and Finnerty, 1984; Vestal, 1984; Leahy and Colwell, 1990; Sims, 1990) and will receive no attention here.

1.3 Bioremediation Options

Numerous technologies to remediate sites exist and many can be used in process control in industries. Some technologies have more applications than others while some are more specialized or contaminant-specific. The following discussion is not an exhaustive review of all the available cleanup methods and design approaches. Instead, the salient aspects of *biological* remediation are given. No one technology is suitable for all sites, since every site and contaminant situation is different.

1.3.1 Bioreactors

Bioreactor technology is broad in application, competitive and, still, innovative. Researchers are constantly striving to achieve the design of the absolute bioreactor: the reactor capable of fitting every possible cleanup situation. The bioreactor sector of the bioremediation industry is one in which engineers and scientists can, through manipulation of the elements of nature, create a near-perfect environment for biodegradation. This environment of total control is achievable because the matrix (e.g. soil or sediment) does not, as in other technologies, govern the success of the process and, thus, the treatment tends to produce quick results under both aerobic and anaerobic conditions. The close ties of this approach to conventional wastewater treatment also aid its favourable perception by both environmentalists and engineers. Simplistically, a bioreactor is a reaction vessel which has a system for delivering oxygen and nutrients and devices for thorough mixing and the adjustment or maintenance of pH. Also, the reactor is normally fitted with influent and effluent pumps and can be run in a batch or continuous mode (**Figure 1.1**).

All bioremediation bioreactors use water to provide an aqueous matrix, whatever their treatment purposes, and they have the flexibility of providing primary, secondary or tertiary treatment.

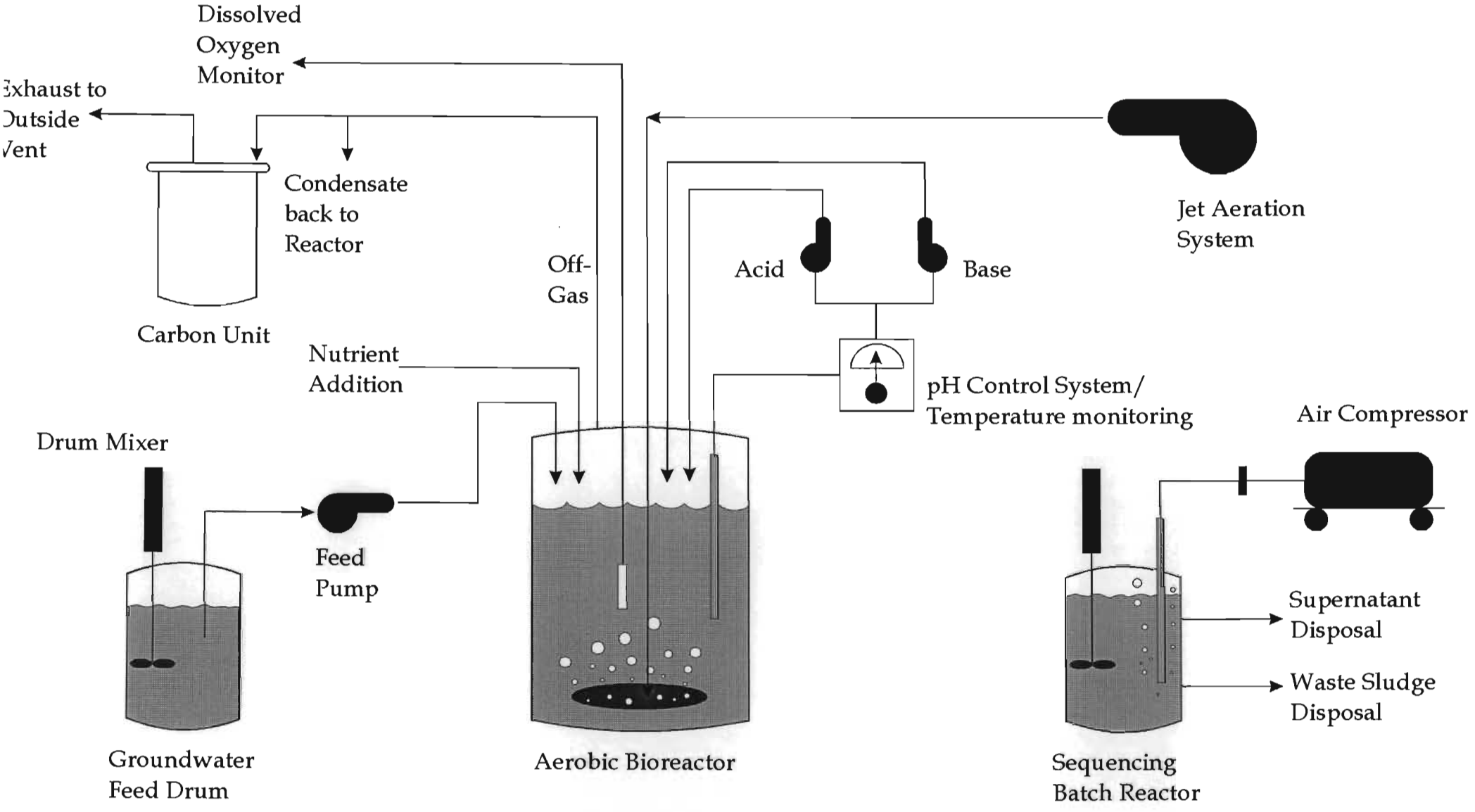


Figure 1.1: Typical Aerobic Bioreactor Design (after Usinowicz and Rozich, 1993).

An added benefit of their use is that they are mobile and can, therefore, be used on site. Two of the major disadvantages of bioreactors are that excavation of soil or the pumping of groundwater is necessary and, during treatment, they all produce a certain amount of sludge or biomass, and/or a volume of off-gas (carbon dioxide, methane, hydrogen sulphide etc.) which must be correctly disposed of. These, unfortunately, increase the treatment costs considerably.

The types of bioreactors used in commercial soil and groundwater bioremediation are numerous and include submerged fixed-film, plug flow, fluidized bed and sequencing batch reactors (King *et al.*, 1992). There are also slurry reactors and reactors which are designed to treat vapour phase contaminants. These all bear a resemblance to other bioremediation options where microorganisms, contaminants, nutrients and (usually) oxygen are all brought into contact in a favourable environment for biodegradation to occur. The preferred above-ground bioreactor designs use suspended microbial growth on a fixed solid support. The principal advantages are reduced sludge production and a more rapid degradation rate, probably due to the greater surface area of the biofilm. However, caution must be exercised when using these techniques as non-biodegraded compounds may be adsorbed onto the biofilm and the support matrix (especially charcoal), making the generated sludge a potentially hazardous material (Tsezos and Bell, 1988).

Although it is always preferable to use indigenous microorganisms for the treatment of soil and groundwater, they are not always successful. Bioreactor systems do, however, lend themselves well to genetically-engineered microorganism technology because of the enclosed design that facilitates greater control of the organisms.

1.3.1.1 Aerobic Bioreactor Technology

(i) Slurry Bioreactors.

One reactor which has particular significance in the bioremediation arena is the soil slurry reactor. Few publications have emerged using this type of reactor

primarily because they are very expensive (Ross, 1991) and the soil needs to be pretreated before entry into the vessel. However, they show great promise.

Silts and clays, or sludges, are most suitable for this type of treatment as the soil particles must be smaller than 60 mesh (King *et al.*, 1992) so that an adequate slurry, with at least 30 % aqueous medium, can be maintained against gravity. As treatment is completed during several days' retention time, the soils must be dewatered by centrifugation or a belt filter press. They are then, depending on the regulations and cleanup levels attained, reused on site as fill material, landfilled or thinspread in road construction. The resulting water may be recycled or discharged.

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

An interesting pretreatment technique that may be considered for hydrophobic contamination involves the use of a soil washing process with biodegradable non-ionic or anionic surfactants. This removes surface contamination and aggressively scrubs soils so that strongly adsorbed contaminants are released. In doing so, soil volume reduction is achieved and there is a separation step in which washed coarse particles are removed and fines exit the system to a slurry reactor. A full-scale slurry-phase treatment has recently been successfully completed at a former wood preserving site. Cleanup operation included material excavation and handling (approximately 14,140 tonnes), soil washing, slurry reactor treatment and slurry dewatering. Four biological slurry reactors, each with a capacity of 210,000 gallons, were operated in batch-mode, treating 150-170 tonnes of material

per batch. Treatment criteria took 8-12 days to be met, after which time the slurry was transferred to the dewatering unit (Jerger, 1995). There seem to be worthy reasons to believe that the combination of soil washing and slurry bioreactors will be a likely process for future development.

(ii) Thermophilic Bioreactor Treatment.

Aerobic thermophilic biological treatment suggests operating temperatures between 50 and 80°C. These temperatures are not usually considered in the application of aerobic biological treatment although recent reports have suggested that high temperatures offer potential benefits for organic destruction. First, under thermophilic conditions, the aerobic metabolic rate is increased and so the reactor size required for treating any organic waste concomitantly decreases. Second, the generation times for thermophiles are less than one tenth of that for mesophiles, resulting in a significantly reduced biomass sludge for disposal (Usinowicz and Rozich, 1993).

Aerobic thermophilic biological treatment can be used as an alternative to anaerobic biological treatment for: sludges or sediments that are highly contaminated with organic compounds; hot streams containing biodegradable organics; and highly contaminated (organic) groundwater. As bacterial metabolic activities are typically exothermic, research has shown that aerobic reactors operate in a thermophilic range as long as the chemical oxygen demand (COD) is at least 30,000 mg l^{-1}, the reactor is insulated and covered to capture the heat generated, and a relatively efficient aeration system is provided (Usinowicz and Rozich, 1993).

(iii) Vapour Phase Bioreactors (Biofilters).

Another area of bioreactor technology receiving a significant amount of development time and funding is the vapour phase filter (Standefer and Van Lith, 1993). In Europe, biofiltration has become an accepted technology for treating volatile organic compounds (e.g. air emissions during *in situ* bioremediation, composting and bioreactor off-gas) and odour-containing industrial exhausts (Duncan, Bohn and Burr, 1982; Prokop and Bohn, 1985). It is now a proven

technology that is very economical for high volume emissions with low concentrations of pollutants. It is especially attractive because of its low energy consumption and low maintenance requirements.

Biofiltration harnesses the processes of decomposition with immobilized microorganisms. The bioreactors are packed with solid material and the microorganisms are attached to the surfaces as a biofilm. Gaseous wastes are passed through, by an induced or forced draft, and the microorganisms catabolize the organic component of the vapour (Kambell, Willson, Read and Stockdale, 1987). Not only do they provide a truly destructive process but they have none of the landfilling or regenerative problems associated with competing processes (incineration and carbon adsorption). Also, they do not generate sludge. They may also be used at the end of a treatment train to deal with organic emissions that could arise from the application of another technology, such as bioventing.

There are two types of biofilters, namely the soil filter and the treatment bed/disc (Holusha, 1991). The former is the simpler in design. Contaminated air is passed through a nutrient-supplemented compost pile which facilitates catabolism by indigenous mesophilic bacteria. In the treatment bed, the waste air stream and filter are humidified as gas is passed through one, two or more beds of compost, wood chips, refuse, sand or diatomaceous earth.

In the disc approach, a series of humidified discs containing activated charcoal, nutrients, peat, microorganisms and compost, is placed inside a reactor vessel. Biodegradation of the contaminants occurs as the gas is vented through the system. Spent filters can be used as fertilizer as they present no hazard. Biofilters are in use throughout Europe receiving and detoxifying emissions containing aldehydes, esters, amines, ethers, acetone, mercaptans, hydrogen sulphide, organic acids, a host of solvents and aromatic compounds (Standefer and van Lith, 1993).

A carefully engineered biofilter must be enclosed, insulated and have a pre-treatment humidification system together with monitors for temperature and

pressure of the waste stream. The filter medium must contain the essential nutrients, carbon, nitrogen and fibre to be properly operational. Although the initial capital costs are high, biofiltration is still an attractive option because of the low operating costs. Biofilters have been built to receive up to 90,000 ft^3 min^{-1} of air flow using filters up to 20,000 ft^2 in wetted area with retention times of between 6 and 80 seconds (King *et al.*, 1992).

(iv) Submerged Fixed-Film, Plug-Flow Reactors.

This reactor type (**Figure 1.2**) has the reputation of being highly effective and adaptable. As a result, it may be successfully used to treat low concentrations of organics found in groundwater, and in the treatment of wastewater where the organic loading can exceed 1,000 mg l^{-1} (Hamoda and Al-Haddad, 1989). Its success lies in its design flexibility. It can withstand extreme fluctuations in organic loading while maintaining an active biomass for long periods of time (Lewandowski, 1990). The critical factor is the C:N:P ratio which must be 100:5:1 to effect biodegradation.

To apply bioreactor technology to groundwater remediation, a water quality study and, perhaps, a pilot study should be made. As with most bioremediation processes, the system requires a multidisciplinary approach (Bourquin, 1989) to identify potential problems such as metal precipitation or toxicity. The degradation capabilities, pH and nutrient preferences of the introduced or indigenous microorganisms should also be investigated.

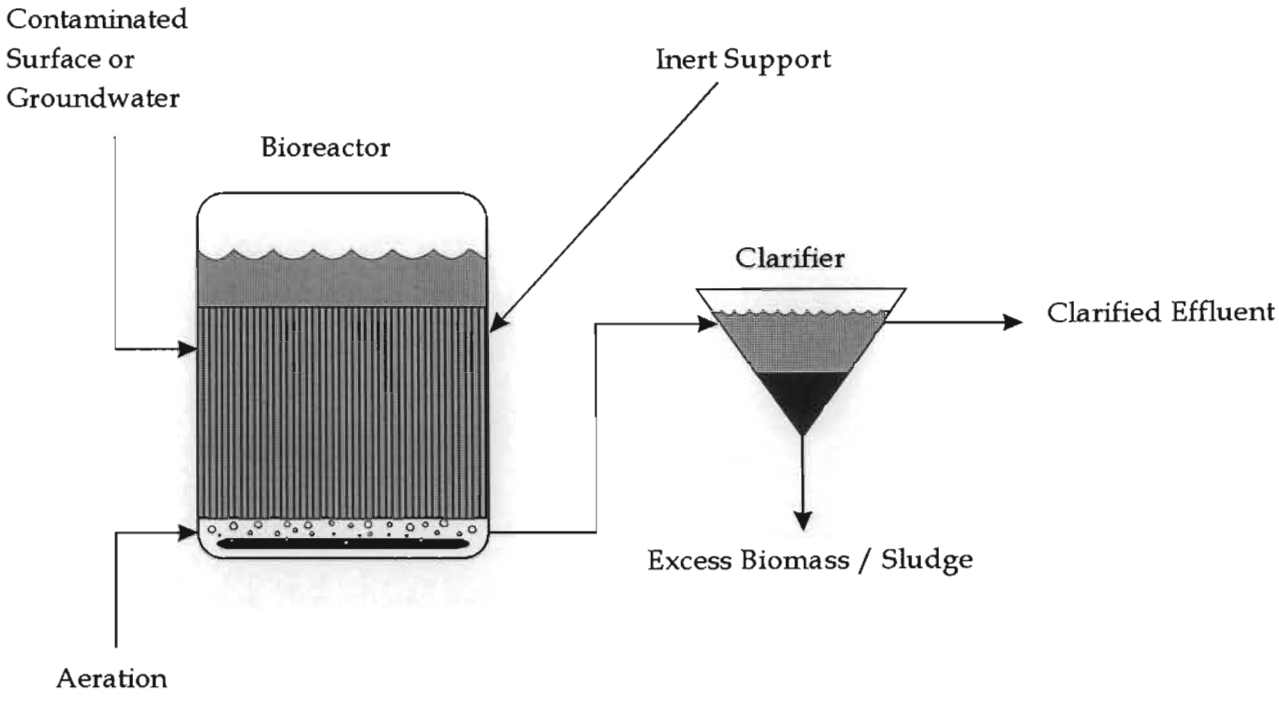


Figure 1.2: Diagrammatic representation of a submerged fixed-film bioreactor

1.3.1.2 Anaerobic Bioreactor Technology

This has many advantages. Anaerobic systems not only retain their biomass longer than aerobic treatment systems but the integrity of the self-sustaining biomass is so strong that contamination is obviated. This means that the biomass produced is extremely efficient in its capacity to degrade high concentrations of waste without high consumption of nutrients. Also, the sludge produced, typically, has a high commercial value as fertilizer. This type of bioreactor operates under the same principles as the aerobic bioreactors and is extremely useful for the treatment of water or soils contaminated with halogenated pesticide residues and chlorinated solvents (Genig, Million, Hancer and Pitt, 1979; Bouwer and McCarty, 1985; Calmbacher, 1991).

As alternatives to bioreactor technologies, solid-state fermentation (composting), land treatment (on-site treatment) and *in situ* bioremediation may be considered.

1.3.2 Composting

Composting is a biological process which depends on the optimal growth, activity and interaction of a mixed population of mesophilic and thermophilic microorganisms. Thus, the composting environment is characterized by elevated temperatures (>50°C), plentiful nutrients, high moisture content (>50%), non-limiting oxygen and a neutral pH. In a sense, this method combines many of the good points of incineration and landfarming and minimizes their disadvantages. Composting as a bioremediation process is similar to the process used for composting of leaves, garbage and food processing residues. The main difference is that the purpose is degradation and loss of specific organic compounds rather than stabilization to produce a mulch or soil conditioner.

The technology involved in composting hazardous wastes falls primarily into three classes i.e. turned Windrow systems, static Windrow systems and in-vessel systems. The process is aerobic, therefore the extent of aeration determines the rate of destruction of the waste and the rise in temperature of the composting

mass. Bulking agents are, therefore, added to improve the overall porosity of the medium, and to enhance the microbial activity by supplying a readily utilisable carbon source, the metabolism of which creates heat. Insufficient aeration leads to anaerobiosis, and an accompanying generation of objectionable odours, although certain pesticides or haloaromatic compounds may be broken down under these conditions (Crawford, Johnson and Goetz, 1993). The most important consideration in the selection of a system for composting a hazardous waste is the control of emissions from the composting operation. The three broad types of composting systems are equally amenable to the effective control of solid and liquid discharges although this equality may not prevail with respect to gaseous emissions.

Williams and Keehan (1993) pointed out that, in composting, the capability of microorganisms to biodegrade specific contaminants may not differ significantly from their capability in the ambient soil environment. The principal difference is the susceptibility of composting to increased control so that the rate and extent of activity can be significantly better than those in landfarming.

The transformation potential differs for a variety of reasons. Firstly, the elevated temperatures increase the process enzyme kinetics. Secondly, the opportunity for co-oxidation may be enhanced due to the range of alternative substrates present. Thirdly, modifications in the physical/chemical microenvironments within the composting mass can increase the diversity of the microflora to which the contaminant is exposed. Finally, the high temperatures will, typically, increase the solubility and mass transfer rates of the contaminants, making them more available to metabolism (Crawford *et al.*, 1993).

There is growing interest in the feasibility of composting as a process for detoxifying, degrading or inactivating hazardous wastes. Unfortunately, little research has been done.

Composting is thought to provide a controlled system for containment of toxic

constituents which may be subject to volatilization or leaching during degradation. Rose and Mercer (1968) found that the insecticides diazinon, parathion and dieldrin degraded rapidly when composted with cannery wastes. The results of composting petroleum refinery sludges were reported by Deever and White (1978) who found that there was a significant reduction in the extractable grease and oil content after composting. Plant potting mixes were prepared from the compost and satisfactory growth was obtained. Studies by Snell (1982) and Epstein and Alpert (1980) showed that several polynuclear aromatic hydrocarbons were degraded during composting; soil contaminated with chlorinated aromatic compounds was successfully decontaminated during a study at the University of Helsinki (Valo and Salkinoja-Salonen, 1986). Research by others (Guenzi and Beard, 1968; Willson, Sikora and Parr, 1983) showed that by interposing specific conditions (e.g. a period of anaerobiosis) two insecticides, initially found to be recalcitrant, could be decomposed.

The methodology involved in the composting of hazardous wastes does not differ greatly from the composting of nonhazardous wastes. Hence, the parameters (particularly aeration, moisture content, temperature and pH) to be optimized are the same. Again, it is essential that nutrients are available in non-limiting concentrations. In the case of hazardous waste, however, emission control is often necessary and, as a consequence, an "in-vessel" system may be required.

If retention time is not a consideration then all but the most recalcitrant of organic wastes would be suitable for composting (Savage, Diaz and Golueke, 1985). The problem is that for some wastes the process is so slow that it entails extended retention times. Despite this, effective composting technology, co-composting of wastes and, perhaps, pre-conditioning of land treatment sites with compost prior to direct waste application could markedly reduce the amount of land required for land treatment systems and greatly minimize the potential environmental impacts of the wastes.

1.3.3 Land-Treatment

Land treatment is a technology developed from the historic refinery practice of landfarming, a method by which oily waste was spread over the soil surface to facilitate, subsequent, natural degradation. Although it is now outlawed in many countries, it was the first demonstration of the use of aerobic microbial processes to surface bioremediate hydrocarbons. Land treatment has been most successful with diesel and crude oils (Willson, Parr, Taylor and Sikora, 1982).

Modern soil bioremediation systems, unlike conventional landfarming techniques, do not rely on large surface areas for spreading contaminated oil and sludge. Instead, solids (i.e. soils or sludge) are placed in Windrows or lined treatment cells (**Figure 1.3**) and atmospheric oxygen is supplied by tilling, forced aeration or negative-pressure systems. Inorganic nutrients (fertilizers) are applied simultaneously either manually or by automated systems installed in the treatment cells. The advantages of biotreatment systems like this are numerous and include a significant reduction in the surface area required for treatment, reduced remediation time because of improved design and increased system control, and the ease of applying treatment to gas emissions if necessary (Hildebrandt and Wilson, 1991). The remediation time can be even further reduced by the constant addition of fermentor-grown degrading microorganisms and weekly turning by a composting machine (Cox, 1995).

The successful operation of a land treatment system is based on a programme that incorporates three phases i.e. a feasibility study; the design and construction of facilities; and the operation and maintenance of the treatment cells.

1.3.3.1 The Feasibility Study

This is usually undertaken to decide whether the chemical, microbiological and hydrogeological conditions are favourable for remediation of the contaminated soil/sediment with a biological treatment system.

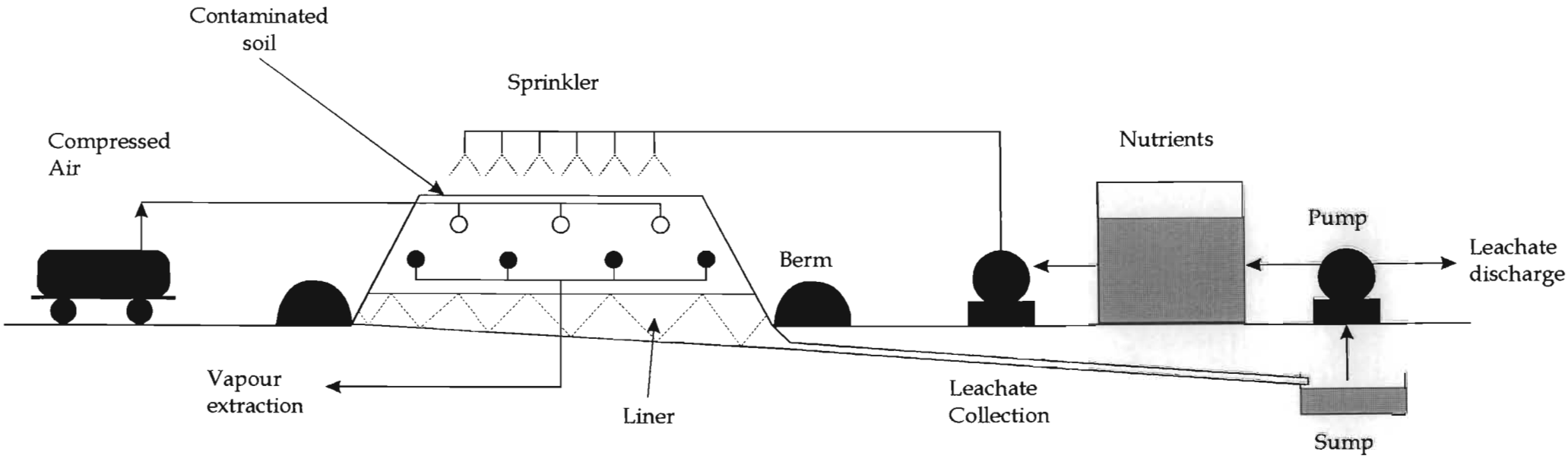


Figure 1.3: Diagrammatic representation of a typical land treatment design (after Hilderbrandt and Wilson, 1991).

Given the simplicity of the technique, it is not surprising that testing to determine whether land treatment should work is relatively straightforward. Usually, a field sampling programme, laboratory analysis and a bench-scale optimization study are included in the programme. Samples are assayed for nitrogen and orthophosphate content, bacterial enumerations of total heterotrophs and specific degraders, soil pH and buffer capacity, moisture content and contaminant concentrations.

The most important part of the feasibility study is confirmation of the presence of indigenous catabolic microorganisms. This is done by bacterial enumeration analyses of aqueous extracts of the solids, following cultivation under carbon-limited conditions. The appropriate nutrient requirements are determined by soil microcosm optimization testing where various types and ratios of nitrogen and phosphorus compounds are added to the contaminated soil and their stimulation effects evaluated. The soil pH is, typically, adjusted to between 6.5 and 8.0. If necessary, water is added to raise the moisture to, approximately, 50% of the field capacity.

Once an active microbial population has been confirmed, tests should be made to identify potential problems regarding air flow and nutrient percolation through the soil matrix. If inadequate mass transfer exists to stimulate the required biodegradation effects, flow rates may be improved through the addition of ameliorants such as wood chips, pine bark (composted) or rice hulls. An accurate estimate of the volume of soil to be treated is also essential.

1.3.3.2 Design and Construction of Above-Ground Biodegradation Facilities

The simplest design is the Windrow, which is a proven design used extensively in the composting industry. Soils for treatment are laid on a synthetic or clay lining in long row-like biopiles about 60 cm thick. Slotted piping is connected to the air system and is installed so that a cylinder-of-influence encompasses each Windrow. Oxygen is either drawn outwards from the centre of the pile (forced-air) or is drawn inwards from the outside atmosphere (towards the piping in a

negative pressure system). Pressure is applied by a regenerative blower to provide air flow. Control of air emissions may sometimes be required if volatiles are displaced by the introduction of air. Nutrients are either mixed with the soil during the pile construction or are supplied by periodic spraying.

Another design which has been used extensively is based on the old landfarming scheme where soil is spread in a number of cells to a depth of up to 50 cm over an impermeable surface/liner. The cells slope towards a sump, for the collection of leachates or runoff water, and a pump redistributes the water back to the treatment cells. Like Windrows, all cells are surrounded by retaining walls (berms). A knowledge of the annual rainfall for the treatment area should indicate whether extra allowance must be made for drainage.

1.3.3.3 Operation and Maintenance of Biotreatment Cells

The soil/sludge is usually tilled, to provide exposure to air, after every significant rain (>25 mm day $^{-1}$) or after two weeks without significant rain (King *et al.*, 1992). The soil water may be maintained at 50% of the field capacity by pumping water from the sump, if necessary. It is important to recognize that inadequate moisture disturbs the osmotic balance between the bacterial association and the medium, while too much moisture reduces air transport by waterlogging.

The condition of liners, pile covers and berms must be checked periodically, and field analyses and laboratory assays must also be made regularly. Soil nutrient, pH and buffering capacity (lime requirement) parameters are monitored and adjusted to ensure optimal bioremediation. Treatment progress is most easily monitored by determining chemical concentrations of samples collected at different intervals and comparing them with the initial concentrations. When the appropriate chemical concentration reduction has been reached, the final sampling and closure steps should be initiated. These steps are different for each country but, generally, data proving that target concentrations have been reached are required to be submitted to the appropriate authorities for consideration.
The costs associated with soil pile bioremediation systems are related primarily to

construction. As with bioreactors, most of the costs are for earth-moving and construction management. Generally, operation and maintenance represent 25 to 30% of the total remediation cost, depending on the volume of soil treated (Hildebrandt and Wilson, 1991).

1.3.4 *In Situ Bioremediation*

In situ bioremediation processes all have a common objective, i.e. to use microorganisms (usually aerobic) to degrade contaminants in soil and/or water with the least disturbance, and are, therefore, recommended in cases where excavation or disposal of soil or groundwater are not possible or economical. This is typically achieved through manipulation of the environmental conditions on site, to stimulate indigenous catabolic microbial associations (Kaufmann, 1986).

The effectiveness of the technique is primarily dictated by the site characteristics. More specifically, the site hydrogeology, climatic factors, soil types and properties, microbiological presence, and the concentration and physical/chemical characteristics of the waste to be treated are key variables. Important hydrogeological characteristics include the direction and rate of groundwater flow, the depth to the water table and/or the contaminated zone, and the heterogeneity of the soil (**Table 1.1**, Sims, Sims and Matthews, 1990).

1.3.4.1 Preliminary Site Investigation

The initial approach is to explore the site history. Information relating to whether site spills were documented, which chemicals or waste materials were stored, used or handled at the site, and whether there were/are leaking tanks present must be gained. Visual observations, such as stained soil, odours and signs of phytotoxicity must all be recorded and can, subsequently, provide the basis for a sampling programme. The initial investigation will usually lead to the installation of groundwater monitoring wells to determine the groundwater characteristics as well as the extent (vertical and horizontal) of the contamination. Cores and water samples collected during well installation are also useful in the feasibility tests.

Well installation must be made under the strict guidance of a qualified geologist/hydrogeologist, who should present a reliable log of the soil stratigraphy and character using a standardized method such as the Unified Soil Classification System (USCS). Logs provide an indication of the sedimentary layers encountered with depth and an estimate of the soil's ability to transport air, water, nutrients, chemical contaminants and by-products through the contaminated zone. Finally, soil samples must also be tested for the presence of heavy metals and other elements which could preclude bioremediation.

(i) Bioassessment Testing .

The investigations so far would give an idea of what is contaminating the site, where it is located and the extent of the contamination. The next step is to determine if bioremediation is feasible by making a bioassessment study. This research may consist of a simple nutrient study or a detailed degradation and stimulation evaluation. The choice depends on the funds and facilities available, and the time constraints.

The main objective of a bioassessment study is to identify factors, other than those realized during the site investigation, which might militate against bioremediation. Initially, soil and/or water nitrogen and orthophosphate concentrations must be determined with microbial characterization tests. Details of the total heterotrophic population and specific degraders are necessary to decide whether there is a suitable indigenous population present *in situ* and if essential nutrients are in plentiful supply. If nutrient supplements, or liming, are necessary then these must be determined by the methods described above (King *et al.*, 1992). A test to determine nutrient permeation/passage through soil would also be appropriate at this stage. Finally, if a chemical oxygen source is required, stability tests of such molecules in the presence of different nutrient concentrations are needed to identify possible transport and decomposition difficulties.

Table 1.1 The site and soil characteristics identified as important in *in situ* treatment of contaminated land (after Sims *et al.*, 1990)

Site and Soil Characteristics Identified as Important for *In Situ* Treatment
Site location/topography and slope
Soil type, and extent
Soil profile properties
boundary characteristics
depth
texture*
amount and type of coarse fragments
structure*
colour
degree of mottling
bulk density*
clay content
type of clay
cation exchange capacity*
organic matter content*
pH*
Eh*
aeration status*
Hydraulic properties and conditions
soil water characteristic curve
field capacity/ permanent wilting point
water holding capacity*
permeability* (under saturated and a range of unsaturated conditions)
infiltration rates*
depth to impermeable layer or bedrock
depth to groundwater*, including seasonal variations
flooding frequency
runoff potential*
Geological and hydrogeological factors
subsurface geological features
groundwater flow patterns and characteristics
Meteorological and climatological data
wind velocity and direction
temperature
precipitation
water budget

* Factors that may be managed to enhance soil treatment

(ii) Pilot Testing.

(a) Microbiology. If the initial bioassessment is favourable, a detailed series of site-specific tests is then required. This treatability study includes a comprehensive investigation to determine the catabolic rates of the target compounds under site (soil and water) conditions. Catabolic optimization is normally achieved through intermittent nutrient and oxygen supplementation in conjunction with target molecule assay and/or by-product appearance in comparison with abiotic controls to monitor non-biological removal rates. Since comprehensive analyses are very expensive, only selected samples should undergo detailed characterization while the rest should be subjected to routine, inexpensive tests. Other test parameters such as pH, nutrient content, oxygen consumption/carbon dioxide evolution may also be useful in monitoring the biological response.

Laboratory degradation rates are likely to be faster than *in situ* rates since microcosm studies (jars, pans, columns or lysimeters) are maintained at near ideal conditions. Realistic extrapolation to field conditions, therefore, necessitates the use of a "fudge factor" (King *et al.*, 1992). This is dependent upon research experience, test conditions and the degree of confidence in site homogeneity. The factor usually varies between 2 and 6. Alternatively, a more detailed study closer to site conditions can be made.

(b) Hydrogeology and Nutrient Delivery. Should the treatment employ groundwater, or if the groundwater is contaminated, then several other tests will be necessary to investigate groundwater behaviour and aquifer characteristics. Simple percolation tests or slug tests provide an estimate of the vertical and horizontal permeability of the aquifer and its ability to convey nutrient-laden water to the region, while a static ring test also provides information about the infiltration rate of water into the soil. The hydraulic conductivity in the area containing the contaminant should be in the order of 10^{-4} cm s^{-1} or greater. Some form of pump test will also be required on at least one well installed in the contaminated zone. Usually, the well is pumped for an extended period, ranging from 6 h to several months, depending on the site hydrogeology and the response

of the aquifer to a sustained pumping rate. These tests yield information on the transmissivity of the system, which is a measure of the volume of water which can be passed through a given cross-section of the subsurface (King *et al.*, 1992). Data obtained from the tests are then used to calculate an approximate flow velocity across the site which is critical for the design of an aqueous injection and recovery system across the contamination plume.

Geochemical testing is recommended at this point to determine the response of the water system to the supplements that will be added during remediation. For example, the intended injection concentration of orthophosphate could cause precipitation of calcium phosphate and the presence of iron, copper and manganese could catalyze the decomposition of hydrogen peroxide. The treatment system must be designed to raise contaminated water to the surface, remove the contaminants, for example, by ion exchange, carbon adsorption or bioreactor treatment, add the right proportions of necessary nutrients and oxygen to stimulate microbial activity in the contaminated area, and re-inject some of the extracted water by well-injection or infiltration galleries (**Figure 1.4**).

It is, therefore, necessary to be able to calculate the pore volume to be treated (i.e. the water volume existing between the injection and recovery systems). Dividing the length of the plume by the groundwater flow velocity gives an approximation of the time required to process one pore volume of water, which is usually targeted to be between 1 and 3 months. Multiplying the expected number of pore volumes by the initial time estimate produces a preliminary estimate of the time required for remediation. It must be clearly recognized, however, that this is only a *prediction*, as few sites are completely homogeneous geologically.

The injection wells and/or infiltration galleries are, typically, placed at the perimeter of the contamination plume, at the highest point of the groundwater gradient, which raises the water table in the immediate area ('mounding'). The water recovery, by contrast, typically occurs at the perimeter of the contaminated area at the lowest point of the groundwater gradient.

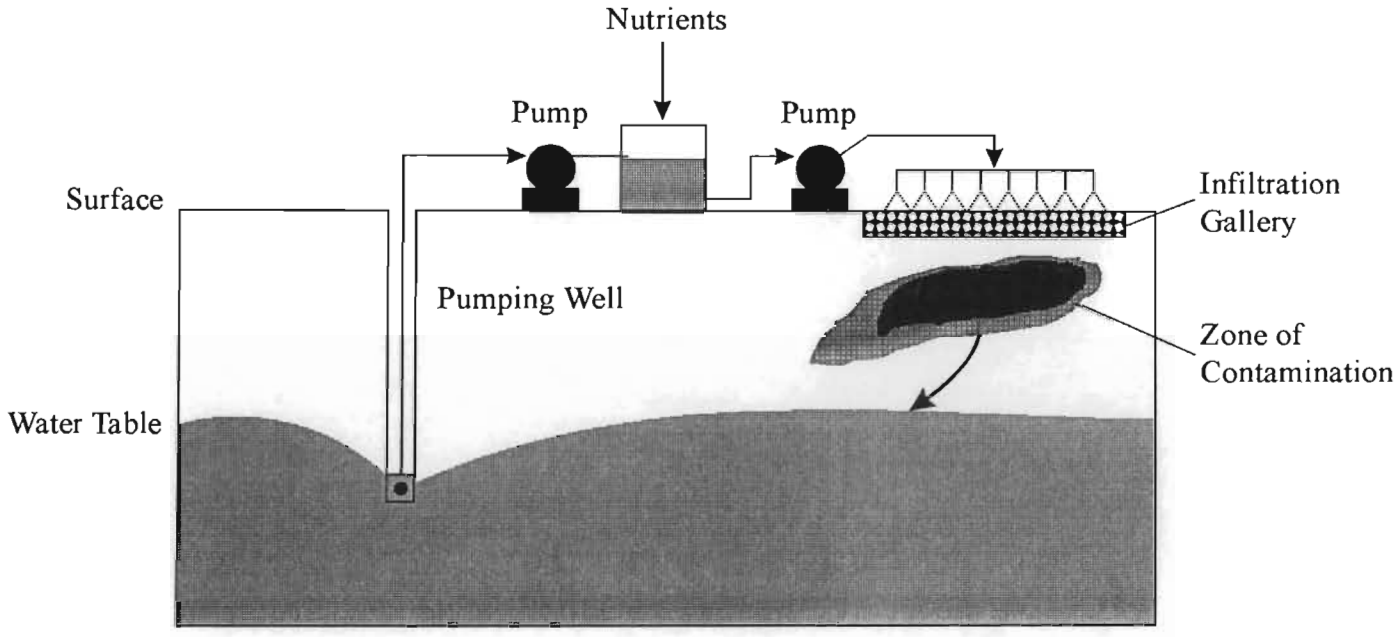


Figure 1.4: Classical *in situ* bioremediation where nutrients are supplied to groundwater which percolates into the subsuface (after Morgan and Watkinson, 1989).

These recovery wells can lower the water table in the immediate area, forming a 'cone of depression', which may pull clean water from areas adjacent to the recovery zone, a situation that should be avoided if possible (Lee, Thomas, Borden, Bedient, Ward and Wilson, 1988). The recovered water is then reintroduced after treatment or supplementation with nutrients upgradient of the biostimulation zone. The rate of nutrient delivery to the contaminated zone, therefore, is often limited by the solubility of the nutrients in water and the reinjection flow rate.

When the nutrients are added to the subsurface, excessive microbial growth may occur around the injection zone, causing significant plugging. A method which has been demonstrated in the field to reduce localized plugging is alternating pulses of electron donor and electron acceptor in the reinjection water. Since both electron donor and acceptor are required for microbial metabolism, advective and dispersive processes within the aquifer must mix the nutrients before conditions promote microbial growth, causing cells to grow dispersed through the aquifer and producing a large biostimulation zone (Semprini, Roberts, Hopkins and McCarty, 1990).

If time and cost factors allow, an *in situ* pilot test of the remediation system is appropriate, especially if the client is inexperienced with bioremediation technology. This affords an opportunity to identify potential problems and modify design/analytical parameters to obtain the best operating conditions. At the same time, confidence is gained to execute the full-scale programme.

1.3.4.2 Full-Scale Implementation and Monitoring

At this stage, the treatment/process design should have been optimized by the expertise of engineers and hydrogeologists. The purchasing and installation/assembly of the necessary equipment is the next phase.

Details of the monitoring phase are different for every situation although there are some common principles. Briefly, field monitoring consists of recording data of

soil and water pH, dissolved oxygen and nutrient concentrations, microbial numbers of heterotrophs and specific degraders (as colony forming units), and contaminant concentrations collected at frequent intervals, particularly during the first few weeks of operation. These will progressively give the project manager a clear indication of the progress of the remediation. Any nutrient or pH adjustments will then be made on the basis of these data. As the remediation proceeds, the sampling will become less frequent until the target contaminant concentrations are reached. At this point, detailed analysis of several samples must be made to confirm that the project objectives have been met.

In situ treatment of contaminated zones is not yet a proven remedy. Although the advantages of attractive economics, minimal site disruption and eliminated liability make it the most promising of all remediation options, it is also the most difficult treatment strategy to control and is chosen when time is not a constraint. Extra investigative work is required to predict its success because of the heterogeneity of most contaminated sites. Neglecting the need for treatability and pilot studies is, therefore, risky. It must be stressed that bioremediation does not work at every site, is not necessarily the least expensive method and may not reach the required cleanup standards in the given time.

1.3.5 *The Oxygen Question*

In situ bioremediation was one of the first technologies with the potential to address both dissolved- and adsorbed-phase organic contamination. Early research demonstrated the feasibility of bioremediation using simple infiltration of nutrient-supplemented water and in-well aeration, and a key finding of this work was the importance of the rate of oxygen delivery (Floodgate, 1973; Zobell, 1973). Where bioremediation was not effective, the failure was often found to be a lack of sufficient oxygen.

1.3.5.1 The Use of Hydrogen Peroxide in Bioremediation

Oxygen supply was thus identified in the industry as the central issue to be

resolved. This challenge led to the first major innovation in bioremediation: the use of H_2O_2 as an oxygen 'carrier'. Hydrogen peroxide was considered promising because it is miscible with water, and each mg l^{-1} can supply ~0.5 mg l^{-1} of oxygen. This meant that the available oxygen could, potentially, be increased beyond the 8-10 mg l^{-1} limit of in-well aeration.

Laboratory studies which investigated the use of H_2O_2 in *in situ* bioremediation (American Petroleum Institute (API) 1987; Brown and Crosbie, 1989) showed that the growth of aerobic bacteria in general, and hydrocarbon-degraders in particular, was significantly enhanced by H_2O_2. Complementary field tests showed that the injection of H_2O_2 solutions into groundwater could increase the dissolved oxygen content at distances of 6 to 15 m from the injection point. Loss of injection as a result of biofouling could be rectified by adding periodic 'spike' concentrations in excess of 2,000 mg l^{-1} to the wells. In 1987, the API demonstrated the successful use of H_2O_2 in a full-scale bioremediation system, where 4, 400 kg of hydrocarbons were removed in 160 days of treatment.

Despite the significant improvement in oxygen supply compared to in-well aeration (air sparging), H_2O_2 has many limitations. Because a basic issue has always been its cost, uncontrolled decomposition or the loss of oxygen equivalents is a serious concern. Therefore, the key to the successful use of H_2O_2 is controlling its decomposition. The conversion to oxygen is most often catalyzed by metals such as Fe and Mn and by the enzyme catalase (which is secreted by many microorganisms). Too rapid decomposition can result in supersaturated water and the subsequent loss of oxygen. Such conditions may result in degassing which causes gas blockage and reduced permeability around injection points.

The potential toxicity of H_2O_2 to microorganisms could be problematic in bioremediation. This issue of toxicity is complex and is influenced by a number of factors which make it impossible to predict the specific threshold toxicity concentration. Most treatments to date have used concentrations <2,000 mg l^{-1}.

A final concern is the precipitation of iron in contaminated aquifers after the introduction of H_2O_2. Such precipitates can plug the aquifer in the vicinity of the injection well thus necessitating frequent maintenance. The precipitation potential can be minimized by adding tripolyphosphate as the phosphorus source or by flushing the aquifer with water which has a low metal content. This may be obtained from a deeper aquifer (Brown, Dey and McFarland, 1991).

Due to the above concerns with H_2O_2 in the unsaturated zone, it was quickly superseded by the development and use of soil vapour extraction technology (Brown and Jasiulewicz, 1992; Brown, Hicks and Hicks, 1993). The original focus of this extraction was to remove volatile organic compounds. Early development work (Thorton and Wooten, 1982), however, showed that increased biodegradation rates were effected in response to the oxygen supplied (Hinchee and Arthur, 1991a). This technology is now commonly referred to as bioventing.

1.3.5.2 Bioventing

Bioventing is an *in situ* remediation technique that combines the physical processes of conventional soil venting with enhanced biodegradation, thus providing speed and flexibility. Soil venting involves the application of a vacuum on vapour extraction wells installed in the unsaturated zone within, or adjacent to, the zone of contamination (English and Loehr, 1991) (**Figure 1.5**). The negative pressure which develops in the soil pores accelerates volatilization of sorbed compounds with high vapour pressures. By lowering the groundwater table simultaneously, by placing dewatering points at and just beneath the water table, additional vapour phase removal can result beneath the normal water table. In addition, the dewatering points facilitate simultaneous removal of groundwater, free product and the vapour phase (Hoeppel, Hinchee and Arthur, 1991).

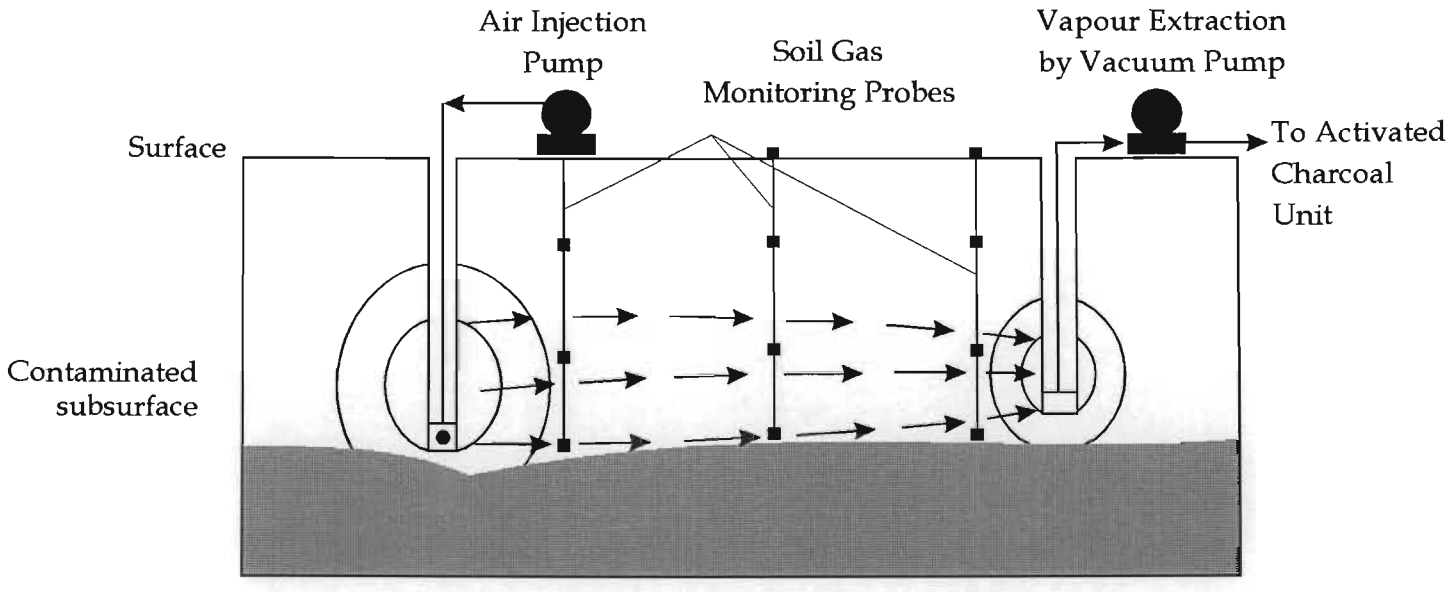


Figure 1.5: Enhanced bioreclamation through soil venting, where air is withdrawn from clean soil (after Hoeppel *et al.*, 1991).

Soil venting increases the rate of air diffusion into the subsurface which, in turn, helps to satisfy the vacuum. Consequently, the air has the potential to diffuse through the soil, displacing the fraction with higher vapour pressures and stimulating aerobic degradation of low volatility compounds (Hinchee, Downey, DuPont, Aggerwal and Miller, 1991b). This is particularly significant in soils with low water permeability because of the greater diffusivities of gases compared with liquids. Since air contains >200,000 mg l^{-1} oxygen, soil venting can overcome the oxygen deficits that often occur in heavily contaminated soils (Connor, 1988).

The limitations of bioventing currently centre on control factors. A primary operational concern is over-pressurizing the sparge system. This can displace both vapours and water and cause the undesired dissemination of contaminated vapours to 'clean' or low pressure areas such as building basements (Brown *et al.*, 1993). Thus, extreme care must be exercised in the design, implementation and control of an air sparging system. As with classical *in situ* bioremediation, the collection of site data and field pilot testing are critical in identifying any irregularities that might restrict airflow or cause accelerated vapour migration.

The number of field demonstrations and pilot applications of bioventing reported in the literature is limited. It has, however, been successful at many sites contaminated with fuels (Downey, Hinchee, Westray and Slaughter, 1988; Hinchee *et al.*, 1991a; Brown and Jasiulewicz, 1992) and shows promise for sites contaminated with halogenated solvents, although the effects of environmental variables on bioventing treatment rates are poorly understood. It is generally known that compounds that are very soluble in water (e.g. alcohols), and which tend to partition in groundwater, are not good bioventing candidates (Reisinger, Johnson and Hubbard, 1993). Also, the tendency of a compound to adsorb to the matrix into which it has been introduced has a bearing on its suitability for soil venting. In these cases, the groundwater is sparged with air and infiltration galleries are built to deliver nutrients into the contaminated subsurface. Sparging would then be conducted with a groundwater capture system to prevent migration of dissolved contaminants. Physical factors such as the thickness of the unsaturated zone, the soil permeability (intrinsic permeability should be greater

than 10^{-6} cm^2), soil moisture content and the macronutrient availability each exert an influence on the success or applicability of this technique.

1.3.5.3 Alternative Oxygen Sources or Electron Acceptors

In designing a bioremediation system, it is important to be aware of the benefits and limitations of all of the available electron acceptors so that the most cost-effective system can be implemented. The injection of water sparged with liquid or pressurized oxygen or ozone, instead of with air, is probably the most obvious alternative to the above techniques and effects a five-fold increase in the concentration of dissolved oxygen (Singh and Medlar, 1992). Another novel approach was investigated by Michaelson and Lofti (1990) who used oxygen microbubbles ("colloidal gas ephrons") created by mixing oxygen under pressure with water supplemented with a surfactant. The resulting colloidal material was a suspension of fine soap bubbles with diameters of 25-50μm that contained up to 65% (v/v) gas, and could occupy up to 55% (v/v) of the pore space in sands.

Other electron acceptors include nitrate, sulphate, carbon dioxide and iron, of which nitrate has been the most extensively researched. This is because it is inexpensive, highly soluble, is not adsorbed to soil matrices and does not decompose as rapidly as hydrogen peroxide. Unfortunately, besides the limitations imposed by water quality standards, nitrate is not as effective as oxygen for the biodegradation of most classes of compounds (Brown and Norris, 1993).

1.3.5.4 Anaerobic *In Situ* Bioremediation

There have been few attempts in commercial bioremediation to use anaerobic conditions for site reclamation. This is an area that has been largely unexplored, and even rejected, because aerobic biodegradation is more energy efficient than anaerobic degradation. Yet, anaerobic systems are prevalent in soil and aquifer environments, and a wide array of reaction mechanisms, including reduction, hydrolysis, dealkylation and dehalogenation, are possible in the absence of

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

When organic matter enters an oxic environment, the indigenous microflora use the contaminants as electron donors to support heterotrophic microbial respiration. Since oxygen is used as an electron acceptor, it is rapidly depleted. This consumption of oxygen provides the selection pressure for other catabolic species. Under anoxic conditions, nitrate, sulphate and carbonate are used as alternative electron acceptors. As a consequence of self-generating redox gradients, one can often observe a spatial separation of dominant metabolic processes, depending on the availability of electron acceptors, the presence of suitable microorganisms and the energetic benefit of each process to the microbial communities. Typically, nitrate reduction occurs first, followed by sulphate reduction and then methanogenesis (Hoeppel, Hinchee and Arthur, 1991). Not surprisingly, different types of biodegradative activities can be observed within depth-related redox zones and research has shown that it may prove possible to stimulate desirable metabolic sequences through the intentional introduction of electron donor or acceptor combinations (Kaake, Roberts, Stevens, and Crawford, 1992; Ramanand, Balba and Duffy, 1993).

Although *in situ* anaerobic treatment would be slow and may be questionable in terms of its overall efficiency in meeting remediation targets, the anaerobic biodegradation of pollutants offers potential advantages over aerobic bioremediation approaches (Semprini, Roberts, Hopkins and McCarty, 1991). Despite the fact that aerobic *in situ* biorestorations provide a cost-effective method

of cleaning up contamination, much of the expense associated with these proceedings is accounted for by the costs of air, ozone, hydrogen peroxide or pure oxygen. Furthermore, biofouling is often a consequence of these treatments. Anaerobic processes, by contrast, are low energy producers and, therefore, generate less biomass which limits biofouling of the system. Also, anaerobic biotransformations sometimes result in metabolic products which are less toxic and more amenable to subsequent aerobic metabolism.

1.4 Conclusions

It is important to recognize that there is unlikely to be a single technology that will work in all situations or that will by itself totally remediate a complex waste. Most likely, waste site cleanups will require 'treatment trains', a sequence of applications of different technologies. The classic use of serialized anaerobic and aerobic degradations in a sequencing batch reactor is a good example. Also, a treatment train for the remediation of creosote or oil contaminated soil and groundwater may involve product removal using a pumping system; flushing with water and surfactants using pump-and-treat technology; and, finally, *in situ* biodegradation of the residual contamination. It should be expected that the uses of complementary technologies will be a common application as part of a sequenced approach to a site remediation programme (Kearney, 1986).

Soils can be treated *in situ* or excavated for bioreactor or land treatment. If time is not a constraint, excavation is impractical, or the contaminants are degradable/moderately volatile, the most cost-effective method is *in situ* bioremediation with soil vapour extraction as an additional option. However, if the quantity of soil to be remediated is relatively small, the soil may be excavated for more timely bioreactor or land treatment. When time is short, conventional landfilling may be considered but with the threat of continuing liability. It is rarely necessary to incinerate soils. From a cost standpoint it is not feasible and there is great public opposition to the potentially toxic gas emissions.

Sludges containing organic or hydrocarbon compounds are amenable to land

treatment, bioreactor processing or composting. Two difficulties associated with sludges are their high moisture content and amorphous structure although, in composting, these difficulties can be ameliorated through the use of a bulking agent such as wood chips. Landfilling or incineration is rarely recommended although, if it appears imminent, a biofeasibility study should be pursued in the interest of economics.

Surface waters and wastewaters containing soluble compounds (organic or inorganic) are particularly suited to biotreatment *in situ* or by means of a treatment train employing bioreactors (aerobic or anaerobic) and/or pump-and-treat technology. Groundwater, by contrast, should always be assessed for *in situ* treatment before consideration of any other alternative. Surface biotreatment, solely, of extracted groundwater could prove to be very expensive due to the volumes of water involved. Simultaneous surface and *in situ* biotreatments are becoming more popular, where product recovery and bioreactor treatment can be coupled with nutrient injection, and subsequent recycling of the produced water.

A very real impediment to applying environmental biotechnology can be the developmental time and costs. Deadlines must be met and frequently the public and the client want the quickest and cheapest treatment available. However, this attitude can be changed through communication and the demonstration that microbial treatment, provided that mineralization is possible, is usually the most rapid and complete treatment system. The true limitations of applying biotechnology to environmental problems are our imaginations and the willingness of microbiologists and engineers to work together.

Microbiological cleanup is a developing technology founded upon basic principles of microbial ecology and physiology. The ability of microorganisms to reduce the potential toxicity of substances to higher organisms through processes of biodegradation, biotransformation and bioaccumulation can easily be demonstrated in the laboratory. In theory, there is no reason why biotechnologies based on such capabilities cannot be successfully developed and applied. In the field, however, these processes may be limited by environmental conditions.

Hence the bioengineering challenge is to realize *in situ* the potential observed in the flask so that biotreatment will become the **first** technology to be considered, not the last.

CHAPTER TWO

BIODEGRADATION OF PETROLEUM HYDROCARBONS IN SOIL ECOSYSTEMS

2.1 Introduction

As pollutants, petroleum hydrocarbons (PHCs) occupy an intermediate position between labile, biogenic and highly recalcitrant, xenobiotic substances. The ecological consequences of petroleum contamination are largely unknown although the molecules are classified as `hazardous' because of their polycyclic aromatic fractions (some of which are known carcinogens) and other toxic constituents. The fate of PHCs after an oil spill, or similar event is, therefore, a matter of some concern and an increasing awareness has prompted stricter national and international regulations relating to public health and safety.

Worldwide, over two billion metric tonnes per annum (mta) of petroleum are produced, and it has been estimated that 1.7-8.8 million mta (0.08 - 0.4 %) of the world production ultimately pollutes the oceans (National Academy of Sciences, 1985). No comparable estimates have been calculated for terrestrial PHC pollution but considering that the greater part of petroleum is produced, refined and utilized on land, the resulting routine, accidental and illegal discharges are likely to equal, if not exceed, the totals cited for the marine environment. Both in the marine and the terrestrial environments, low-level routine discharges account for probably over 90% of the total PHC pollution (Bartha, 1986). Production and transportation accidents such as tanker disasters account for only a small percentage of the total PHC discharge but because of their drastic and highly visible local effects, press coverage and public attention is focused disproportionately on these, predominantly, marine incidents.

The behaviour of spilled oil is very different on water to that on land.

Although oil spreads over a water surface to cover very large areas (which is often alarming to environmentalists) this strongly favours evaporative and photooxidative losses, and may result in 25 - 35 % of the oil being lost through volatilization and photodegradation during the first few days of a marine oil spill (Ward, Atlas, Boehm, and Calder, 1986). In contrast, on land PHCs are subject to rapid vertical movement through soils, therefore evaporative and photodegradative losses are minimal, amounting to only 1 - 2 % of a spill (McGill, Rowell and Westlake, 1981). Infiltrated PHCs move downwards through the soil, where scarcity of oxygen, nutrients and viable organisms restrict biodegradation to extremely low rates (Bartha, 1986). Their persistence is, therefore, assured for years. It is for this reason that this discussion focuses on PHC contamination in soils rather than in marine environments, for which voluminous literature is available.

At present, the legally acceptable disposal methods for refinery wastes and PHCs, that are classified as hazardous, are deep-well injection, burial in a secure chemical landfill, steam/air stripping, encapsulation, vitrification/pyrolysis, incineration and bioremediation (Bossert and Bartha, 1984). Of these alternatives, only pyrolysis, incineration and bioremediation inactivate the hazardous constituents (Bartha, 1986).

2.2 The Biodegradative Approach

It is difficult to follow transformations in natural environments and great caution needs to be exercised if biodegradative routes are to be identified *in situ*. It is, therefore, vital that one has a complete understanding of the process, in order to design a strategy which will be applicable to that specific environment. Most contemporary attitudes and experimental strategies found in the study of biodegradation stem from the Koch tradition of microbiology (Bull, 1980) where the approach is to study a single organism which has been selected on the basis of its ability to utilize a given organic compound as a sole source of carbon and energy. Although much has been accomplished via this approach, it engenders a simplistic

and often misleading view of biodegradation and, at the same time, has tended to obscure many other essential issues. Only very exceptionally do monospecies populations of microorganisms, growing on a single substrate, develop in nature.

Undoubtedly, fundamental studies have provided data that are relevant to the understanding of biodegradation in the environment but, unfortunately, there are serious drawbacks to the monoculture approach e.g. the biodegradative capacity of microbial associations may be greater, both quantitatively and qualitatively, than monocultures. Similarly, the degree of resistance to toxic substances like PHCs may be significantly higher in microbial associations than in the individual species comprising them. An increasing number of examples are being reported in which widely different xenobiotics are degraded by associations of microorganisms (Gunner and Zuckerman, 1968; McLure,1973; Jensen, 1975; Senior, Bull and Slater, 1976; Balba, Al-Sarraj and Senior, 1982; Senior and Balba, 1984). Most of the biodegrading communities studied to date are composed of, or dominated by, bacteria, but a few have far greater complexity and contain bacteria, actimomycetes and fungi (McLure, 1973; Senior *et al.*, 1976). This disproportionate emphasis on bacteria is surprising since the filamentous fungi often constitute the largest fraction of the total microbial biomass in soils (Davies and Westlake, 1979).

The ability to degrade PHCs is not restricted to a few microbial genera since a diverse group of bacteria and fungi have been reported to have this ability (Zobell, 1946). Cerniglia and Perry (1973) found that several fungal species exhibited greater PHC biodegradative activity than selected bacterial species. Research continues to expand the list of microbial species which have the ability to degrade PHCs.

It is known that microorganisms require specific growth conditions which will contribute to the generation of a sufficiently large biomass during biodegradation of PHCs. In this regard, four contributory factors must be

considered i.e. (a) innumerable carbon sources are present in the soil which may be utilized in preference to the contaminating PHCs, or suppress induction of metabolic pathways; (b) competition with indigenous species for the available nutrients, minerals and growth factors may occur; (c) many catabolic species are unable to degrade the PHCs at a sufficient rate to be useful; and (d) key elements essential for mineralization such as nitrogen, phosphorus and oxygen may be absent.

The fate of PHCs in the soil environment is largely determined by abiotic factors which influence the breakdown (including biodegradation) of the oil. Biodegradation of PHCs in natural ecosystems is complex and depends on the nature of the oil, the nature of the biological community and the variety of environmental determinants which influence the microbial activity.

2.3 Chemistry of Petroleum Hydrocarbon Biodegradation

2.3.1 *Biodegradation of Mixed Petroleum Hydrocarbons*

The composition of crude petroleum is not only complex but also shows considerable variation, even in samples from the same locality. Typically, 200-300 different hydrocarbons are present, including n-alkanes, branched chain alkanes, alicyclics and aromatics (**Figure 2.1**).

The proportion of ring compounds (alicyclic and aromatic) is extremely variable (2-40 % of the total) (Higgins and Burns, 1978). Crude petroleum also contains a variety of low molecular weight alkane vapours and some non-hydrocarbon components.

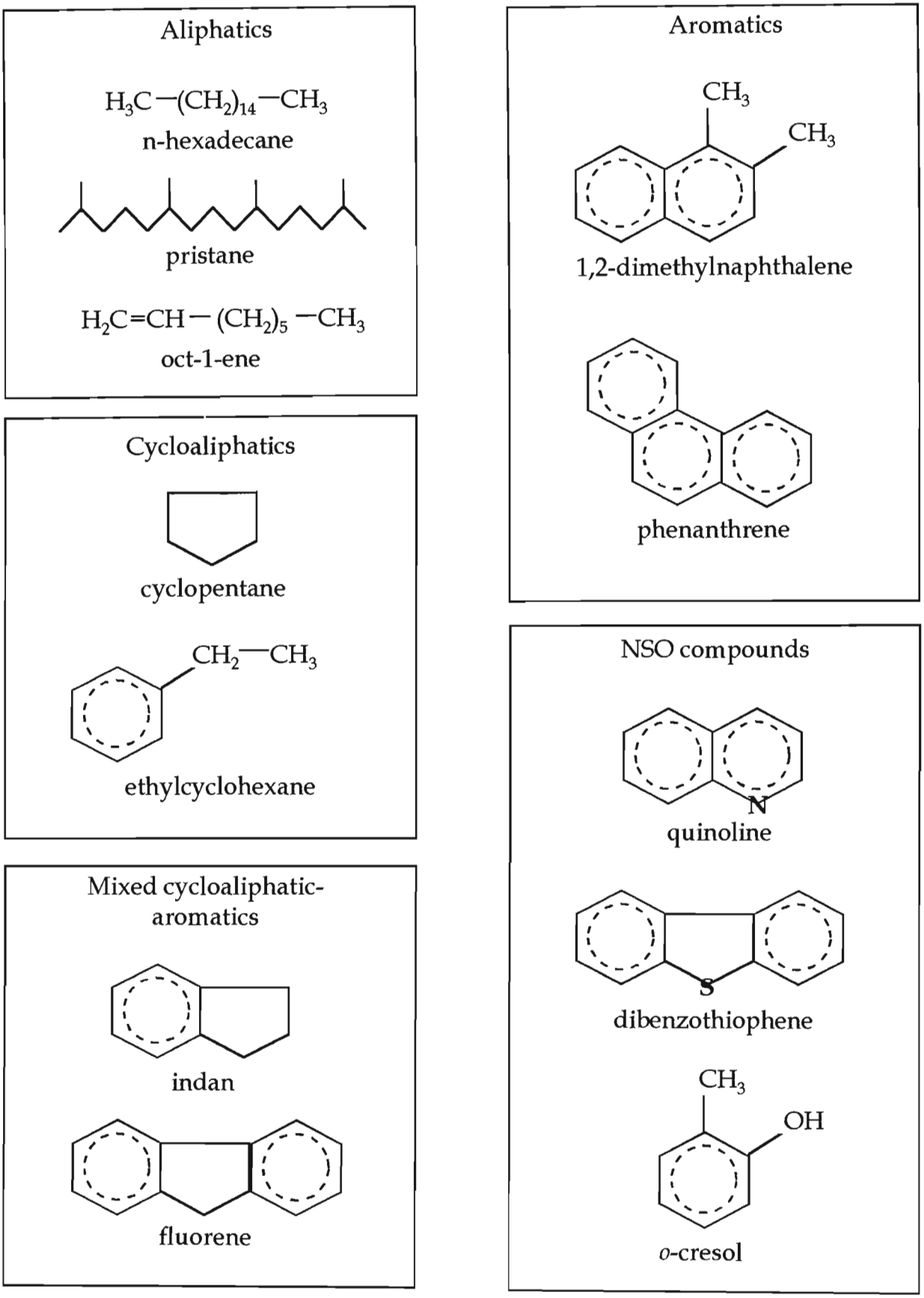


Figure 2.1: The chemical complexity of oil

Approximately 1% is composed of sulphur-containing compounds and 0.01% of nitrogen-containing compounds. Several studies have determined the metabolic pathways for the degradation of these compounds (Foster, 1962; Treccani, 1964; McKenna and Kallio, 1965; Gibson, 1971;1979; Perry, 1977,1979; Trudgill, 1978).

The qualitative hydrocarbon content of the petroleum mixture influences the biodegradability of the individual hydrocarbon components. Several researchers (Mulkins-Phillips and Stewart, 1974a; Westlake, Jobson, Phillipe and Cook, 1974; Walker, Colwell and Petrakis, 1976) have examined the susceptibility of hydrocarbons in different crude and fuel oils to microbial degradation. Westlake *et al.* (1974) and Walker *et al.* (1976) reported major differences in the susceptibility of each of the components (identical compounds) within the context of the different hydrocarbon mixtures of the oils tested. The ability of mixed microbial populations to utilize the hydrocarbons as sole carbon sources was found to depend not only on the composition of the unsaturated fraction but also on the asphaltic fraction. They further pointed out that the chemical composition of a crude oil not only has a marked effect on its biodegradability characteristics but also on the types of bacteria which will metabolize the oil, and their growth characteristics.

Each of the oils supported a unique population of bacteria, filamentous fungi and yeasts with respect to generic composition. This observation may be explained in terms of the concepts of succession (Horowitz, Gutnik and Rosenberg, 1975).

Two processes, both occurring within the context of a petroleum spillage should be considered in the metabolism of mixed petroleum hydrocarbons, namely (a) sparing and (b) co-oxidation.

2.3.1.1 Sparing

LePetit and Tagger (1976) found that acetate, an intermediate product in hydrocarbon biodegradation inhibited the utilization of hexadecane. A diauxic phenomenon has been reported for the degradation of pristane, in which pristane was not degraded in the presence of hexadecane (Pirnik, Atlas and Bartha, 1974). The basis for this sparing effect was not defined and there is speculation as to whether this is an example of classical catabolite repression. These sparing effects have a marked influence on the persistence of particular hydrocarbons within a mixture.

2.3.1.2 Co-oxidation/Co-metabolism

This term is used to describe the process in which a microorganism oxidizes a substrate without being able to utilize the energy derived from this oxidation to support growth. This concept has been expanded to include dehalogenation reactions frequently carried out by microbial species (Horvath, 1972).

Evidence indicating the ecological importance of co-metabolism is rapidly accumulating. Work on dichlorodiphenyltrichloroethane (DDT) (Guenzi and Beard, 1968), previously described as 'recalcitrant', because of repeated failures to isolate organisms capable of utilizing it as a sole source of carbon and energy for growth, has shown that co-metabolism of this molecule can occur under laboratory conditions. Reports concerning co-metabolism *in situ* are few in number although microbial populations are clearly capable of degrading pollutants and a petroleum hydrocarbon mixture, with its multitude of potential primary substrates, provides an excellent chemical environment in which co-oxidation can occur. It may be especially useful for the partial breakdown of the complex branched and cyclic hydrocarbons (Perry, 1979). Jamieson, Raymond and Hudson (1976) found that the degradation of hydrocarbons within a high-octane gasoline was not in

agreement with the degradation of individual hydrocarbons by monocultures. They concluded that co-metabolism played a major role in the degradation of the hydrocarbon mixture. However, in mixed population studies, synergism could be an alternative hypothesis to explain the observed result.

2.4 The Physical State of Hydrocarbon Pollutants

The rate and extent of biodegradation depends not only on the chemical state but also on the physical state of the PHC contaminants. The physical state of the PHC pollutant determines the surface area available for bioremediation. The tendency of PHCs to percolate into the subsoil is strongly related to their viscosity and the porosity of the soil. Heavy lubricating oils and oily sludges exhibit little or no vertical movement, while light petroleum infiltrates very rapidly into porous soils under the same conditions. Spills of PHCs which enter the subsoil and percolate down to the water table pose a special problem since evaporation and photodegradation play no role in this environment and, although absorption of PHCs by plant matter and soil particles limits their spread, the solubility of the PHCs becomes an important consideration. The aromatic fractions are perhaps the most important group of chemicals from an environmental point of view. Benzenes, toluenes and xylenes (BTEX) each have densities less than that of water (<1), and are very soluble.

Nyns (1967) proposed that the rate-limiting factor in the oxidation of PHCs is their solubility. The number of carbon atoms present in a compound has a major effect on its properties. Alkane chains and cycloalkanes up to C_{17} are liquids and have densities less than that of water. Alkanes with 18 or more carbons are actually solids at room temperature and are commonly referred to as waxes. Alkane solubility and vapour pressure rapidly decrease as the number of carbons in the compound increases while the boiling points increase with increasing molecular weights (Johnson, 1964). At very low

concentrations, most PHCs *are* soluble in water although, unfortunately, most oil-spill incidents release PHCs in concentrations far in excess of their solubility limits (Harrison *et al.*, 1975).

The short chain alkanes ($<C_9$) are toxic to many microorganisms and this toxicity is usually attributed to their greater solubility and higher concentration in the aqueous phase. These toxic PHCs are also the most volatile and it is likely that they will evaporate readily after an oil spill. It is probable that their toxicity is not a serious consideration in the biodegradation of oil (van der Linden, 1978), provided that the regional weather conditions facilitate evaporation.

Temperature may be a factor contributing to the solubility and mobility of a PHC contaminant. At low temperatures, most pure PHCs are very viscous or solid. Atlas (1981) observed that hexadecane supported only marginal bacterial growth at 5°C when the compound was in solid form. However, if the hexadecane was solubilized in another liquid hydrocarbon or crude oil, extensive degradation occurred at the same temperature, despite the obvious implications of low temperatures on bacterial metabolism. This may explain the persistence of PHCs in cold Arctic soils.

2.5 Environmental Factors Influencing the Biodegradation of Hydrocarbons

2.5.1 *Temperature*

Hydrocarbon degradation can occur over a wide range of temperatures and psychrophilic, mesophilic and thermophilic hydrocarbon utilizing microorganisms have been isolated (Jobson *et al.*, 1979). The role of temperature in determining the physical state of PHCs, and the influence of the physical state on the rates of microbial degradation have been discussed

earlier (**Section 2.4**).

It has been shown that there are seasonal shifts in the compositions of the microbial communities which can be reflected in the rates of hydrocarbon metabolism at a given temperature (Atlas, 1975). Ward and Brock (1976) observed that, although hydrocarbon-degrading microorganisms persisted during the year, there were seasonal variations in the rates of hydrocarbon oxidation. Rates of PHC biodegradation were, therefore, correlated with temperature. Dibble and Bartha (1979b) showed a definite correlation between the rates of disappearance of hydrocarbons from an oil-contaminated field with mean monthly temperature. This research indicated that the rates of degradation showed a definite climatic shift. The temperature range for optimal microbial growth in aerobic systems has been found to be from 20°C to 37°C, within a pH range of 6.0 to 8.0.

2.5.2 *Nutrients*

In addition to suitable temperature and pH conditions, microorganisms require specific inorganic nutrients (nitrogen (N), phosphorus (P) and trace-metals), as well as a carbon (C) and energy source (E), to survive. Many organic contaminants provide the C and E and thus serve as primary substrates.

Nitrogen and P are the nutrients most frequently present in limiting concentrations in soils. However, they are essential for incorporation into biomass and must be readily available. The hypothesis that a nutrient deficient condition can be responsible, in part, for the persistence of oil in soil was substantiated by data presented by Jobson *et al.* (1974). The application of N and P as urea-phosphate resulted not only in a rapid increase in the numbers of bacteria present but also an accelerated rate of disappearance of the n-saturate fraction of the crude oil. Similar results were obtained by Westlake, Jobson and Cook (1978) who also noted a rapid and

continuous loss of n-alkanes and *iso*prenoids as a result of the supplementations.

A significant proportion of the total biodegradative activity involves co-metabolism (Horvath, 1972; Jensen, 1975). Provided that the co-metabolic products do not have toxic effects, or increased recalcitrance, a preferential C source (citrate or glucose) should be added to the soil. Such an addition could also be made when low concentrations of contaminants are present which are insufficient to sustain an active microbial population. The addition of low concentrations of amino acids (for example, yeast extract) could also promote biodegradation (Lehtomäki and Niemelä, 1975).

2.5.3 *Oxygen*

Both aerobic and anaerobic biodegradation have been shown to reduce the concentrations of several components of petroleum (Kuhn, Zeyer, Eicher and Schwarzenbach, 1988; Swindell *et al.*, 1988b; Aelion, Dobbins and Pfaender, 1989).

The importance of oxygen for hydrocarbon degradation is indicated by the fact that the major degradative pathways for both saturated and aromatic hydrocarbons involve oxygenases and, hence, molecular oxygen. Although aerobic metabolism is more energy efficient than anaerobic metabolism, it is vital to consider anaerobic systems as having an equal role in biodegradation. Mineralization is dependent upon the simultaneous occurrence of both processes.

A considerable body of research indicates that methanogenic associations are active in the subsurface and are capable of degrading certain organics (Ehrlich, Godsey, Goelitz and Hult, 1983; Suflita and Gibson, 1984). Most notably, they are able to degrade low molecular weight halogenated aromatics and polynuclear hydrocarbons which cannot be degraded by

aerobic or other respiratory processes. However, the degradation of PHC straight-chain and branched alkanes and alkenes is not possible under methanogenic conditions. The disadvantage of methanogenic activity is that it requires a very low redox potential (≤-250 mV).

There have been few reports on the anaerobic degradation of hydrocarbons in natural ecosystems. Hambrick, DeLaune and Patrick (1980) found that at pH values between 5.0 and 8.0, mineralization of PHCs in estuarine sediments was highly dependent on oxygen availability. The rates of PHC degradation decreased with decreasing redox potential and they concluded that the PHCs would persist for longer periods than they would in aerated surface layers. In support of this, Ward and Brock (1976) found that hexadecane was rapidly mineralized in sediments under aerobic conditions but that little hydrocarbon mineralization occurred under anaerobic conditions. Addition of nitrate and sulphate failed to increase the mineralization under anaerobic conditions.

Oxidation is a process in which the oxidation state of a substance is increased by the removal of electrons or the addition of oxygen. This process may result in the transformation, degradation or immobilization of a substance. Certain compounds (for example, aldehydes and aromatic amines) are more oxidizable in soils than others (halogenated hydrocarbons and saturated aliphatic compounds). Augmentation of the contaminated soil system with oxidants, such as oxygen, hydrogen peroxide (H_2O_2) and ozone (O_3), has been shown to improve the degradation of PHCs (Nagel, 1982).

Oxygen may be added to a soil by tilling and draining the soil. This is easily accomplished and is currently practiced at full-scale hazardous waste land treatment sites (Song, Wang and Bartha, 1990). Alternatively, air can be added to extracted groundwater before reinjection, or it can be injected directly into aquifers. The use of *in situ* aeration wells is a more suitable

method for injecting air into contaminated plumes. A bank of aeration wells may be installed to provide a zone of continuous aeration (Ward *et al.*, 1986).

Hydrogen peroxide and O^{3-} have been demonstrated to cause an increase in microbial activity and to result in direct or partial degradation of PHCs which are otherwise degraded more slowly. These oxidizing agents may be applied in water or nutrient solutions, directly onto the soil surface, injected into the subsurface, or applied through injection wells, depending on the depth and location of the contaminants. Augmentation of soil with H_2O_2 or O_3 may, however, result in the oxidation of non-target organic material, causing a decrease in the sorptive capacity of the treated soil. Also, depending upon the amount of soil requiring treatment, and the type and amount of organic material in the soil, the cost of treatment may be very high.

Natural soil catalysts promoting oxidation of constituents in soil systems include iron, aluminium, trace metals and adsorbed oxygen. More water-soluble compounds should be more readily oxidized in clay-catalyzed systems because sorption to the hydrophilic clay mineral surface (the oxidation reaction site) precedes the oxidation process. Also, greater oxidation of chemical contaminants is expected in less saturated soils, therefore control of soil moisture by drainage and/or addition of uncontaminated clays may be necessary (Sims and Sims, 1986).

Anaerobic ecosystems are of considerable significance and are widespread, although studies of biodegradation and persistence under anaerobic conditions have been neglected. It is known that different pathways for xenobiotic degradation operate under aerobic and anaerobic /microaerophilic conditions. Just as aerobic co-metabolism can give rise to products with greater toxicity and recalcitrance than the parent compound, anaerobic transformation of xenobiotics can produce analogous results

(Guenezi and Beard, 1968). It is now known that biodegradation of several xenobiotics can proceed anaerobically via photometabolism, fermentation and anaerobic respiration, and that a wide array of reaction mechanisms are possible in the absence of molecular oxygen (Singer and Finnerty, 1984).

Microorganisms interact with one another whenever they come into contact or close proximity and, consequently, the study of microbial interactions is of vital importance in biodegradative studies. The methods used to analyze interactions are important since the interpretation of the way in which microorganisms interact can depend upon the precise technique used to study the interaction. Interface phenomena and the characterization of microorganism-microorganism interactions are facilitated by the development of relevant laboratory models and microcosms.

2.6 Surfaces and Interfaces

The three dimensional organization (`soil fabric') of soil particulates determines the nature and extent of the solid-liquid interfaces in soils. The soil fabric includes the pore spaces (voids) that are surrounded by solid-phase components. Voids may be partly or completely filled with water thereby providing the aqueous medium required for microbial activity. Any discontinuity in the aqueous phase produces an interface or surface. Such interfaces can be defined as phase or density gradients which provide unique environments not found in either of the neighbouring phases, and in which microbial activity may be significantly altered (Bull, 1980).

There are many situations where aerobic and anaerobic conditions prevail within very close physical dimensions (at the sediment and water interface, for example), and it may be that this conspicuous heterogeneity plays an important part in the considerable activity often associated with such environments. The biodegradation of some xenobiotics may even *require*

fluctuating redox conditions (Pfaender and Alexander, 1972).

Clearly, interfaces are common features of natural environments. Yet few biodegradation studies have considered interface effects and the importance of surfaces and aggregates. Evidence has shown that the retention of microorganisms at a stable interface confers a greater tolerance to environmental perturbations (Genig *et al.*, 1979), and that sorption onto sediments can modify microbial interactions through the frequency of genetic exchange (Roper and Marshall, 1974). Furthermore, surfaces provide spatial arrangements which may play an important role in the development of microbial associations, particularly in determining the outcome of predator-prey relationships (Bull and Slater, 1981). The effects of the metabolic activity of closely associated microorganisms over a prolonged period may also provide the bacteria with an opportunity to respond to the adjacent metabolic activity and develop a complementary metabolism.

Is the availability and rate of transformation of a compound enhanced or reduced at an interface? A number of studies have revealed enhanced rates of degradation when a microbial population is associated with a surface or interface (Pawlowsky and Howell, 1973; Lee *et al.*, 1975; Lee and Ryan, 1979). Zobell (1946) reported that by dispensing hydrocarbons adsorbed onto the surfaces of inert solids, the microbial attack on the most resistant hydrocarbons was greatly increased, particularly those hydrocarbons which initially seemed to be invulnerable to microbial attack. Gannon, Manilal and Alexander (1991) posed a contrasting view, stating that sorption between the microbial cells and soil particles limits the extent of microbial movement through contaminated soils, thereby hindering bioremediation.

It is well known that partitioning effects can occur at interfaces. The consequences for microbial degradation are important but little studied. Sayler and Colwell (1976) made a study of the partitioning of highly insoluble aromatic hydrocarbons and PCBs in crude oil and suspended

sediment. They found partitioning effects which profoundly influenced the microenvironment. Both oil and sediment strongly partitioned mercury with the result that a toxic environment developed. Such environments are likely to inhibit microorganisms capable of degrading the individual chemicals, rendering them more recalcitrant. Similarly, chemical pollutants can be isolated from the environment and made unavailable for degradation by bioaccumulation (Grimes and Morrison, 1975).

2.7 Concluding Remarks

The subject of oil pollution is probably one of the most emotive subjects in any discussion on environmental pollution. It is not usually recognized, however, that considerable amounts of oil and hydrocarbon material have always existed in the ecosystem, through seepage from oil fields, or from biosynthesis (Kanada, 1968). Thus, the ecosystem has always been able to contend with low concentrations of naturally-occurring hydrocarbons. The major problems arise when the environment is faced with massive local pollution over a short time period, particularly as a result of uncontrolled or poorly managed disposal of hazardous wastes.

The chemical compositions of oil and oil waste are extremely variable, consisting of more than 200 different compounds and stereoisomeric forms differing in their volatilities, solubilities, sorptive characteristics and subsurface transport capabilities. This means that no two spillages are identical in terms of microbial substrate. Hence, *in situ* treatment of contaminated soils must be based on an intimate knowledge of the factors and processes which determine the behaviour of PHCs in soil systems. Specifically, an evaluation of the physical and chemical properties, biochemical processes and environmental factors influencing the fates of chemicals in soils is required.

Before beginning *in situ* remedial actions to treat PHC-contaminated soils,

relevant site characteristics must be identified and evaluated. Soil characteristics that affect water movement (infiltration and permeability), and factors that affect contaminant mobility, are the most important considerations.

Bioremediation utilizes the indigenous soil microorganisms to degrade and detoxify hazardous constituents. The soil microfauna are a vast mixture of microorganisms encompassing thousands of species, the proportions of which change in response to environmental perturbations. The two major mechanisms in soil which are involved in the degradation include aerobic and anaerobic treatment though, initially, abiotic processes such as photo-oxidation, auto-oxidation and volatilization may be significant. Parameters affecting the biodegradation of constituents in soil systems are of two types i.e. those that determine the availability and concentration of the constituent to be degraded and those that affect the microbial population size and activity and, hence, the reaction rate.

Even if substantial microbial populations are present, the wastes are biodegradable, and there are parameters that can be altered to optimize biodegradation *in situ*, bioremediation will not be feasible if added substances react with the soil components. For example, the addition of oxidizing agents to the subsurface could result in the precipitation of iron and manganese oxides causing blockage of pores. Similarly, the addition of phosphates could result in the precipitation of calcium or iron phosphates, rendering these nutrients unavailable to the microorganisms. These limitations would be minimized if aeration could be provided by burrowing animals such as earthworms. These could provide natural perturbations, while their wastes and secretions would supplement the available nutrients in the soil. This method could be very effective, provided that the organisms are contaminant-tolerant.

It is apparent that the microbial degradation of oil pollutants is a complex

process and requires considerable information and understanding concerning site/soil/waste interactions. The fates of PHCs, the degradative pathways which are active in the environment, the importance of co-oxidation in natural ecosystems, and the use of models and microcosms in biodegradative research have been discussed. Although a number of rate-limiting factors have been elucidated, the interactive nature of microorganisms ensure that, through a series of manipulations, biological cleanup of oil-contaminated land could become a rapid and cost-effective strategy.

CHAPTER THREE

THE IMPORTANCE OF BIOREMEDIATION IN SOUTH AFRICA

3.1 Introduction

It is a recent understanding that man is destroying the natural environment and action must be taken to minimise, indeed prevent, continued mistreatment of the biosphere, acknowledging that 'damage prevention' is a better policy than one of subsequent restoration. Unfortunately, this realisation has not been sufficiently expeditious to prevent the accumulation of many different categories of polluted, disturbed or seriously altered natural environments. In some cases (e.g. the disposal of hazardous wastes) these problems were predicted. In other cases, despite a stringent regulatory framework, incidents (e.g. accidental oil spills) are consequential risks of permitted commercial activity which cannot be eliminated but nevertheless need to be remedied once they occur. The root cause of most situations seems to be the activity of mankind, as stated by Slater and Somerville (1979), "originally man was a minor component within the biosphere: his activities and wastes had little effect on the quality of the environment. Since the nineteenth century, waste treatment has increased in complexity, with a large number of novel problems as well as logistic difficulties associated with the large increase in waste production. One of the major contemporary problems has been the appearance of thousands of new chemicals released into the biosphere as products of the synthetic chemical industry, particularly the many novel compounds used in agriculture as pesticides and herbicides."

3.2 The State of the Environment in South Africa

It is now universally accepted that, according to present trends, we must expect the world, and South Africa, to become more crowded, more

polluted, ecologically more variable and more vulnerable to natural hazards in the years ahead (Fuggle, 1992). These trends are leading to an obvious degradation of the environment, of which there are two components. One is the depletion of essential resources for the maintenance of present-day life styles. The other is the deterioration and destruction of natural processes which ultimately sustain life on Earth. Both are aggravated by the increasing human population and subsequent industrial development.

For far too long, polluting industries in South Africa have been continuing their operations without sufficient financial liability for environmental damage caused, or for the administration costs accruing to the public for pollution regulation and monitoring. National economies generate large quantities of hazardous emissions, effluents and solid wastes. In addition, certain waste types (especially toxic waste) may be imported or exported, and there are often other forms of transboundary flow of pollutants. Different waste types and forms are disposed of in different ways, ranging from dedicated engineered waste disposal facilities, to dilution in the natural environment (air, water or soil). According to reports complied by the Council for Scientific and Indusrial Research (CSIR) in 1991 and 1992, the volume of waste generated amounted to between 340 and 480 million tonnes annually, and these figures are growing. Of these wastes, hazardous wastes were estimated at about 1.9 million tonnes per year, or less than 1% of the total. However, their toxicity is such that these wastes require particularly careful treatment and disposal. Aside from solid waste, approximately 1.2 million tonnes of effluents are discharged to estuarine, freshwater and marine environments. There are a number of policy issues relating to hazardous wastes which need attention. South Africa lacks a coherent regulatory system for hazardous waste. Hazardous waste producers tend to regulate themselves. Furthermore, hazardous waste is often not properly identified, handled or disposed of (CSIR, 1992).

According to official figures (Bridges, 1991), the extent of the pollution problem in the United Kingdom is reported to be over approximately 300 sites, covering an estimated 10,000 ha. It has been suggested, however, that this is a considerable underestimate and that there may be 50-100,000 contaminated sites, affecting an area of up to 100,000 ha (House of Commons Environmental Committee, 1990). In the United States, 25,000 sites have been identified by the USA Environmental Protection Agency (USEPA, 1990), 1,211 of which are on the National Priority List for urgent action. In the Netherlands, 6,000 sites are being reclaimed; Denmark has over 3,115 sites, and the former West Germany has 5,000 - 6,000 suspected sites covering an area of 40,000 ha. One can only hope that South Africa is not in a similar predicament. However, the limited facilities available for the treatment and disposal of wastes, the rising costs, and the lack of a powerful regulatory (environmental) system in this country only serves to support the uncontrolled dumping of wastes, particularly by smaller concerns that do not have the available funds to deal with their wastes responsibly.

Since the Department of Environment Affairs and Tourism (DEA&T) embarked on the issuing of waste permits in 1985, approximately 200 waste sites have been visited or advised of permit conditions and requirements. Permits became enforceable by August, 1990. Of the 9 Class I landfill sites (which are permitted to receive hazardous waste) identified in South Africa, 3 have permits and 6 have concept permits. Nine Class II sites (domestic waste and some industrial waste) have been isssued with permits while the majority of the remaining sites are in possession of "concept" permits. This means that approximately 28 permits are issued each year and if the number of waste sites were to remain static, then the 1,600 sites identified by the CSIR (1991) would take ~50 years to permit.

Some 1250 recorded/identified waste sites are not yet permitted and little is known about their suitability in terms of limiting impacts on the environment. Some of these sites could be causing serious pollution

problems resulting in a loss of valuable groundwater resources. Even though admirable, the permit process is at present far too slow to avert a potentially major crisis (Parsons, 1992).

The fact that the environment has been low on the agenda of the South African Government can be explained, in part, by the urgent quest for social, economic and political development. This has left little space to reflect on the environment. Perhaps this was most apparent in the minimal involvement of South Africa in the informal and formal meetings surrounding the United Nations Conference on Environment and Development (UNCED) (IDRC, 1994). However, the election of a new government means that new policies are being developed and a new set of international relationships is emerging, as objectives are redirected towards alleviating poverty, job creation and the meeting of basic human needs for the majority of South Africans. Within this context, it is critical to define clear policy objectives in the area of environmental quality and the use of natural resources (e.g. soil and water). The new constitution provides a powerful safeguard in shaping future development in an environmentally sustainable way since it decrees as one of the *fundamental* rights of citizens that :

"Every person has the right

(a) to an environment which is not detrimental to his or her health or wellbeing; and

(b) to have an environment protected, for the benefit of present and future generations, through reasonable legislative and other measures that

> ***(i) prevent pollution and ecological degradation;***
>
> ***(ii) promote conservation; and***
>
> ***(iii) secure ecologically sustainable development and use of natural resources while promoting justifiable economic and social development."***

(Quote : Constitutional Assembly, 1996).

This indicates that the South African intention is to create a proactive "green" economy, which is partly the function of the Reconstruction and Development Programme in the implementation of economic and social goals; for example, the emphasis on small-scale sustainable agriculture and the requirement for new mining projects to incorporate rehabilitation costs and to undergo environmental impact assessments. The ANC has committed itself to integrated environment and development policies, but there are some major oversights, such as no mention of clean technologies or a proactive "green" trade policy (IDRC, 1994).

3.3 Major Problems Identified in the Current South African Environmental Management System

3.3.1 Fragmentation of Policy

One of the most difficult aspects of environmental policy is that it cannot be effectively demarcated to its own domain; it touches on all other areas of policy and public administration, whether agriculture, trade, energy, industry or land use planning, because each of these sectors is using environmental resources and services to achieve its own objectives. In South Africa, the degree of fragmentation is very high; almost every central government department is involved in some aspect of environmental administration, and both resource allocation and pollution control are subdivided between different legislation and different departments. Furthermore, environmental management is a joint responsibility of the central and provincial departments, while certain aspects are under the jurisdiction of local authorities (IDRC, 1994). In effect, no-one is managing the parallel national 'budget' of environmental goods and services. Without a coherent environmental policy, each of these sectors is likely to exploit these resources.

3.3.2 *Ineffective Enforcement*

One obvious problem with current environmental management is that of ineffective enforcement of the legislation which is in place. The penalties specified in legislation are often ludicrously low and act as no deterrent. The capacity of the responsible government body to monitor for infringements of environmental legislation has also been largely inadequate. The recommended 'polluter pays' principle would ensure that the costs of a regulatory system would be covered from the fees that are charged to individual polluters, and that stiff fines for violators of regulations are stipulated and enforced. Unfortunately, there are also critical shortages of trained technical staff in key areas such as the Inspectorates, as well as professional staff in the responsible government departments.

3.3.3 *Lack of Adequate Accountability*

In many cases, it appears that the responsible government body does not have relevant information regarding pollution levels generated by industry, but is reliant on that obtained, and owned, by the industrial and private sector. The greater awareness and demands of environmental organisations and unions are revealing a general situation of inadequate accountabilty to the public through the environmental management system. As a result, the extent of contamination in South Africa is imprecisely known; information is scant or unavailable. Many sites of former polluting industries and landfill dumps have long been forgotten and often the contamination has re-emerged to pollute water supplies, or redevelopment surveys have revealed the hazard.

3.3.4 *A Weak "Champion" for the Environment*

It is generally thought that a strong Department of Environment is required, supported by a legislative system that has "teeth" (IDRC, 1994). The present

Department of Environment Affairs and Tourism has weak legislative authority, exercising little executive power, and lacks an adequate complement of professional and technical staff. It is highly reliant on the co-operation of other departments, Provincial government, and the private sector whose mandates are often not, first and foremost, the environmental health of the country. A powerful advocate for environmental sustainability that is supported by far-reaching legislation would be the ideal, with a system, perhaps, modelled on a concept like the USEPA. The envisaged agency should have large budgetary and staff resources, as well as significant political "muscle".

3.4 Waste Disposal to Protect Groundwater - Are We Doing Enough ?

The impact of waste disposal on the environment is a major problem to a country such as South Africa which is threatened with serious water shortages in the future. Groundwater resources are an integral part of the country's water supply and their role in the national water supply strategy is expected to increase. The American experience has shown that once groundwater pollution has occurred, it is almost impossible to clean the aquifer. However, through their well-funded and legislatively enforced efforts, many proactive and reactive measures have been tested. Clearly, aquifers and current waste disposal activities cannot coexist.

3.5 How Does Bioremediation Fit into All of This ?

There is clearly an urgent need to develop cost-effective technologies to treat contaminated soils and groundwater in South Africa. Technologies that are based on simple principles and are flexible enough to treat mixtures of organic compounds, preferably on site or *in situ*. Bioremediation may be such a technology and it is currently one of the fastest growing areas of research in the world. This is because other techniques, by themselves, are inadequate, do not permanently solve the problem, or have the potential to

be very expensive. The principal cause for the heightened interest in biological remediation is its potential for significantly reducing site cleanup costs.

There are, apart from the cost factor, many other advantages to this process e.g. it can be used when other processes cannot, i.e. when excavation is impossible; both soil and groundwater may be treated in one step; treatment can follow the contamination plume in the subsurface; and, if done correctly, hazardous waste products are not generated. In fact, to date, no major health, pollution or other hazards arising from the use or enhancement of microbial processes in soil have been identified, although it is conceivable that not all potential hazards have been considered. In the case of *in situ* bioremediation, there is the added benefit of minimal site disruption and the reduced potential for public exposure. This is no small consideration on built-up sites, where the costs of lost production due to excavating could prove to be as costly as the remediation itself. Another important consideration is the relative simplicity of the technique compared to the other technologies. Operational sophistication may be dramatically lower than on-site incineration (for which South Africa has no facility), solidification, vitrification, or soil washing systems and the soils can be rehabilitated to a near-natural ecological state. Multi-disciplinary teamwork *is* required, but there is no shortage of professionals capable of its operation.

Finally, and probably most importantly, the technology is infinitely appealing to the public as an 'environmentally friendly' technique, an image which could easily be exploited by the South African industrial sector. Even so, exporting industries are already under pressure to conform to stricter environmental policies, and the introduction of clean technologies and innovative waste or pollution management strategies may secure economic competitiveness in the long term. Bioremediation is also compatible with the so-called concept of "sustainable development" since it allows later use of the land, once the soil and/or groundwater have been rehabilitated.

Bioremediation, as an emerging technology which can compete effectively in the waste management arena, holds tremendous potential for contaminated site remediation and for future land use/management. In this country, its success is very dependent on new legislation (particularly the formulation of soil pollution guidelines or standards), but there is every indication that South Africa will follow the lead of the USA, UK and other European countries in the encouragement of technologies or innovations which improve the quality of life. Although not fully proven to date, it is the subject of much research and development, especially with respect to a 'Third World' approach. Clearly, the implications for the future of land reclamation in South Africa are significant, and the technique is thus worthy of consideration.

CHAPTER FOUR

PRELIMINARY SITE INVESTIGATION

4.1 Site Location and History

Cera Oil S.A. (Pty) Ltd is located on Rem 6 of Lot 11 No. 1609, which is situated in Hammarsdale in the County of Pietermaritzburg, approximately 42 kilometers from Durban **(Figures 4.1 and 4.2)**. The installation comprises an oil recycling plant, purchased from Lubex (Pty) Ltd in 1986, and which was previously operational for a period of approximately two decades. In August 1986, Cera Oil, in seeking to upgrade the site, discovered four buried drum caches. The drums were removed and landfilled at a Class IIA Sanitary Landfill in Welbedacht (Waste Services) (Loudon and Partners and Lombard and Associates, Proposal, 1990). The management of Cera Oil S.A.(Pty) Ltd. subsequently decided to close the refinery and rehabilitate the impacted soil, which was, in some areas, heavily contaminated **(Plate 4.1 A-F)**.

The preliminary site investigation was carried out by A.A. Loudon and Partners (Consulting Engineers), who conducted a geotechnical investigation and assisted in the physical examination of the strata. Subsequently, bioassessment studies (this research) were undertaken to assess the feasibility of an *in situ* bioremediation programme to clean up the site. The area requiring remediation covered two hectares.

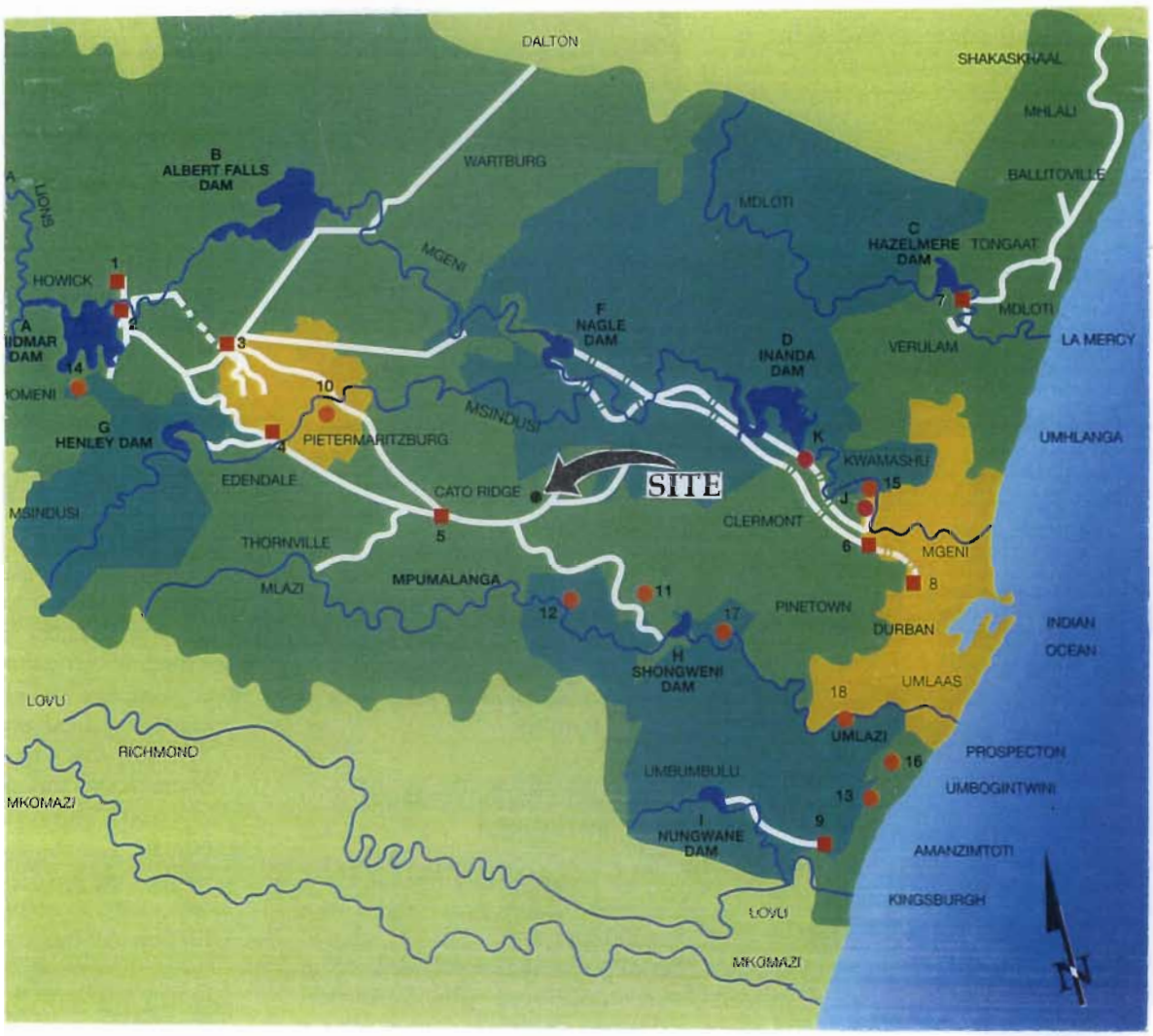


Figure 4.1: Map of KwaZulu Natal, South Africa, showing the general location of the experimental site.

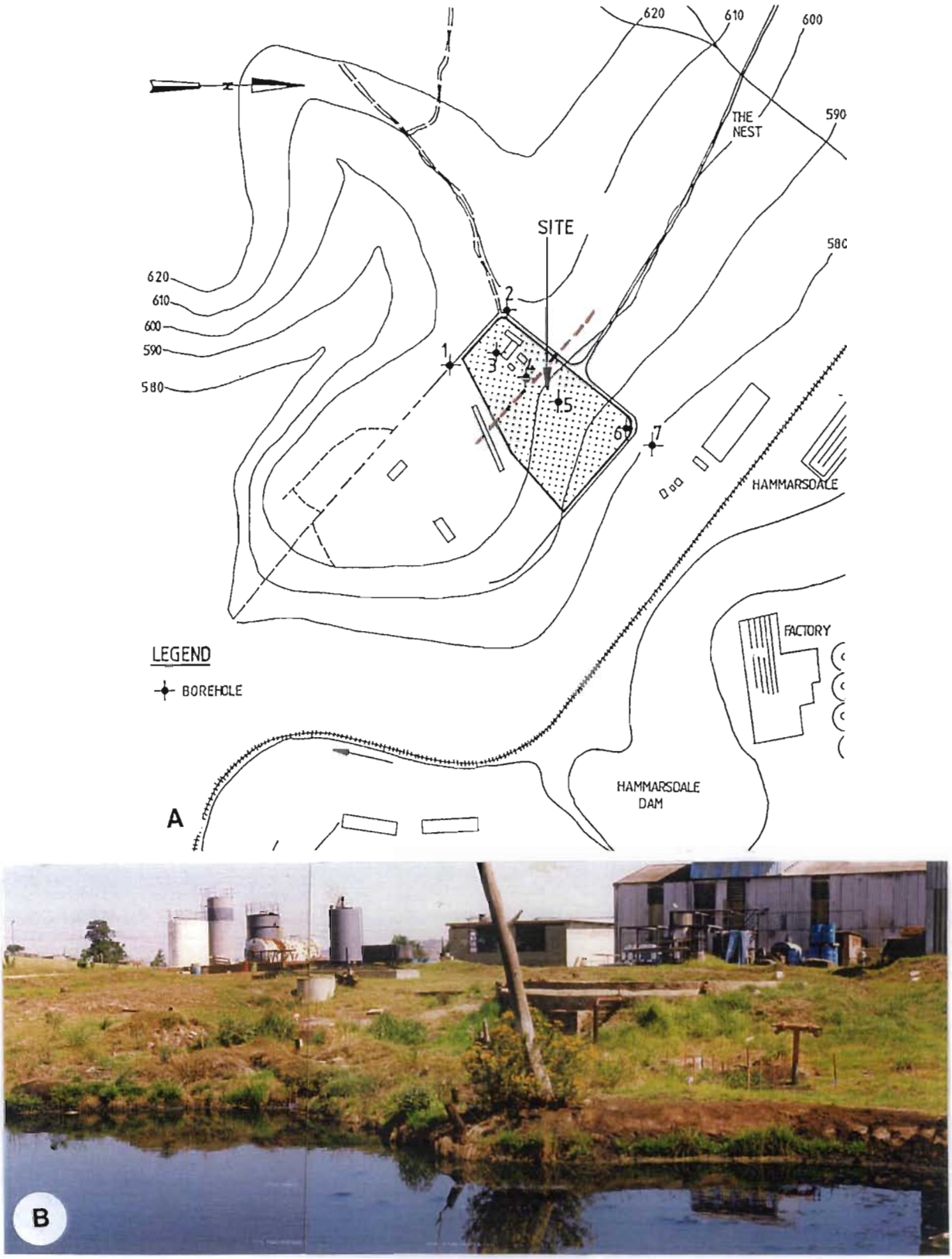


Figure 4.2: (A) Aerial map of the experimental site (Hammarsdale). (B) A photograph of the site, facing southeast (taken from the location which is indicated by an arrow).

PLATE 4.1 (A)-(F) : The experimental site.

(A) A photographic view of the eastern corner of the site (at the end of the initial site investigation).Tanks and equipment used in oil recycling were hydrocleaned and sold after the plant was closed.

(B) The south-south eastern portion of the site (at the back of the warehouses) was a drum storage area and was heavily contaminated with oil at the surface.

(C) A wall formed the boundary to the property in the north east, below the effluent dam. Overflow from the effluent dam and seasonal seepage drained into a ditch which lead to a sump on the adjacent property.

(D) Heavily contaminated seepage or overflow water was treated using Drizit™ bags which absorbed oil.

(E) The effluent dam, northeast below the water reservoir, was unlined and heavily contaminated with a thick, black, tar-like sludge (F).


A
B
C
E
D
F

4.2 Soil and Site Characteristics

Before beginning *in situ* remedial actions to treat polluted soils, relevant site characteristics must be identified and evaluated. Soil characteristics which affect water movement (infiltration and permeability), nutrient availability and factors that affect contaminant mobility were believed to be the most important considerations.

4.2.1 Visual Inspection

The nature and extent of the hydrocarbon contamination of the soil was determined by an examination of the physical and chemical factors. The outcome of this was to be used in an economic evaluation of the problem, in order to select the most environmentally acceptable and practical option for the effective rehabilitation of the site.

Physical evaluation of the soil horizons clearly identified the zones of contamination and a subsequent, more detailed, investigation demonstrated that the depth to which the contamination occurred varied across the site. Thirty seven test pits were excavated by hand across the site to an approximate depth of 1.2 m (some are shown in **Figure 4.3**). The test pits were profiled in accordance with standard practice and the various soil horizons were sampled for further laboratory testing (**Plates 4.2 A-D** and **4.2 E-H**). Due to time constraints, the site was not subjected to a random sampling regime (S. Jewaskiewitz, personal communication). Pollution of the surface water extended beyond the immediate site into stormwater drains through blocked soak-pits, heavily contaminated site roadways and drains.

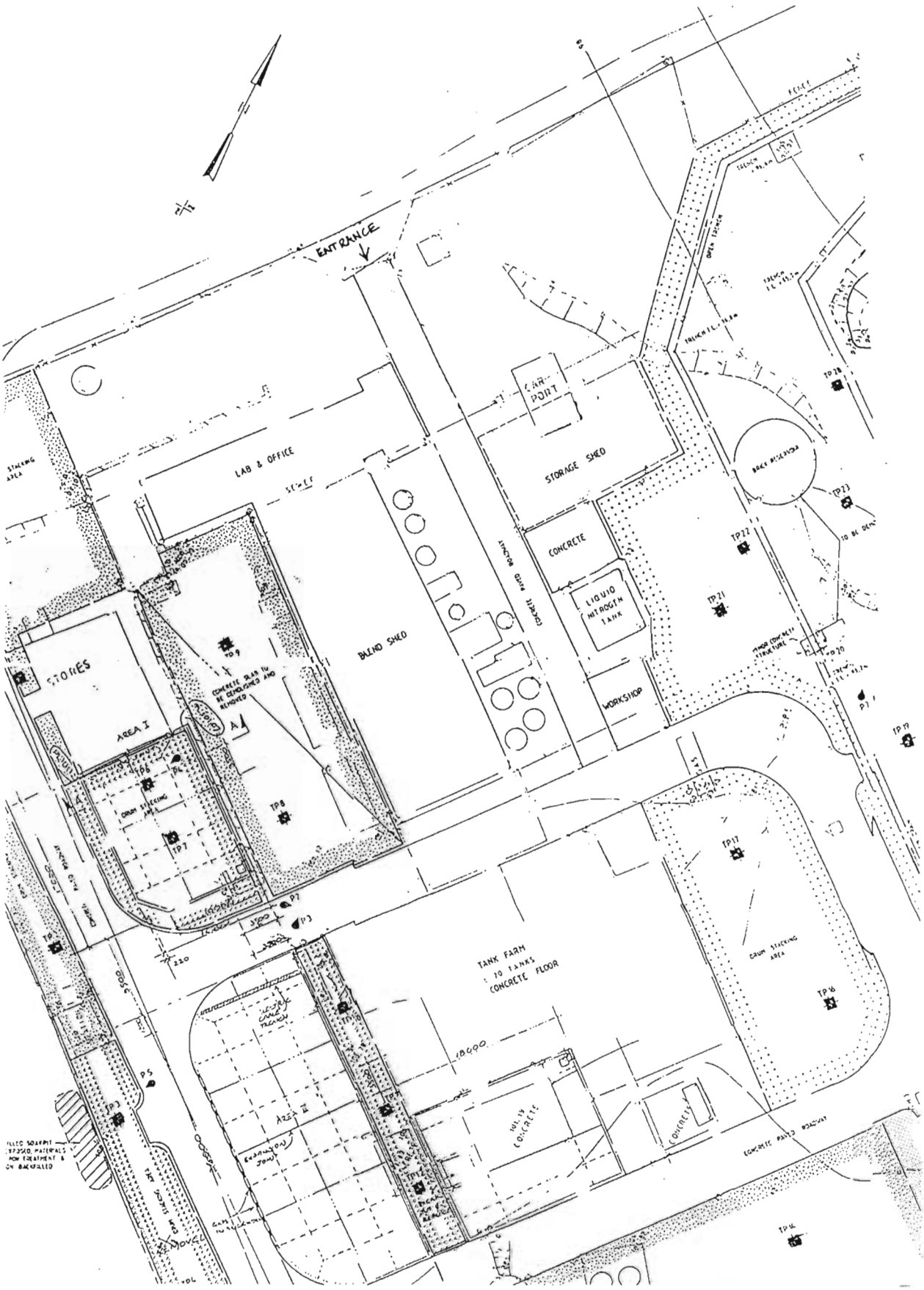


Figure 4.3: A site plan which indicates the location of some of the 37 test pits which were excavated across the site.

PLATE 4.2 (A)-(D) : Photographic views of selected test pits.

(A) Test pit 3, excavated at the top end of the site (south boundary fence) near the soakpit in the drum stacking area. Heavy surface contamination was noted (water table : 900 mm).

(B) Test pit 23, located in the highly contaminated area above the effluent dam, was visibly contaminated and had highly polluted groundwater (water table: 600 mm).

(C) Test pit 29, excavated below the effluent dam (north boundary). The entire pit profile appeared to be contaminated and there was strong groundwater seepage (highly polluted). (Note the oil accumulation in the root zone).

(D) Test pit 32, located within the roadway access and overflow drainage area below the effluent dam. Note the groundwater seeping through the sandy clay, which is heavily contaminated and indicative of the contamination plume downgradient of the site.

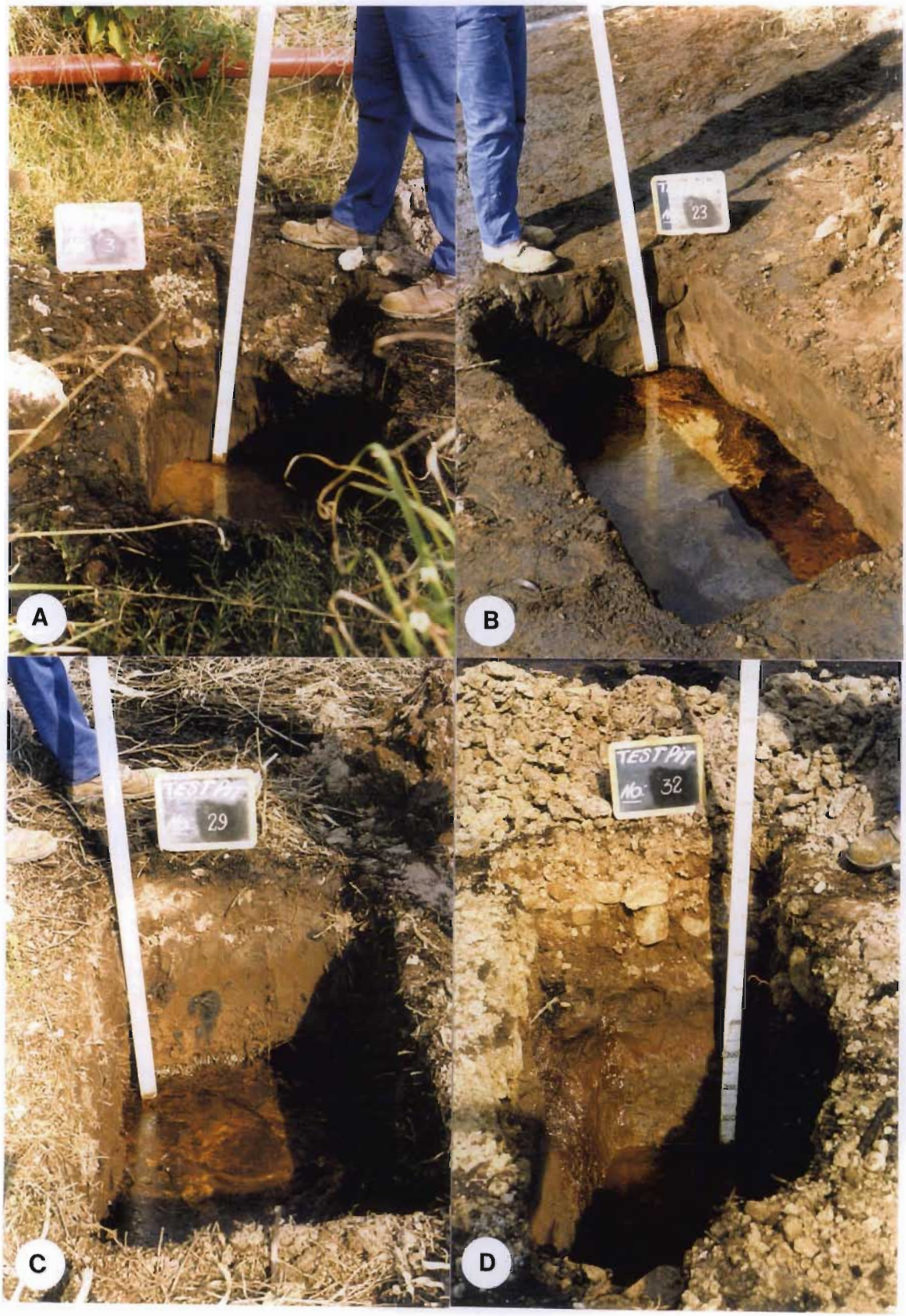

A
B
TEST PIT
No. 23
C
TEST PIT
No. 29
D
TEST PIT
No. 32

PLATE 4.2 (E)-(H) : Photographic views of selected test pits.

(E) Test pit 30, located at the base of the effluent dam, was heavily contaminated and filled with water from the perched water table (at 400 mm) as soon as it was excavated.

(F) Test pit 33, excavated close to test pit 32, was noted for its heavy surface contamination and very high water table.

(G) Test pit 36, located adjacent to the stormwater surface drain at the bottom of the adjoining property (~ 100m below the effluent dam). Groundwater seepage appeared contaminated (plume) and an oily odour was noted.

(H) Excavations in the area between the effluent dam and the water reservoir, to locate buried drums, revealed soil beneath the surface which was black in colour, very moist and evidently very polluted.

E

F

G

H

4.2.2 *Site Hydrogeology and Borehole Installation*

Generally, the geological profile was found to consist of up to 1.6 m of silty sands and fine sands, overlying a stiff, sandy clay of varying thickness (0.5 - 4.0 m) which, in turn, overlies highly weathered sandstone of the Natal Group Formation (**Figure 4.4**) (Moore, Spence, Jones and Partners, 1991). A light grey or light pink horizon on the residual clay or bedrock interface was indicative of seasonal seepage (T. van Niekerk, personal communication). It was found during this initial investigation that a large volume of groundwater was present on the site (>20,000*l*), although a dry period was being experienced (July, 1991) (**Plate 4.2 E-H**).

The groundwater appeared to be perched, was highly polluted and flowed essentially within the upper silty sands and across the silty sand/stiff sandy clay interface. The rate of seepage of this groundwater into the test pits indicated that the silty sand and fine sand overburden was highly permeable, and that the shallow water table was in hydraulic continuity with features such as the effluent dam and the drainage soakway adjacent to the southern boundary (**Plate 4.1 B**). The geology of the area suggested that a well-established groundwater plume could be emanating from the site in a northerly direction (T. van Niekerk, personal communication), towards the Sterkspruit stream (**Figures 4.2** and **4.4**).

A total of 6 x 30m, and a single 70m, -deep monitoring boreholes (BH1-BH7, **Figures 4.5** and **4.6**) were installed across the site in accordance with the "Standard Specifications for Geotechnical Investigations" as specified by the National Transport Commission (Geomeasure cc., 1991). This was achieved with a rotary drill. The cores, on retrieval, were placed in plastic sleeves, and stored in core boxes, and marked top and bottom with the appropriate depths (**Figure 4.7**).

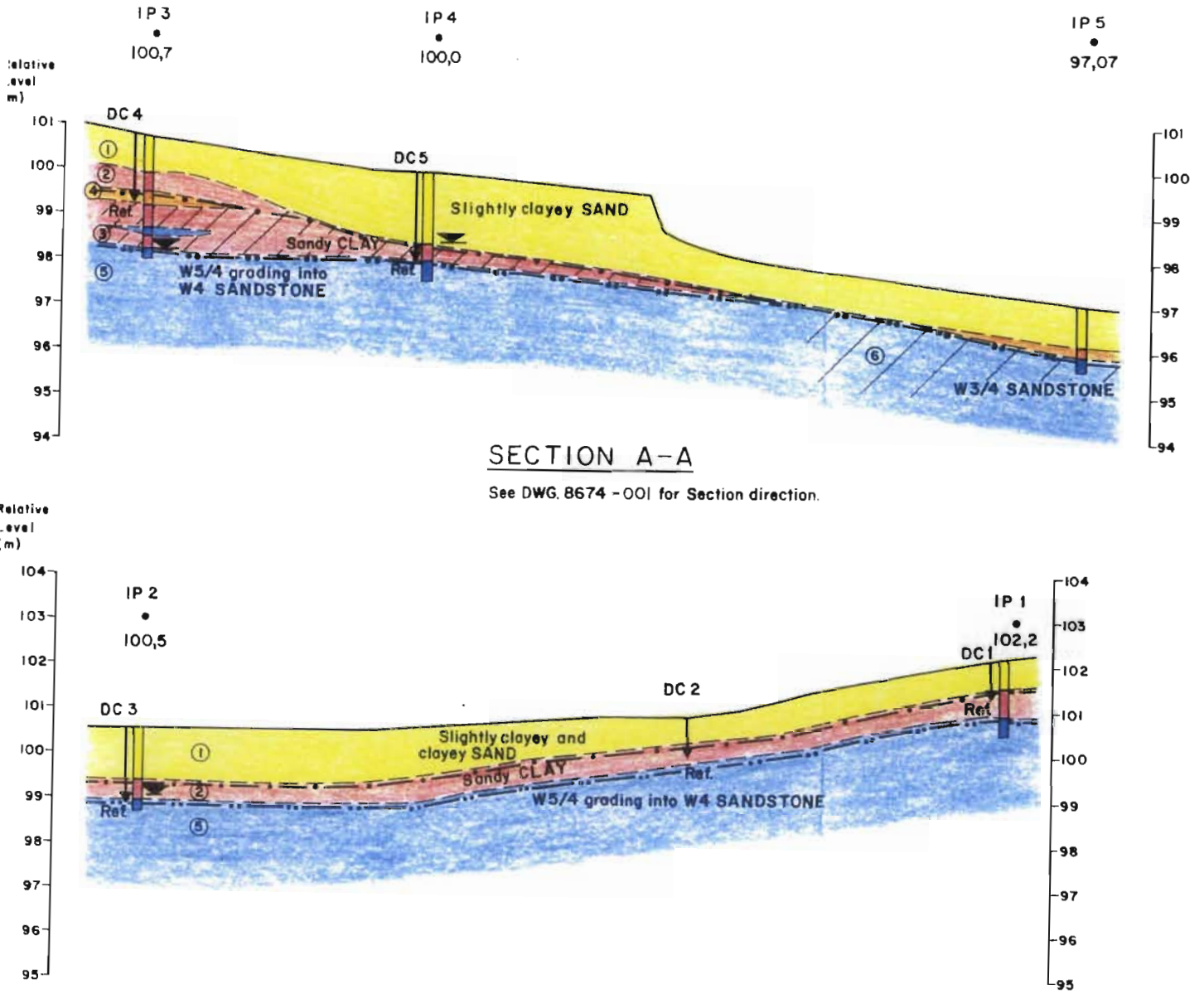


Figure 4.4: Site hydrogeology (Moore, Spence and Partners)

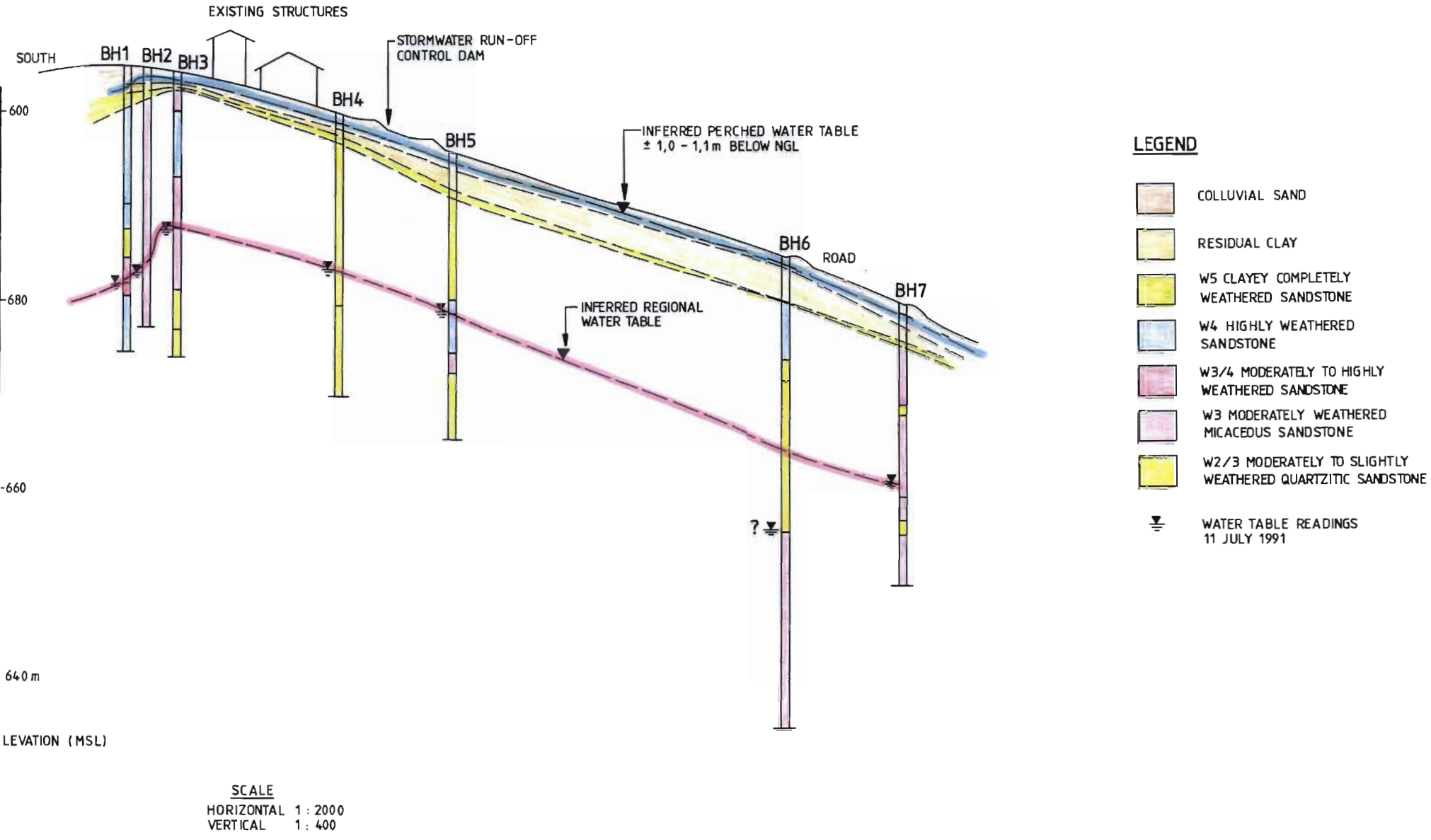
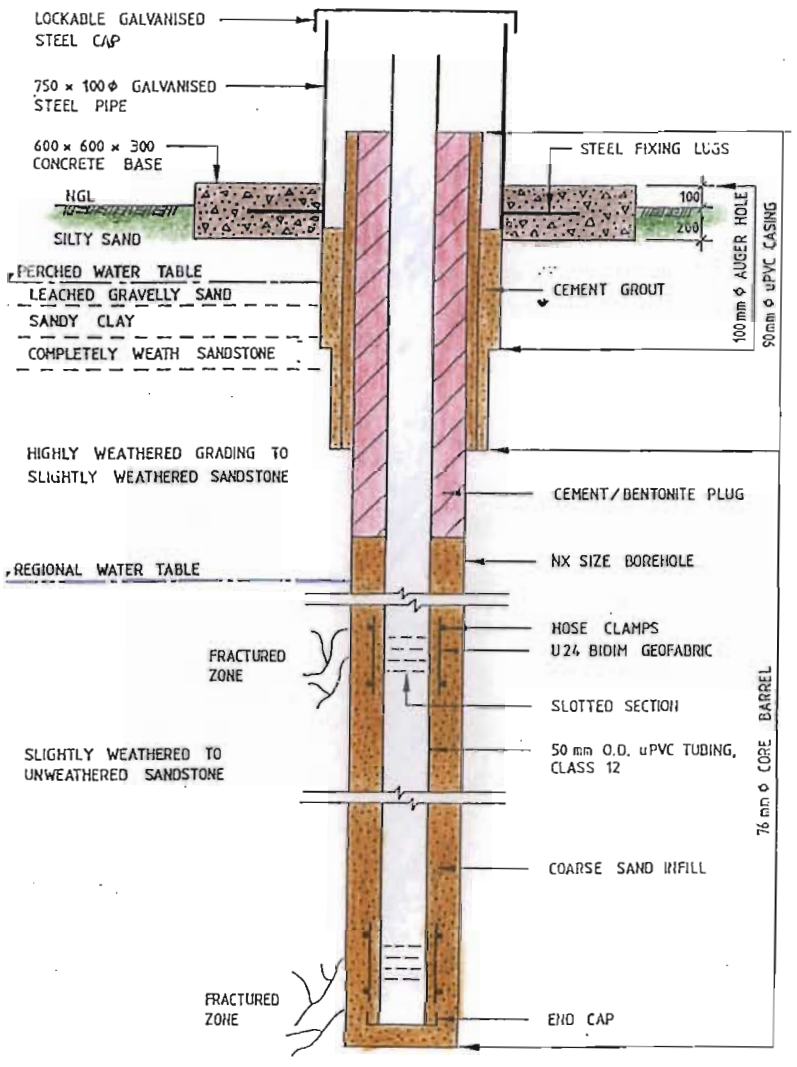
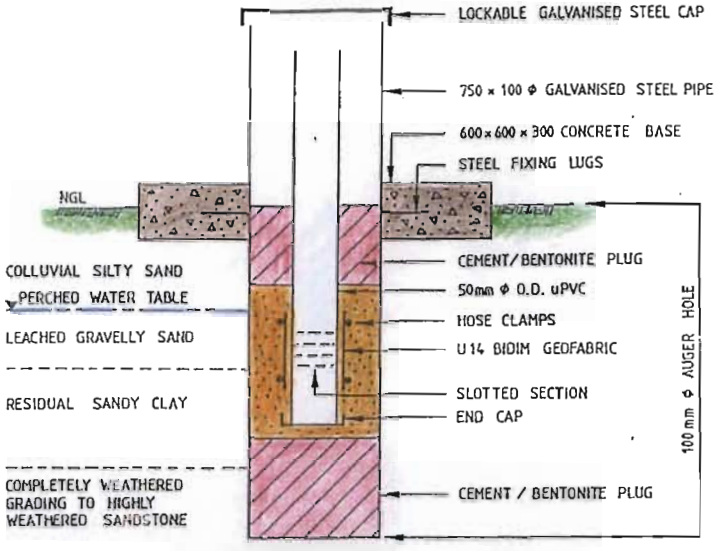


Figure 4.5: Plan section showing the boreholes, water table and geological profile (A.A. Loudon and Partners).



DEEP MONITORING BOREI



SHALLOW MONITORING BOREHOLE

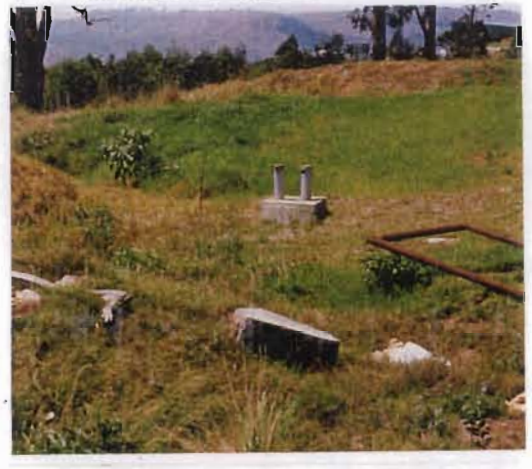


Figure 4.6: Section diagram of the deep and shallow monitoring boreholes. The insert photograph shows the concrete base and the capped boreholes as they appeared at the surface.

The drilling procedures were adjusted so that any contamination of the regional water table during drilling operations and installation of the monitoring boreholes was avoided. Shallow wells (2-5m), consisting of augered holes into which a slotted 50 mm (diameter) liner was inserted, were installed adjacent to each deep monitoring well to test the quality of the perched water (**Figure 4.6**).

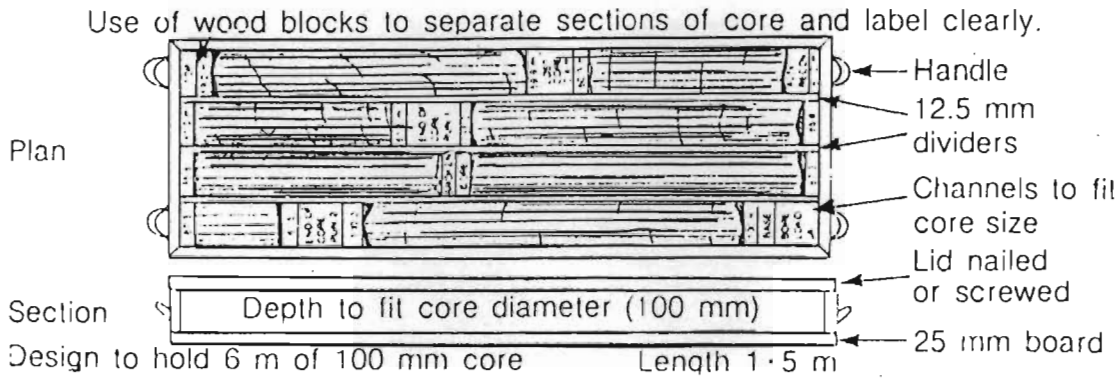


Figure 4.7: A core box plan and section (Clarke, 1988)

Coring is relatively expensive compared with other drilling practices but can provide invaluable information for the geologist. Samples were taken from the fresh cores of all 7 boreholes, at regular depth intervals. The samples were immediately placed on ice and were subsequently stored at 4°C in sealed jars and bags, to prevent biodeterioration. Data from the logs of soil borings were used to delineate the stratigraphy of the subsurface

It was apparent, from a visual examination of the cores, that numerous faults occur in this area and these have caused extensive shattered zones in the sandstone. Numerous smaller shear zones/lineations were also apparent. Some of the sandstone beds also displayed cross-bedding and parted readily along kaolinised bedding planes. This is of significance, as it is along these routes that oil is likely to travel. The kaolin is absorbent and lines the path of least resistance through the rock which may be otherwise impermeable.

Contamination was noted to depths of 5.85m (BH7), 11.66m (BH3) 12.95m (BH1), and 16.88m (BH2). Black-stained cavities were also observed at 48.45 m in the core samples from BH6. The area in the northwest corner of the site, in the vicinity of the effluent dam, appeared to be particularly badly contaminated. After heavy rains, underground manifestations were in the form of oily patches which emerged at the surface in various locations adjacent to the effluent dam. Large amounts of oil tend to deplete the oxygen reserves in the soil, and a high water table contributes to the development of anoxic conditions. Odours characteristic of anaerobic metabolism were noticeable in this area.

4.2.3 Water Table Levels and Permeability

A total of nine double and four single 'Packer' water pressure tests were carried out at different depths in the boreholes at three pressure increments, to determine rock mass permeability (Geomeasure cc., September 1992). The results of the water pressure tests (**Table 4.1**) indicated that the rock mass could be classified as jointed rock with some clay-filled joints, with moderate discharge and medium drainage (T. van Niekerk, personal communication). Furthermore, a Packer test carried out at BH3 indicated the continuous nature of the sub-vertical joints as water from the test emanated from joints exposed at the surface. In addition to this, water table levels in the area were measured (July 1992) from the boreholes. The regional water table was found to be between 16 and 29 m below ground level (**Table 4.2**).

The results of these tests gave hydraulic conductivities that ranged from 10^{-2} to 10^{-3} cm s^{-1}. These hydraulic conductivities were measured across the site in all boreholes tested, and are indicative of very permeable sandy soils and fractured rock.

Thus, the bulk of the site had sufficient permeability and apparent hydraulic interconnection to enable circulation of biorestoration fluids. The conditions were found to be ideal for *in situ* bioremediation. Unfortunately, they were also perfect for the migration of hydrocarbon contaminants.

Table 4.1 : Water Pressure Test Results.

BH No.	DEPTH RANGE (m)	PACKER TEST		CONSTANT HEAD (KPa)	WATER TAKE ($l min^{-1}$)	PERMEABILITY k ($cmsec^{-1}$)
		DOUBLE	SINGLE			
1	7-9	*		100	2.2	1.15×10^{-2}
	7-9	*		200	2.7	1.42×10^{-2}
	7-9	*		300	3.4	5.96×10^{-3}
	18-20	*		100	2.4	1.26×10^{-2}
	18-20	*		200	3.2	8.41×10^{-3}
	18-20	*		300	3.9	6.83×10^{-3}
2	8-10	*		100	1.4	7.36×10^{-3}
	8-10	*		200	2.9	1.52×10^{-2}
	8-10	*		300	3.9	2.05×10^{-2}
	10-20	*		100	2.2	1.15×10^{-2}
	18-20	*		200	2.7	1.42×10^{-2}
	18-20	*		300	2.9	5.08×10^{-2}
3	9-11	*		200	1.5	7.88×10^{-3}
	9-11	*		250	2.0	4.20×10^{-3}
	15-17	*		250	3.0	6.31×10^{-3}
	28-30		*	100	4.2	2.21×10^{-2}
	28-30		*	200	4.9	1.28×10^{-2}
	28-30		*	300	5.4	9.46×10^{-3}
4	9-11	*		100	1.7	8.93×10^{-3}
	9-11	*		200	2.1	5.52×10^{-3}
	9-11	*		300	2.6	4.56×10^{-3}
	15-17	*		200	1.7	4.47×10^{-3}
	15-17	*		300	2.9	5.08×10^{-3}
	28-30		*	200	2.4	6.31×10^{-3}
	28-30		*	300	2.9	5.08×10^{-3}
5	15-17	*		100	2.2	1.15×10^{-2}
	15-17	*		200	4.0	1.05×10^{-2}
	15-17	*		300	13.0	2.27×10^{-2}
6	10-70		*	100	19.0	5.60×10^{-3}
	10-70		*	150	25.0	4.91×10^{-3}
	10-70		*	250	55.0	6.48×10^{-3}
	10-70		*	300	59.0	5.80×10^{-3}
7	15-30		*	100	9.0	9.51×10^{-3}
	15-30		*	200	20.0	1.05×10^{-2}
	15-30		*	300	29.0	1.02×10^{-2}

Table 4.2 : Water Table Levels (BH1-7).

BOREHOLE No.	DEPTH (m)	
	Deep Boreholes	Shallow Boreholes (Perched)
1	22.92	2.71
2	21.90	1.08
3	16.30	1.00
4	16.74	1.10
5	16.90	1.10
6	29.06	1.00
7	19.40	1.10

The relationship between immobilisation of chemical constituents in soil systems (based on chemical properties) and chemical class (based on chemical structure) is summarised in **Table 4.3.** Generally, nonionic constituents of low water solubility and cationic constituents have low mobilities and leaching potential. Acid constituents at neutral and high pH values are most easily leached from soil systems (Sims *et al.*, 1984).

Table 4.3 : Immobilisation of Compounds with Different Chemical Properties in Soil Systems (Sims *et al.*, 1984).

	CHEMICAL CLASS							
	NONIONIC			IONIC				
LEACHING	WATER SOLUBILITY			BASIC		CATIONIC	ACIDIC	
POTENTIAL	HIGH	MED	LOW	LOW pH	NEUTRAL pH		LOW pH	NEUTRAL pH
LOW			X *	X *		X		
MED		X			X		X *	
HIGH	X							X

* = Site Conditions

Table 4.3 indicates which compounds are most likely to migrate through soil under various pH conditions. Clearly, hydrocarbon classes which have

low water solubilities and are ionic, have low to medium leaching potentials under acidic soil conditions, such as those found at this particular site. This is extremely favourable in terms of contaminant migration in the long-term.

If *in situ* bioreclamation technology is to succeed, the hydrogeology of the site must be well defined and have sufficient permeability to allow effective transport of injected solutions, thus resulting in hydrocarbon degradation within a reasonable time frame. Therefore, the rate of groundwater movement, which is determined by rock permeability, establishes the maximum rate of *in situ* bioremediation by controlling the rate of nutrient and oxygen transport. If the site hydraulic conductivities and gradients are low, *in situ* treatment may not be technically feasible or cost-effective.

The data thus far indicated that bioremediation was feasible. To promote rapid degradation of subsurface hydrocarbons by native bacterial populations, the metabolic activities of these microorganisms must be stimulated. This is often accomplished by injecting fluids containing nutrients and an oxygen source to bacteria residing in the subsurface. These solutions may solubilise the hydrocarbons so that they are available to the bacteria as a carbon source. The enrichment of appropriate indigenous microorganisms and their stimulation was the next logical step in this investigation.

4.2.4 Soil pH

The soil samples retrieved from the hand augers were subjected to a variety of tests (**Tables 4.4** and **4.5**). With one exception of 8.6, the majority of the soils exhibited pH values of between 4.2 and 6.9 (**Table 4.6**). pH values in the region of 5.0 and 5.2 are typical of the weathered Natal Group Sandstone-derived soils (T. van Niekerk, personal communication). It was observed that the presence of the oil resulted in an increase in pH, when compared to the control (uncontaminated) sample, the pH of which was 5.0.

4.2.5 Soil Elements

Two samples of uncontaminated soil (0.25 - 0.5 m depth) were taken from the top (south) of the site (**Figure 4.2**) and sent to the laboratories at SASTECH, Secunda. The samples were analyzed with respect to nutrients and particle size (**Table 4.4**).

The soil was of a sandy loam texture and was consistent with the previous description. It was noted that this soil was very high in iron, calcium and, to a lesser extent, aluminium. It was also very low in nitrogen, phosphorus and sulphur.

Table 4.4 : Element and Particle Size Analyses of Two Soil Samples from the Experimental Site.

ELEMENT (mg kg^{-1})	SAMPLE 1	SAMPLE 2
Nitrogen	0.24	0.53
Phosphorus	10.64	11.67
Potassium	21.02	19.83
Calcium	154.94	136.36
Magnesium	13.17	10.87
Iron	111.38	111.89
Manganese	4.81	4.64
Zinc	16.39	15.15
Copper	6.00	5.57
Chloride	3.60	5.20
Aluminium	68.00	69.94
Sulphur	7.10	9.11
TEXTURE Sand (%)	78	78
Silt (%)	18	18
Clay (%)	4	4
Exchangeable Acidity (mg l^{-1})	0.10	0.10
pH	4.60	4.56

4.3 The Nature and Concentration of the Contaminants

4.3.1 Heavy Metals

Heavy crude and fuel oils contain potentially toxic trace metals, especially Mn, Cu, Pb and Zn, which may accumulate in contaminated soils (Bossert and Bartha, 1984). Of particular interest is the fact that heavy metals are more readily mobilized in acidic soils and may leach into groundwater (M. V. Fey, personal communication). It was, therefore, necessary to test the soil samples for heavy metals and other toxic residues. Concentrations of As, Zn, Pb, Ni and Cu were determined by atomic absorption (AA) (Langet Laboratories, Durban) because of the possibility of the presence of organometallic additives in some of the oil residues on site. The presence of these metals are, except in rare circumstances, severe inhibitors of microbial metabolism and their presence above acceptable physiological thresholds could retard or preclude the natural bioremediation of the soil and groundwater.

The concentrations of heavy metals (**Table 4.5**) in all samples tested were low (often below the detectable limits for the AA sensitivity selected) and were, consequently, regarded as insignificant.

Table 4.5 : Chemical Analyses of Selected Samples of Oil-Contaminated Soil.

ELEMENT OR COMPOUND	CONTROL SAMPLE ($mg\ kg^{-1}$ soil)	CONTAMINATED SAMPLE RANGE ($mg\ kg^{-1}$ soil)
OIL	Trace	650 - 158,000
ARSENIC	0.2	<0.1
ZINC	<10	<10 - 60
LEAD	<20	<10 - 40
CADMIUM	<10	<10 - 20
NICKEL	<10	<10 - 20
CHROMIUM	<20	<20- 40

The key problems in dealing with a pollutant are the prediction of the migration rates or velocity, the area spread and the residual concentrations of hydrocarbons which remain immobile. A more subtle problem is the estimation of the extent to which the soil components can partition into air and water phases (MacKay, 1988) since it is crucial that the hydrocarbons are available to the microorganisms for degradation. The only method of approaching this problem is to consider in some detail the chemistry of the oil, in the hope that if the composition, concentration and the properties of its components can be determined, the bulk properties of solubility and vapour pressure (MacKay, 1988) and how these properties will change with time in the subsurface environment can be deduced.

In the light of this, the soil strata from the boreholes and test pits, which were visually contaminated and/or had a strong smell, were sampled and stored at 4°C. The oil concentrations of a set of samples were determined (Langet Laboratories) using a classical 6 to 8 cycle Soxhlet extraction procedure, followed by evaporation of the dichloromethane solvent, and extraction with cold, normal hexane. The concentrations of oil found in the samples ranged from 650 mg kg^{-1} to 158,000 mg kg^{-1} soil (**Table 4.5**). The South African limit for mineral oil in water is 50 mg kg^{-1}. In the absence of a similar limit for soils, this value was set as the base-line concentration. As revealed by the GC analysis, all the samples tested were above the acceptable limit (**Table 4.6**).

4.4 Chemical Analysis of Contaminating Oil

A heavily contaminated sample was subjected to the same solvent extraction regime for qualitative analysis. This extract was then filtered through a layer of anhydrous Na_2SO_4 and collected in a 50 m*l* conical flask. The original flask was then rinsed twice with 10 m*l* portions of methylene chloride and these were pooled with the extract. The volume of the extract was then

raised to 40 m*l*. A 10 m*l* aliquot of this solution was concentrated to a volume of approximately 2 m*l* by overgassing with oxygen-free nitrogen in a fume cupboard.

Table 4.6 : Analysis of Contaminated Soil Removed from the Test Pits at the Experimental Site.

TEST PIT NO.	SAMPLE DEPTH (mm)	pH	OIL (mg kg^{-1} soil)
CONTROL	400-700	5.0	TRACE
2	50-200	6.1	158,000
3	900-1100	5.0	700
4	200-400	6.6	16,000
7	750	8.6	46,000
14	650-750	6.2	600
15	400-600	6.6	700
17	650	5.2	67,000
17	1000-1200	6.3	1200
18	0-200	6.3	8000
18	1200-1300	4.2	8000
23	500	6.2	70,090
28	600-700	6.8	800
30	600-800	6.2	1500
31	1000-1100	6.5	1600
32	900-1100	6.5	650

A Varian 3600 Gas Chromatograph was fitted with a custom-designed glass capillary column (46m x 0.3mm) with an OV73 film (0.2µm)[Chromtek (Pty) Ltd]. Nitrogen was used as the carrier gas at a flow rate of 150m*l* min^{-1}, and the injector port and detector were maintained at 270°C and 280°C, respectively. The oven temperature was held at 70°C for 5 minutes, then programmed to increase at a rate of 10°C $minute^{-1}$ to 260°C, at which point it was held for 15 minutes. An injection of 1µ*l* at a split ratio of 50:1 (range 10^{-11} x 8), gave a GC trace of the oil components. The trace suggested that the composition of the polluting oil consisted of more than 200 different

compounds and stereoisomeric forms. These included n-alkanes, branched alkanes, alicyclic and aromatic molecules.

4.5.1 Choice of Key Organic Molecules (Markers)

Despite the high efficiency of the glass capillary column, which allows the alkanes and the *iso*prenoids (e.g. pristane and phytane) to be resolved, it leaves a multitude of unresolved saturates and aromatics. Thus, major compounds (components in the extract) are usually used as markers to determine the extent of degradation. n-hexadecane and pristane were found to be major components of the oil extract. Because of their properties and degradation differences, they were selected as representative markers in the biodegradative profile.

n-hexadecane [$CH_3.(CH_2)_{14}.CH_3$] is a straight-chain normal alkane. It has a molecular weight of 226.45 and a specific gravity of 0.77 at 20°C. It is extremely well documented in biodegradative studies. Evidence suggests that it is relatively easy to degrade under aerobic conditions, yet recalcitrant under anaerobic conditions.

Pristane (4,6,10,14-tetramethylpentadecane) is a high molecular weight alkane which contains *iso*-branched termini. Pristane is frequently used as a marker in degradative studies and some of its degradative pathways have been elucidated. Oxidation of this compound is known to occur, albeit slowly (Nakajima *et al.*, 1974; 1987). It is thought to be more easily degraded under anaerobic conditions.

Carbon dioxide always results from the microbial dissimilation of hydrocarbons (Zajic, Supplisson and Volesky, 1946). However, besides producing carbon dioxide and biomass, most species of hydrocarbon-

oxidising bacteria produce organic acids as intermediate products of metabolism (Zobell, 1946).

The bacterial oxidation of hexadecane by an *Acinetobacter* sp. was accompanied by the extracelluar accumulation of the wax ester, cetyl palmitate, during the stationary phase of growth (Stewart *et al.*, 1959; Stewart and Kallio, 1959; Makula *et al.*, 1975). Cetyl palmitate is derived from the condensation of cetyl alcohol and palmitic acid as determined by ^{18}O-labelling (Stewart *et al.*, 1959) and by chemical degradation of the ^{14}C-palmitate moiety of the wax ester isolated from ^{14}C-hexadecane-labelled cells (Finnerty and Kallio, 1964). Only small amounts of free fatty alcohols or free fatty acids accumulate in hexadecane-grown cells although free fatty acids accumulate extracellularly (Makula and Finnerty, 1972; Makula, Lockwood and Finnerty, 1975).

The bacterial oxidation of the *iso*prenoid, pristane has been studied by a number of investigators (Nakajima *et al.*, 1974; Pirnik *et al.*, 1976). McKenna and Kallio (1971) isolated a *Corynebacterium* species from soil which oxidised pristane to 4,8,12-trimethyltridecanoic acid and 2-methylglutaric acid. The isolation and identification of these two acids indicated the presence of dichotomous pathways in this organism : ß-oxidation; and ω-oxidation followed by α-oxidation. Nakajima *et al.* (1974) isolated a soil *Nocardia* sp. which oxidised pristane to 2,6,10,14-tetramethylpentadecan-1-ol and 2,6,10,14-tetramethylpentadecanoic acid as major products, with minor products of pristylpristanate and pristylaldehyde. Further evidence for a dichotomous pathway was found when it was shown that catabolism proceeded through pristanic acid, with subsequent oxidation to either pristandioic acid, followed by sequential ß-oxidation, or 4,8,12-trimethyltridecanoic acid, followed by α-oxidation (Pirnik *et al.*, 1974).

The metabolism of branched alkanes has received relatively little attention in contrast to microbial n-alkane oxidation. The nature of metabolic pathways in the microbial metabolism of branched alkanes has been primarily deduced from the isolation and characterisation of products arising from such oxidations (Nakajima, 1987). The ability of diverse microorganisms to oxidise and/or to grow at the expense of branched-chain hydrocarbons has been found to be variable, with the indication that 2-methyl-branched alkanes are usually good growth substrates, whereas 3-methyl-branched alkanes are attacked by very few organisms. It is difficult, however, to establish any clear metabolic similarities in the biodegradability and/or oxidizability of branched alkanes among a broad diversity of hydrocarbon-utilising microorganisms (Singer and Finnerty, 1984).

Consideration of the regulatory aspects of n-alkane versus branched alkane metabolism indicates that branched alkane oxidation is repressed by n-alkane oxidation (Pirnik *et al.*, 1974). The mechanism of repression as well as the possible involvement of independent enzyme systems remains unresolved. It is clear, however, that dibasic acids result from the oxidation of branched alkanes.

CHAPTER FIVE

AN ASSESSMENT OF THE INDIGENOUS SOIL MICROORGANISMS CAPABLE OF CATABOLISING OIL COMPONENTS

5.1 Introduction

The aim of studying microbial associations is to understand how they function *in situ*. It is, therefore, contradictory to study these communities *in vitro* as monocultures or dual and triple cultures. This is particularly important in biodegradative studies since it is apparent that mineralisation of most xenobiotics is the result of community activity (Bull and Slater, 1976).

The classical batch enrichment culture technique is routinely used in the isolation of microorganisms which degrade xenobiotic substances. Typically, microorganisms with such abilities are relatively few in environmental samples and their numbers require amplification prior to isolation by giving them a selective advantage over other more abundant microbial forms (Slater and Bull, 1982). As in all enrichments, all non-selective growth requirements such as temperature, moisture, aeration and pH are kept near their known or presumed optima (Bartha, 1986). Soil with a long contamination history increases the likelihood of finding appropriate degradative species and it is thus logical to start the enrichment process with such a sample.

Batch cultures have been criticised as a method of enrichment since, in an environment which is nutrient limited, the important microorganisms may be those which are able to utilise low substrate concentrations and have low specific growth rates (Jannasch, 1967). However, it is not often realised that when contamination occurs in the environment, the dormant or opportunistic microorganisms grow rapidly and successful competition is

determined by similar kinetic and nutritional characteristics as those governing classical, substrate-excess, batch cultures. Explicit here is the ability to cope with a continuously changing environment due to the metabolism of the substrate (Bull and Slater, 1978).

The isolation of monocultures has traditionally been the microbiologists' principal tool to establish causal connections between certain microbial strains and their activities. The enrichment of hydrocarbon-degrading microorganisms in liquid culture mostly results in the isolation of mixed populations and the characterisation of the components of these mixtures and their relationships to each other is often possible through their isolation on solid media and electron microscope examination.

5.2 Materials and Methods

5.2.1 Sampling

Two 1000 g samples each of lightly and uncontaminated soil were sampled on the experimental site using a hand auger (15-30 cm depth). The samples were placed in plastic bags which were sealed and immediately placed on ice for transportation to the laboratory where they were stored at 4°C until required.

5.2.2 Media

5.2.2.1 Mineral Salts Medium (IM) (after Coutts, Senior and Balba, 1987)

Every litre of medium contained: K_2HPO_4, 1.5g; $MgCl_2.6H_2O$, 0.2g; $NaH_2PO_4.2H_20$, 0.85g; Na_2SO_4, 1.4g; NH_4Cl, 0.9g; $NaHCO_3$, 0.5g; Na_2CO_3,0.2g; 1mM solution of $NiCl_2.6H_2O$, 1 m*l*; Vitamins, 1 m*l*; Trace Element Solution A, 1 m*l*; Trace Element Solution B, 1 m*l*.

(i) Vitamins (mg l^{-1} distilled water) : biotin, 10; *p*-aminobenzoic acid, 10; folic acid, 10; pyroxidine HCl, 20; thiamine, 20; riboflavin, 30; nicotinic acid, 50.

(ii) Trace Element Solution A (mg l^{-1} distilled water) : $FeCl_2.H_2O$, 1500; NaCl, 9000; $MnCl_2.4H_2O$, 197; $CaCl_2$, 900; $CoCl_2.6H_2O$, 238; $CuCl_2.H_2O$, 17; $ZnSO_4$, 287; $AlCl_3$, 50; H_3BO_3, 62; $NiCl_2.6H_2O$, 24; conc. HCl, 10 m*l*.

(iii) Trace Element Solution B (mg l^{-1} distilled water) : $Na_2MoO_4.2H_2O$, 48.4; $Na_2SeO_3.xH_2O$ (31% Se), 2.55; $Na_2WO_4.2H_2O$, 3.3.

Volumes of medium were prepared in three stages : For every litre of medium, a mineral salts solution deficient in Na_2CO_3, $NaHCO_3$, trace elements and vitamins was diluted to 900 m*l* with distilled water. This was dispensed into flasks, closed with cotton wool bungs and aluminium foil, and autoclaved at 121°C (15lb psi) for 15 minutes.

Appropriate masses of $NaHCO_3$ and Na_2CO_3 were added to 97 m*l* of distilled water, and sterilized by autoclaving, as above. Finally, prior to use of the medium, 1 m*l* each of trace element solutions A and B, and vitamins were filter sterilized by passage through 0.2µm Millipore membrane filters, and added to the medium. All solutions were stored at 4°C. Dispensing was under aseptic conditions with sterile glassware on a laminar flow bench (Labaire).

5.2.2.2 Soil Extract Agar

Soil (1000g) was autoclaved at 121°C (15lb psi) with 1000 m*l* of distilled water, in a 2*l* Erlenmeyer flask for 30 minutes (flask 1). In a separate 1*l* flask, 1.5 g K_2HPO_4, 0.5 g KH_2PO_4, and 20 g agar (Difco) were made up to 500 m*l*

with distilled water and autoclaved as above (flask 2). 0.5 g $(NH_4)_2SO_4$and 0.2 g of $MgSO_4.7H_2O$ were added to a third flask containing 400 m*l* of distilled water and 100 m*l* of the autoclaved soil extract (flask 3). After sterilization, the contents of flask 2 and 3 were aseptically mixed. The medium, after cooling to 50°C, was poured into standard 100 mm plastic petri dishes. When the plates had set, 50 µ*l* of the representative alkane was dispensed onto the surface and spread with a sterile glass spreader.

5.2.2.3 Mineral Salts Agar (IMA)

Mineral salts medium for the plates was prepared as described for IM (**Section 5.2.2**), with the addition of $20gl^{-1}$ agar (Difco) to 900 m*l* medium, before autoclaving. After cooling (50°C), the vitamins and trace elements were filter sterilised (0.4µm) into the agar, which was then thoroughly mixed before pouring into 100mm petri dishes. All operations were made under a laminar flow hood (LabAir). The plates were stored at 4°C until required. Prior to use, 50 µ*l* of pristane/hexadecane were filter sterilised and dispensed onto each plate with a syringe fitted with a 0.2µm disposable filter, and spread over the surface using a sterile glass spreader.

5.2.3 *Chemicals*

The hydrocarbons used were n-hexadecane (99.9% purity) and pristane (98% purity).

5.2.4 *Enrichment of Hydrocarbon Catabolising Microorganisms from Soil*

A series of liquid enrichment cultures was prepared in 250 m*l* Erlenmeyer flasks which each contained 100 m*l* of sterile mineral salts medium (IM). The inoculum for each flask was 2.5 g of lightly contaminated soil from the experimental site. The individual cultures were then overlaid with 0.05%,

0.10%, 0.50% or 1.0% (v/v) of pristane or n-hexadecane. The flasks were closed with cotton wool bungs, and incubated at 29-30°C on a rotary shaker (150 rpm) for 21 days.

These primary cultures were subsequently used as the inocula for the next set of identical flasks. One m*l* of the culture was withdrawn (aseptically) from each flask and inoculated into the corresponding flask. This procedure was repeated after a period of 21 days and the subsequent cultures were used as the inocula for the growth experiments.

All flasks were incubated in the dark to exclude the growth of phototrophs.

5.2.5 *Isolation of Hydrocarbon Degraders from Enrichment Cultures*

Isolation from the enrichment cultures was accomplished by making a serial dilution (10^{-1} to 10^{-8}) and inoculating (0.1 m*l*) soil extract plates which were then overlaid with the respective alkane. Soil extract agar is a classic nutrient-poor medium for the isolation of microorganisms from surface soils. These "master plates" were incubated at 29-30°C for 21 - 28 days and checked daily for the appearance of new growth. Drying of the agar over the protracted incubation period was prevented by pouring deep plates and wrapping them in thick plastic bags before sealing with masking tape.

5.2.5.1 Isolation of Catabolic Strains

Colonies on soil extract agar tend to be very similar in appearance therefore a general swab was taken from each "master plate" and subcultured onto nutrient-rich agar (IMA) overlaid with the respective alkane.

When not too crowded to permit isolation, all of the colonies were examined and recognizably distinct types (based on standard morphological traits

such as colour and surface) were noted and described. (Nutrient-rich media are useful for the isolation of different microbial types since the colonies generally produce more pigments and differentiate more extensively than on nutrient-poor media, thus making it comparatively easy to recognise the distinct types). Purification of the desired microbial types was then attempted by inoculating from each colony of interest onto a fresh plate of the same medium.

5.2.6 *Scanning Electron Microscopy (SEM)*

Cellular aggregates were aseptically removed from the enrichment cultures of both pristane and n-hexadecane for SEM examination. The treatment of both types was identical i.e. 3% (v/v) of buffered gluteraldehyde was added to the cells and the specimens were stored in the fixative overnight at 4°C. The fixative was withdrawn with a Pasteur pipette and the specimens were washed (30 minutes) twice in 0.05 M Cacodylate buffer. The specimens were then dehydrated with an alcohol series (30%-90%), by exposure to each solution for 10 minutes, after which they were washed three times (10 minutes each) in 100% alcohol.

The granules were then transferred to critical point drying (CPD) baskets under 100% alcohol. Critical point drying, at 1072lb psi and 31°C, was performed for 1 hour. The specimens were placed, using a low-power microscope, onto metal stubs with double-sided tape. The stubs were sputter coated with a film (5-15 nm) of electron-dense material (gold-palladium) and viewed under a Hitachi 570-S SEM.

5.2.7 *Growth Curve Preparation (Batch Cultures)*

A 1% (v/v) inoculum, taken from the enrichment cultures, was transferred to a series of sterile 250 m*l* Erlenmeyer flasks, containing 100 m*l* of sterile

nutrient medium (IM). The substrates (0.1 - 1.0%, v/v) were transferred through Millipore filters (0.2μm) to a set of flasks corresponding to the enrichment batch cultures. Since the microbial oxidation of hydrocarbons is usually accompanied by acid production, the presence of phosphate in the medium was considered desirable to poise it at a favourable pH. To test the efficacy of this, and to see whether there was a preference for a particular pH value, duplicate flasks were set up, with phosphate buffer (0.2M) as the diluent. Four discrete pH values 6.2 ; 6.8; 7.1; and 7.8 were examined. The flasks were incubated at 29-30°C on a rotary shaker (150 rpm), in the dark, for the duration of the experiment.

At time zero, and at hourly intervals, 2 m*l* samples were withdrawn from each flask with sterile pipettes. The optical densities of the samples were determined at 620 nm (Milton Roy Spectronic 301) against blanks of sterile medium after which their pH values were measured with a calibrated ion analyzer (Crison Micro pH 2001). The planned length of the experiment was 14 hours but this was later extended to 47 hours to accommodate a lag phase. The absorbance and pH results were plotted against time.

5.3 Results and Discussion

5.3.1 *The Enrichment of Catabolic Species in Contaminated Soil*

The enrichment of indigenous microorganisms which were capable of degrading representative alkanes (n-hexadecane and pristane) was successful. Within eight hours of inoculation, a visible change in turbidity of the, previously, clear medium was apparent. This was especially obvious at the oil-water interface where cells became attached to droplets despite the agitation of the shaker. Studies have indicated that bacterial attachment to alkane droplets may be an important mechanism for the transport of the alkane to the cell, until a critical amount of surfactant or enzyme is produced to emulsify the alkane (Neufield *et al.*, 1980). Between 24 and 48

hours, growth was observed on the walls of the flasks and as aggregates (or "flocs") which floated on the surface of the medium. These aggregations persisted and continued to grow in size for the duration of the experiment.

This phenomenon was previously observed by Jannasch (1960) who postulated that heterogeneous conditions were generated in microbial clumps and that increased uptake of oxygen within these aggregations led to the formation of anaerobic pockets. Alkane-grown yeasts also form flocs consisting of cells, alkane and air bubbles. This enables the cells to remain in close contact with each other and the alkane, thus creating a type of microenvironment (Blanch and Einsele, 1973; Mallee and Blanch, 1977). A similar phenomenon was observed by Kennedy *et al.* (1975) and Jørgensen (1975). Clearly, this represents an environmentally-relevant enrichment condition, as in the natural environment there are a number of situations in soil where aerobic and anaerobic conditions juxtapose, and it may be that this marked heterogeneity plays an important part in the high activity often associated with such environments.

It has been suggested by Marshall and Devinny (1980) that oil/water interfaces provide the opportunity for spatial arrangement, which plays an important role in the development of microbial communities. The close association of microorganisms over a prolonged period allows them to respond to an adjacent metabolic activity and develop either a complementary metabolism or increase the frequency of genetic exchange. Environmental variations within several μm are significant in relation to individual microorganisms because of their small size, limited mobility and ability to secrete metabolic by-products that may rapidly alter their microhabitat (Marshall and Devinny, 1980). The spatial organisation of microorganisms may thus be crucial in the regulation of their activity and the interactions (both physical and chemical) within these habitats, and may lead to the establishment of stable, mixed microbial communities representing a variety of catabolic activities. Unfortunately, the interactions

occurring in heterogeneous communities such as these are often difficult to study.

Scanning electron microscopic examination of the microbial aggregates revealed that pristane and n-hexadecane were metabolised by markedly different species of microorganisms. The aggregates/flocs isolated from the hexadecane batch cultures revealed a dominance of filamentous forms (**Plate 5.1 A**), including at least three predominant species of fungi and their spores, which were tentatively identified as *Aspergillus, Penicillium and Fusarium* spp. (**Plate 5.2 A-D**). It is postulated that filamentous fungi enhance biodegradation of oils in soil indirectly by their mycelial invasion providing an increased surface contact area for bacteria capable of initiating the degradation (Davies and Westlake, 1979). The orientation of bacteria and fungal hyphae within the aggregates on **Plate 5.3 C-D** illustrates that this may, indeed, be the case.

In contrast, the pristane aggregates were dominated by bacterial species (**Plate 5.1 B**) although one fungal species was present. Both pristane and n-hexadecane-degrading populations existed together in a honeycomb-like matrix, resembling layers of glycocalyx (**Plates 5.1D** and **5.3A**), and cells were frequently shown to be held closely together by an intricate network of extracelluar fibres (**Plate 5.1C**).

This observation is consistent with the findings of Käppelli and Fiechter (1980) and Scott and Finnerty (1976) who showed that alkane-utilizing microorganisms are generally characterised by the presence of extracelluar projections and secretions. The role of this polysaccharide-fatty acid polymer in alkane transport is thought to be for increasing cellular hydrophobicity which allows passive binding (partitioning) of the alkane to the cell surfaces, a proposed "first step" in alkane uptake (Käppelli and Fiechter, 1980).

PLATE 5.1 (A)-(D) :

Scanning electron micrographs of the diverse microbiological species which were present in (A) the n-hexadecane ; and (B) pristane enrichment cultures.

The flocs consisted of closely connected microbial associations bound to one another by a network of extracellular fibres (C) and a glycocalyx-like matrix (D), both of which may be responsible for the maintenance of a specific spatial arrangement within the aggregates.

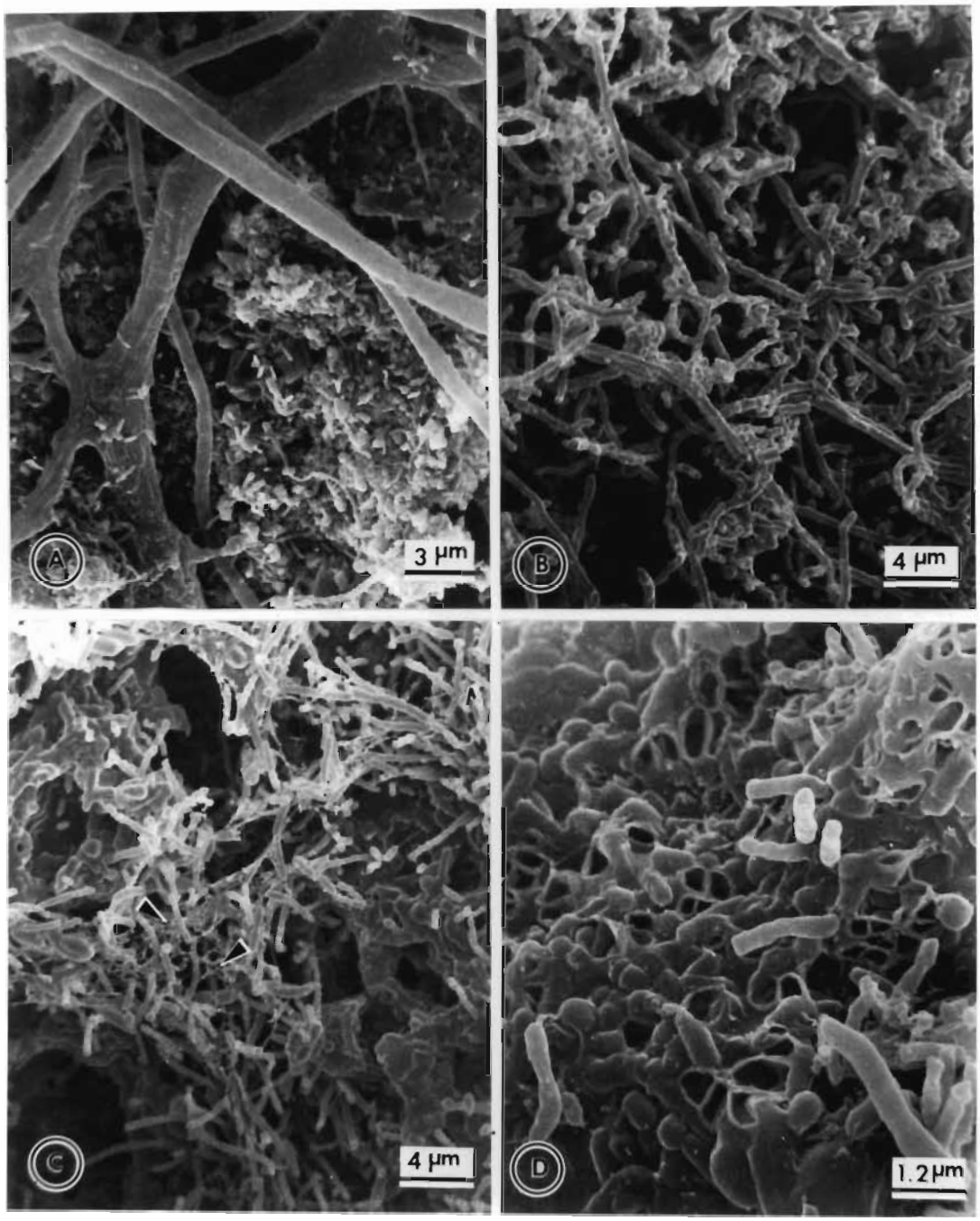

A
3 µm
B
4 µm
C
4 µm
D
1.2µm

PLATE 5.2 (A)-(D) :

Scanning electron micrographs of fungal reproductive structures, tentatively identified as a : (A) *Fusarium* sp., (B) *Aspergillus* sp. ; and (C) *Penicillium* sp.in the cellular flocs of an n-hexadecane enrichment culture.

(D) A structure believed to be a chlamydospore of one of the fungal species existing within the flocs of an n-hexadecane enrichment culture.

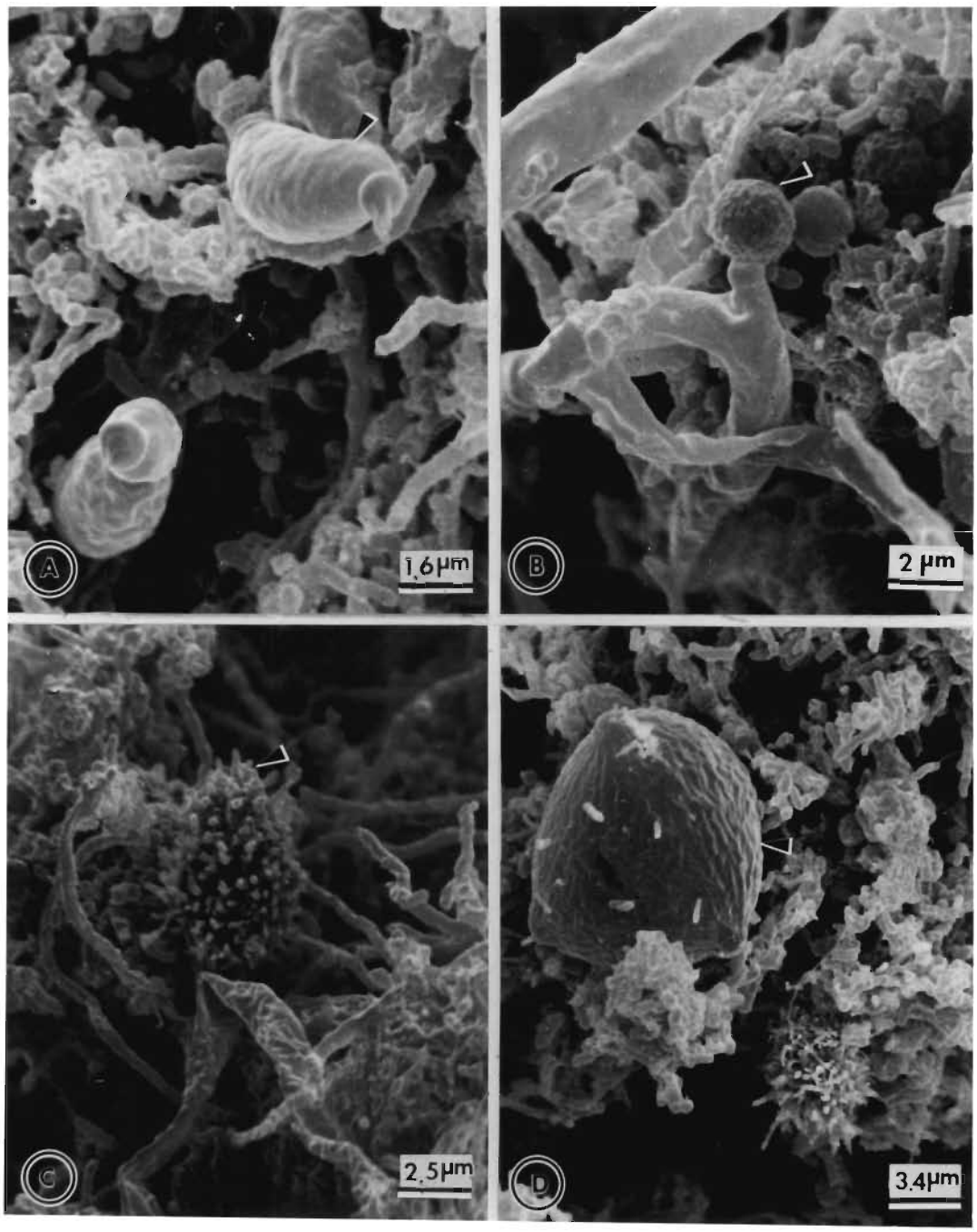

A
1,6µm
B
2 µm
C
2,5µm
D
3,4µm

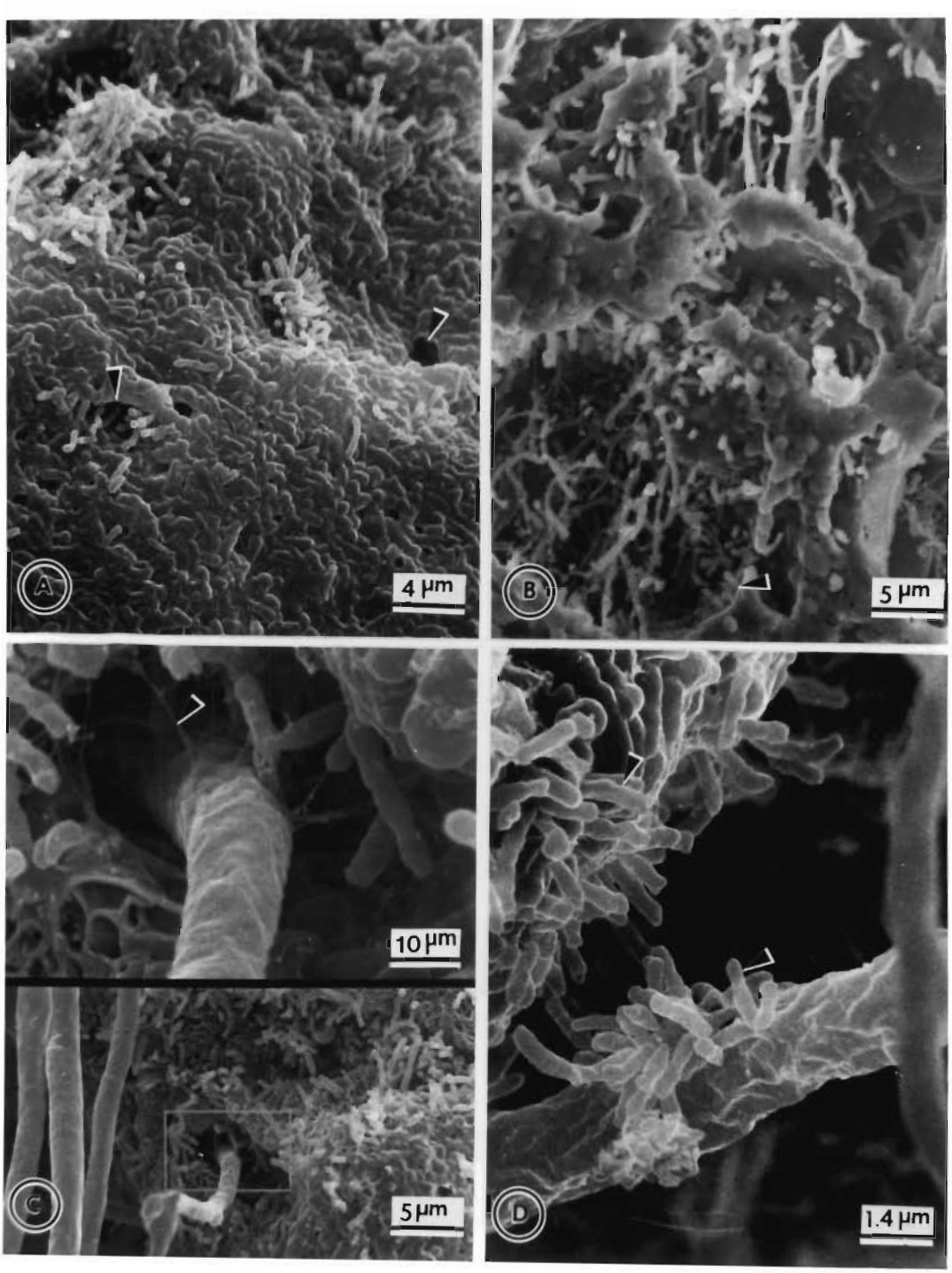
PLATE 5.3 (A)-(D) :

(A) Scanning electron micrograph of the surface of a floc isolated from the pristane enrichment culture. Arrows indicate the presence, within the matrix, of pores or air spaces.

(B) Scanning electron micrograph of the same floc which had been freeze-fractured to examine the relationship of the microorganisms with the oil droplets. The arrow shows filamentous microorganisms within an air space, droplet or anaerobic microenvironment.

(C) Bacterial cells within a floc isolated from an n-hexadecane culture were often observed to be attached to fungal hyphae by extracellular fibres (arrow).

(D) Bacterial cells were also frequently observed in this perpendicular orientation (arrow).


A
4 µm
B
5 µm
10 µm
C
5 µm
D
1.4 µm

Surfactant properties (e.g. partitioning to the hydrocarbon/water interface) have been studied in some detail with *A. colcoaceticus* RAG-1. The interplay of at least four variable components was found to be responsible for the adhesion, desorption and emulsifying ability of this hydrocarbon-degrading bacterium : (a) thin fibrils; (b) an emulsan capsule; (c) an outer membrane; and (d) extracellular emulsan (polysaccharide). Fibrils, similar to the ones shown in the SEM plates, are believed to be the major organelle responsible for adhesion of cells to hydrophobic surfaces (Rosenberg *et al.*, 1982).

These findings are also consistent in several strains of *Pseudomonas aeruginosa* that produce extracellular rhamnolipids during growth on alkanes, which effectively decrease the aqueous surface tension, thus serving as an emulsifier to stimulate the growth of this species on n-hexadecane. An additional extracelluar component (protein) was isolated from the same culture medium which appeared to act as an "activator", by stimulating growth and oxygen uptake on the alkane in cooperation with the rhamnolipid. The presence of these structures may be indicative of the mechanisms involved in alkane uptake by the cells (Käppeli and Fiechter, 1981). Since the enzymes necessary for hydrocarbon oxidation are reportedly on the cell membrane (Rosenberg *et al.*, 1982) it is, therefore, vital that the microorganism come into direct contact with its substrate. Electron micrographs of freeze-fractured yeast granules have shown the presence of numerous, radially-oriented fibrous projections (Käppelli and Fiechter, 1978) which were thought to represent the polysaccharide-fatty acid complex responsible for the binding of the alkane. Similar projections were observed (SEM) in alkane-grown *Candida* species (Scott and Finnerty, 1976) and in this study (**Plate 5.3 A**).

It is widely accepted that the heterogeneous microbiota of most non-contaminated soils include naturally-occurring hydrocarbon-degrading populations (Perry and Scheld, 1968; Odu, 1978; Pinholt, Struwe and Kjoller, 1979). This inherent characteristic imparts a large hydrocarbon

assimilatory potential to most soils. Because utilizable organic carbon is in chronically short supply in most soil environments, petroleum addition selectively enriches that sector of the microbial community able to adapt and utilize the new substrate as a source of carbon and energy (Bossert and Bartha, 1984). A change in geophysical environment, creating additional selective pressures, may ensue.

An important consideration, also, in any assessment of the effects of petroleum pollutants is the recognition that oil can serve as both a microbial nutrient and a potential toxicant (Pfaender and Buckley, 1984). Petroleum percolation through the soil reduces aeration and distrurbs the carbon/inorganic nutrient balance for the indigenous populations. Toxic components of petroleum may inhibit members of the community, producing shifts in population size and species diversity within the soil. Hence, the response of the community is a function of both the nature of the community and the composition of the polluting hydrocarbons (Pfaender *et al.*, 1984).

Even with the support of experimental evidence, it is difficult to generalise about the microbial response of soils subjected to oil contamination. The literature contains numerous reports that at times appear to be contradictory because of the great diversity of soils and petroleum studied in different geographical regions that do not lend themselves to comparison (Bossert, Kachel and Bartha, 1984). Certain trends are apparent, however. For example, in general, microbial numbers and activity are enhanced in contaminated soils (Bossert *et al.*, 1984) and stimulation of microbial activity is positively correlated to increasing concentrations of hydrocarbons (Odu, 1972; Jensen, 1975; Dibble and Bartha, 1979).

5.3.2 *Isolation of Hydrocarbon Degraders*

The process of isolation is crucial for subsequent analysis although it is often difficult to identify species because features such as colony shape and colour can change when an organism is isolated from the community or as a result of repeated subculture [although continued subculturing of the isolates on agar plates containing the complex substrate can provide a good indication of primary-degrading organisms (E. Senior, personal communication)].

Over 150 strains which appeared to exist in stable microbial associations were isolated on solid media from both the pristane and n-hexadecane batch cultures. A number of these different species' habitat domains appeared to overlap closely and the isolation of some of the community members, therefore, proved difficult by these procedures. This may have reflected close metabolic interdependence and the presence of a 'core' community. The use of a medium containing different growth factors and possible substrates (e.g. palmitic acid/hexadecanol, or analogues) supplied by the other members of the association could facilitate the isolation of these fastidious species and thus provide information for determining the mechanism of community interdependence.

It seems probable that the synergistic activities of the bacterial and fungal components of these associations resulted in the utilisation of, initially, the representative molecules of n-hexadecane and pristane, and, later, the mixed oil components as sources of carbon and energy.

Isolation allows a more detailed study of pathways, enzymes, degradation intermediates and products. In the case of 'oil', which is typically composed of a number of compounds degradable by different organisms at different rates, the succession of organisms and processes occurring with time would present the researcher with a study of such complexity that it would only be possible to determine which microorganisms were responsible for

degradation at a single point in time and under very specific conditions. This would not represent a realistic picture of the processes occurring in natural situations. Thus, although isolation could be used as an auxillary technique in gathering basic information about the ecology, taxonomy and catabolic diversity of the participating microorganisms, it was not the primary aim of this research.

Although comprehensive taxonomic analyses were not made, Gram staining of microorganisms taken from the enrichment cultures after 24 hours revealed a greater proportion of Gram-negative than Gram-positive species. It is likely that they included members of the *Arthrobacter, Corynebacterium, Brevibacterium, Micrococcus, Mycobacterium* and *Nocardia* populations, all of which have been reported to show positive responses to oil contamination. Soil pseudomonads represent another bacterial group with a major role in oil biodegradation because of their great metabolic diversity. A number of actinomycetes would also be expected, although these organisms do not appear to compete successfully in soils due to their slower growth rates (Jensen, 1975). Alternatively, their slower growth rates may infer a more dominant role in the later stages of the degradation. Austin *et al.* (1977) described some general properties of the petroleum-degrading bacteria which they isolated. The majority of strains were Gram-negative rods and a large proportion of the Gram-positive strains were either filamentous or mycelial. Just over half of the strains were pigmented and, of these, most were either orange or yellow and a few (3%) possessed a red pigmentation on solid medium. These observations were very similar to those made in this study, and the taxa which were identified included all of the groups previously mentioned.

5.3.3 Growth of Hydrocarbon Associations

According to Jannasch (1967) *"it is the aim and the art of the enrichment culture technique to control those selective conditions that quickly and reproducibly lead to*

the predominance of the population of one or more special organisms, thereby facilitating their isolation". pH changes in the batch system enrichment culture are the rule rather than the exception. This means that the period in which the pH is optimal for the organisms to be enriched, is often limited. Among the factors causing, in particular, a decrease in pH are (Veldkamp and Jannasch, 1970) :

(a) Application of NH_4^+ as a nitrogen source;

(b) Production of organic acids by heterotrophic bacteria; and

(c) Production of inorganic acids by autotrophic bacteria.

It was apparent in both the pristane and n-hexadecane cultures that the bacteria tended to respond more rapidly to the alkane while the colonisation by fungi was somewhat delayed. It has been postulated that fungi may be initially inhibited (Pinholt *et al.*, 1979). Conversely, the fungi tend to persist long after bacterial activity has declined. The changes in pH which occurred during the growth of the associations in hexadecane concurred with this since the pH values favoured by fungi are mostly lower than those favoured by bacterial species. The pH was initially above neutral (bacterial dominance) but fell as a result of the emulsification of the hydrocarbons and the possible accumulation of metabolic products (organic acids), thereby selecting for fungal dominance (**Figures 5.1** and **5.2**). Bushnell and Haas (1941) suggested that, in general, hydrocarbon oxidisers were not highly sensitive to changes in hydrogen ion concentration within the range of pH 6-9.5. This was confirmed by Zobell (1946), who observed the active growth of hydrocarbon oxidisers between pH 5 and 7.9, with optimal growth between pH 7 and 8.

In soil, the pH will determine which type of microorganism can participate in hydrocarbon degradation. In fertile soils, bacterial biomass may comprise 0.015-0.5% of the soil mass, the fungi occupying 80% of that total (Atlas, 1981). It is not known whether soil fungi participate to a similar extent in the biodegradation of hydrocarbons (Dibble and Bartha, 1979b) but evidence

supports the hypothesis that in acidic soils fungi play a significant role (Anderson and Domsch, 1975). However, it has been found that the overall rate of degradation under low pH conditions is lower than the rate attainable by a mixed bacterial and fungal community at neutral or slightly alkaline pH (Bossert and Bartha, 1984).

The formation of long-chain fatty acids may have accounted for the emulsification of oil samples undergoing microbial decomposition (Makula and Finnerty, 1972). This is characterised by a drop in pH, the emulsification of oil and the appearance of a residue. Makula, Lockwood and Finnerty (1975) interpreted changes in pH and emulsification of oil in aqueous media as evidence that long-chain organic acids were being formed during hydrocarbon degradation (Käppeli and Fiechter, 1981).

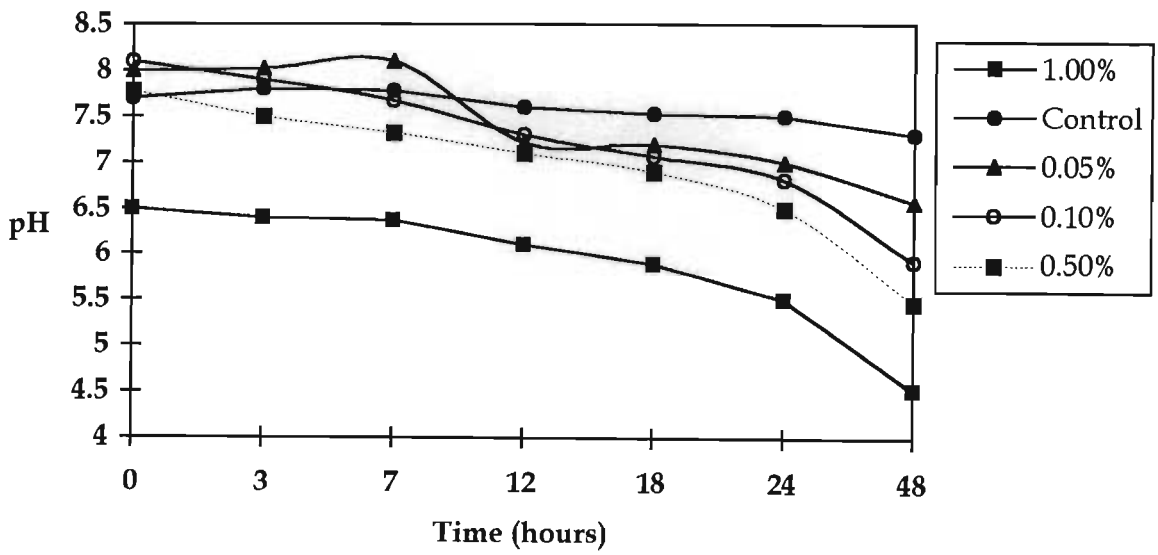


Figure 5.1: pH changes which occurred in batch cultures of soil microorganisms in the presence of different concentrations of n-hexadecane as the sole carbon source, over a period of 48 hours (n=3)

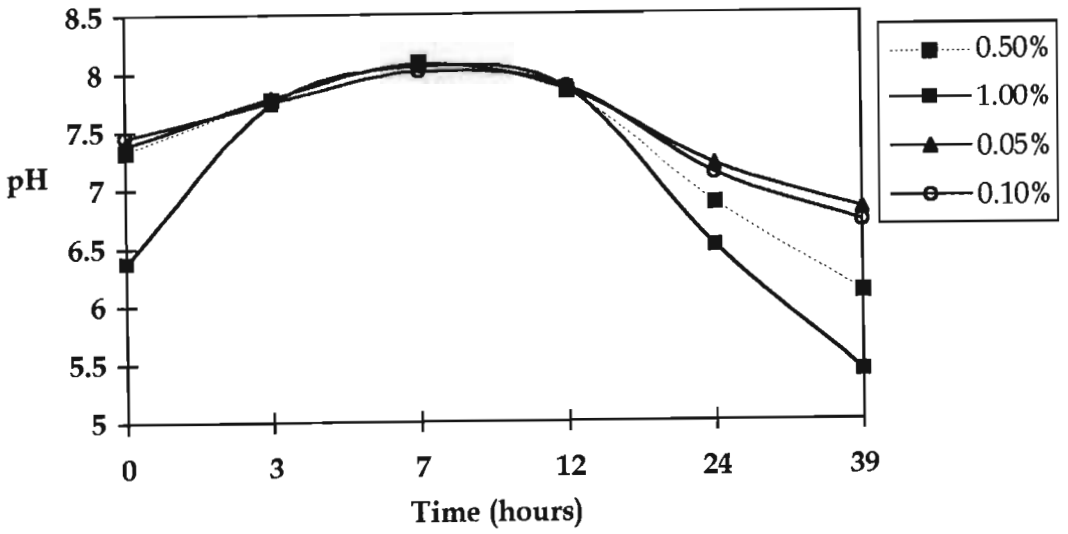


Figure 5.2: pH changes which occurred in batch cultures of soil microorganisms in the presence of different concentrations of pristane as the sole carbon source, over a period of 39 hours (n=3).

A difference in the pH reaction of n-hexadecane and pristane in batch culture was observed after approximately 40 hours, particularly at concentrations of 1.0%. The medium containing n-hexadecane became more acidic than that containing pristane. An explanation for this may be that the n-hexadecane is more readily degraded than pristane due to its relatively uncomplicated linear form, resulting in a higher concentration of organic acids after the same time period. Similar trends were obtained when the same enrichment cultures were grown on an oil substrate (mixed hydrocarbons extracted from soil at the site) with nutrient supplementation (**Figure 5.3**) at different C:N ratios, where the pH of the culture medium dropped from approximately 6.5 to 4.5 This change seemed to only occur when a nutrient supplement was added between C:N ratios of 10:1-20:1. The change may be due to competition between the nitrifying bacteria in the soil samples and the hydrocarbonoclastic bacteria, however, it is more likely that microbial activity was stimulated by the addition of nutrients (thereby restoring the C:N:P balance) which resulted in the metabolism of the hydrocarbon substrate. The pH change in the batch culture containing C:N 5:1 was not significantly different from the Control (**Figure 5.4**), and it was

concluded that the addition of such large amounts of nitrogen may have had a toxicity effect on the microorganisms.

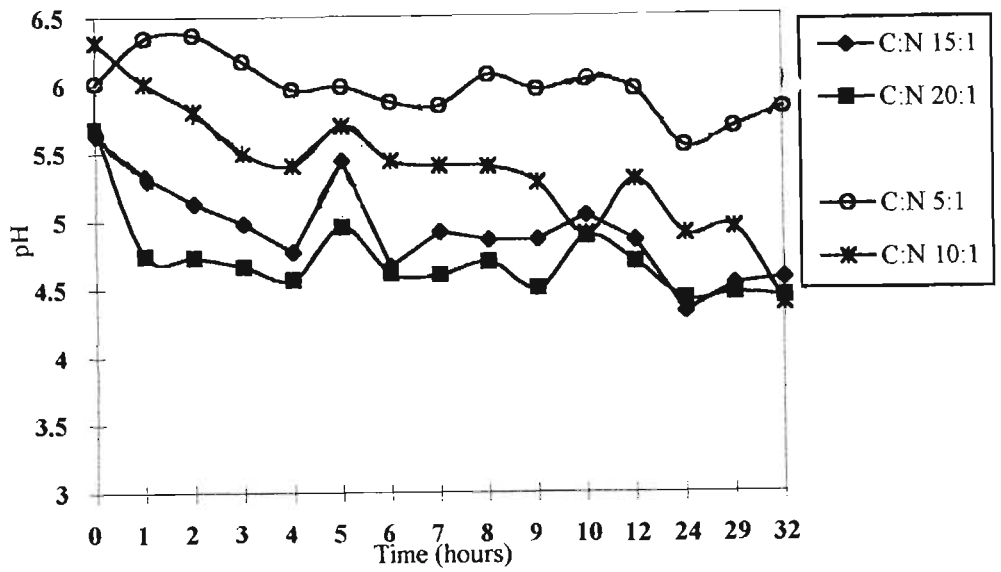


Figure 5.3: pH changes which occurred in batch cultures of soil microorganisms enriched with a mixed hydrocarbon substrate and with different carbon:nitrogen ratios (n=3).

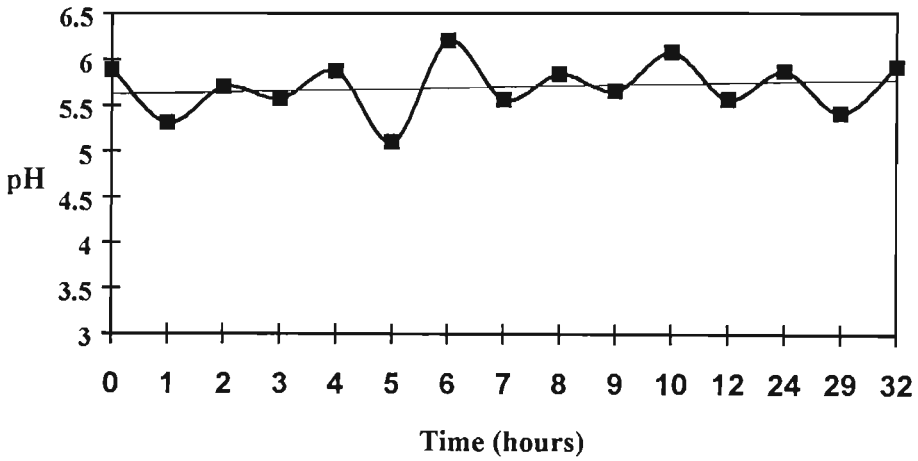


Figure 5.4: pH changes which occurred in the control batch culture receiving water but no nutrients (n=3).

The growth of the associations was measured by spectrophotometric techniques. The associations did not appear to need an acclimation period although a 14 hour lag period was observed during which time the pH did not change. This behaviour was reported by Käppeli *et al.* (1980) as a "mobilisation" period in which large amounts of membranous material were produced by the cells for the formation of vesicles. These were observed in cells grown on alkanes and serve to partition the alkane during uptake.

After 24 hours, growth in the exponential phase of both sets of cultures showed a three-fold increase in biomass (**Figures 5.5** and **5.6**) although the specific growth rates of the pristane cultures were significantly lower than the hexadecane cultures.

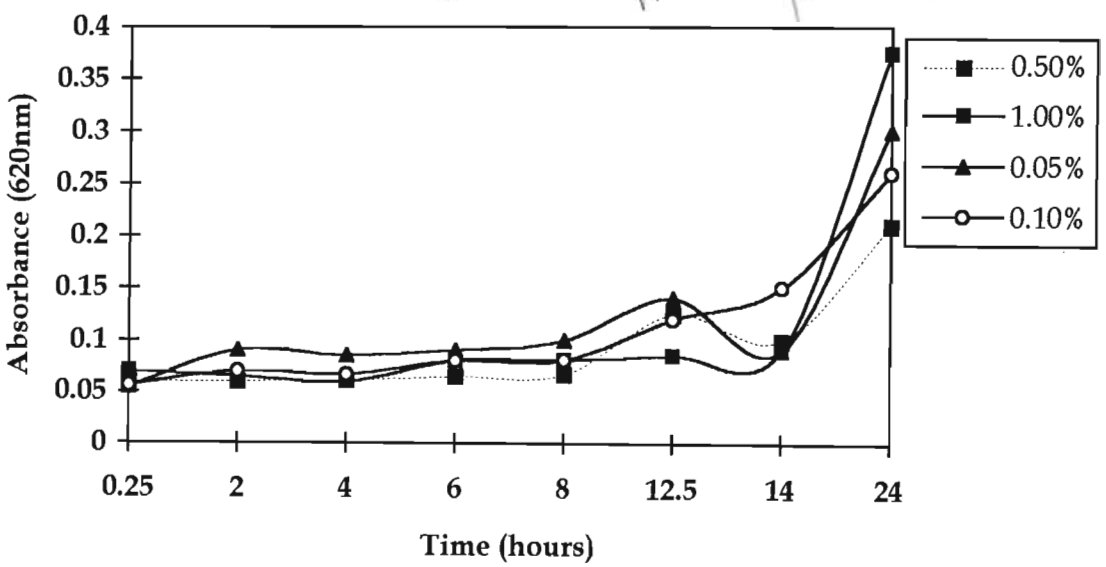


Figure 5.5: Changes in absorbance (A_{620}) of batch cultures supplemented with n-hexadecane in concentrations of 0.05 - 1% (v/v).

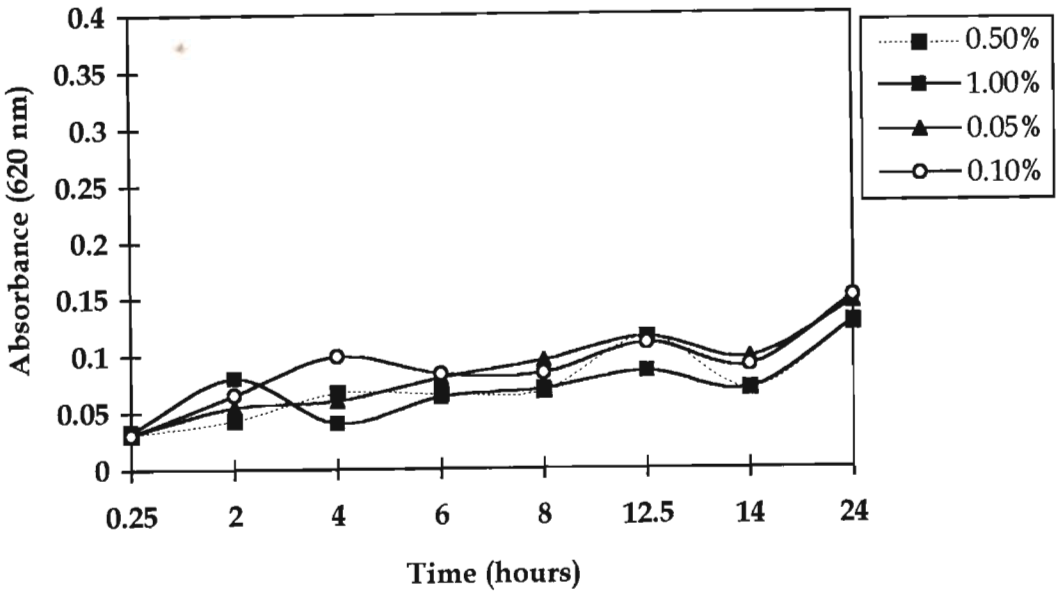


Figure 5.6: Changes in absorbance (A_{620}) of batch cultures supplemented with pristane in concentrations of 0.05 - 1% (v/v).

It was observed (data not shown) that the addition of phosphate buffer (0.2 M) did not significantly alter the growth patterns of either the pristane or hexadecane cultures and no preferential pH was identified. After 24 hours, the cells began to flocculate making accurate absorbance readings very difficult. As a result, the growth curves generated by this procedure were not reproducible, which suggested that the technique requires further refinement. The use of absorbance as a measure of growth is appropriate for most monocultures but the biomass of a mixed population of microorganisms with different species sizes may produce a very confusing set of data. Also, extensive multiplication can occur without rendering the medium turbid. This is because some organisms are intimately associated with solid surfaces or with the hydrocarbon (Zobell, 1946). The apparently haphazard growth patterns that were recorded may have been indicative of the processes of succession occurring within the degradative communities since it seems most likely that the hydrocarbon biodegradation was due to the cooperative metabolism of mixed microbial associations.

Viable count estimations of growth were attempted, but the number of plates required to determine growth for each hydrocarbon, in four different concentrations and seven dilutions (10^{-1} to 10^{-7}) at each time interval was prohibitive (56 plates per time interval), and certainly could not be replicated. This initial attempt did show, however, that the 0.5% (5,000mgl^{-1}) n-hexadecane concentration was the most favourable for growth (**Figure 5.7**). The general trend was as expected and the cell numbers increased with time as the hydrocarbon concentration increased. For both chosen concentrations inhibition was not observed although, if the experiment had been monitored for at least 72 hours, falls in activity may have been recorded as a result of by-product accumulations. Within the experimental time frame, the pristane cultures did not grow appreciably on the plates. This was, however, in agreement with the previous results which showed a 14-hour lag period before exponential growth ensued (**Figure 5.6**).

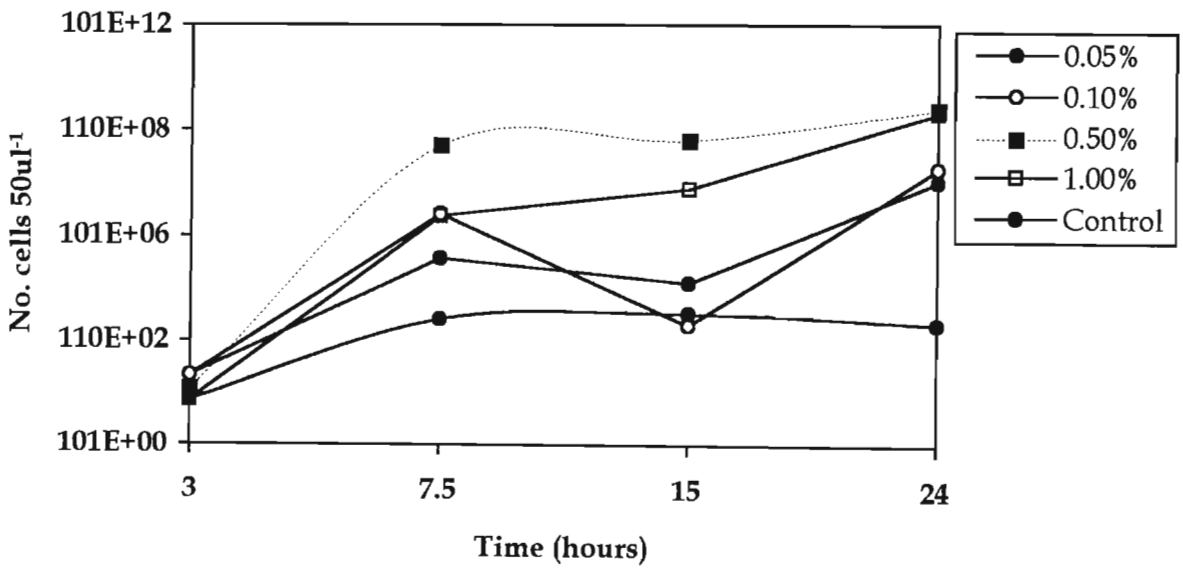


Figure 5.7 : Growth of n-hexadecane on nutrient (IM) plates with a 50μl hydrocarbon overlay, and an indigenous soil inoculum (50μl) from enrichment batch cultures.

5.3.4 *Methodology Development*

The use of hyrocarbons as substrates for microbial growth presents special problems to both the microorganisms using them as a source of carbon and energy and to the investigators in the field of hydrocarbon microbiology. The isolation methods employed are dependent on the solubilities of the hydrocarbons, their physical state (solid, liquid or gas), and toxicity. In all cases, the heterogeneity of the system complicates sampling, enumeration and growth measurement procedures.

Petroleum-degrading bacteria tend to adhere to hydrophobic materials (Rosenberg *et al.*, 1983). Thus, unless the bacteria are displaced from the material and dispersed prior to enumeration, only minimum cell numbers can be obtained. The choice of carbon source is an even more serious problem. Petroleum is an extremely complex mixture of hydrocarbons. Because certain bacteria may grow only on minor components in the oil, it would be necessary to incorporate large quantities of petroleum into the growth medium to ensure sufficient substrate for these bacteria. However, high concentrations of petroleum and mixtures of petroleum cannot be used because they are toxic to bacteria (Vestal *et al.*, 1984). Thus, the enumeration of hydrocarbon-degrading bacteria with petroleum as the carbon source selects primarily for the bacteria capable of degrading major components of the oil mixture. Often, pure hydrocarbons and fractions of oil are used in replacing petroleum as the carbon source in the isolation media. This was the approach taken in all of the initial experiments.

Hydrophobic substances present problems with respect to handling and quantification. Microorganisms have access to them only at the restricted hydrophilic-hydrophobic interface. The best strategy for biodegradation is, therefore, to increase the interface by dispersion or to use an inert hydrophilic carrier. Zobell (1946) found that by dispersing hydrocarbons adsorbed to the surfaces of inert solids, the microbial metabolism of most of

the hydrocarbons tested was greatly increased. In fact, certain hydrocarbons, which were usually recalcitrant, were rapidly catabolised when dispersed throughout the medium on the surface of inert solids (e.g. asbestos fibres and sand). Solid particles of small dimensions such as kaolin or talcum powder are not beneficial because they consolidate at the bottom of the culture vessel and the adsorbed oil completely fills the interstitial spaces so that relatively little surface is available to the microorganisms. With this in mind, the enrichment of hydrocarbon degraders was initially carried out with the hydrocarbons adsorbed to glass wool. This was achieved by dissolving the alkanes in methylene chloride in sterile MacCartney bottles each containing 0.1g of glass wool. The solvent was then evaporated off in the fume cupboard to leave the hydrocarbon adsorbed on the surface of the glass wool.

Each adsorbed substrate was added, using sterile forceps, to the appropriate flasks (100 m*l*) containing mineral salts medium (IM). The flasks were inoculated and incubated at 30°C for 21 days. This method was, however, abandoned as the hydrocarbon was released from the glass wool as a result of the prolonged and vigorous shaking of the cultures, which was necessary for aeration. In addition, subculturing the enriched microorganisms is difficult since the microorganisms remain firmly attached to the surfaces. The technique could also not be used in the growth studies as most of the microbial activity occurs on the surfaces. Therefore, the sampling of the medium (free-living species) would give the results considerable bias.

Sonication failed to produce a stable emulsion of n-hexadecane or pristane in aqueous medium. Thus, the growth kinetic studies may not have been valid since the glass pipettes used for the sampling of the medium withdrew more than the required substrate from the culture vessel, as a result of the affinity of the chemical for the glass.

Problems were also experienced in the preparation of solid media. n-hexadecane and pristane were individually incorporated into mineral salts agar plates by blending them into molten agar cooled to 50°C. Poured immediately after mixing, the agar solidified before the emulsion could break. However, the distribution of each chemical was patchy and the droplets were not uniform in size. This method was abandoned due to the fact that it was not reproducible. Also, one could not confidently conclude whether the organisms were utilizing the alkane rather than merely utilising components of impurities in the atmosphere, in the agar, or organic additives such as the vitamins. Similar difficulties were experienced with hexadecanol and palmitic acid which are possible intermediates in the degradative pathway of n-hexadecane and may thus be utilized by secondary species.

Both compounds (hexadecanol and palmitic acid) are insoluble in aqueous solution and are solid at room temperature. They dissolved readily when heated and so solubilised when added to molten agar. Unfortunately, when the agar cooled and solidified both compounds precipitated forming white crystals in the medium. These crystals were not uniformly distributed and, since the plates were surface inoculated, it was concluded that the substrates would not be readily available to the microorganisms. Since both compounds are soluble in methylene chloride, they were dissolved in a small volume of solvent and transferred to an aspirator. The solution was then sprayed onto the surface of the plates, leaving a fine, white film of substrate after the solvent had evaporated. The idea was based on the assumption that microorganisms utilising the substrate would leave a zone of clearing in the film. Unfortunately, after a few plates had been sprayed, the aspirator repeatedly became blocked. This method was, subsequently, abandoned.

A recently developed method is the double-layer agar technique, where agar containing the concentrated substrate (opaque) in a uniform layer

(subjected to sonication and/or heating) is poured over pre-prepared mineral agar support plates before the mixture can separate or precipitate. Surface streaking or spreading techniques are then used to separate microorganisms which are identified by zones of clearing. To aid isolation, a magnifying colony counter or low-power microscope is used. The double-layer technique ensures that clearing of limited depth is detectable without danger of the thin plates drying out during lengthy incubation periods.

Various practical problems in the laboratory have indicated the need for the refinement of existing techniques and the development of others to determine the growth rates of the associations and their biodegradative potentials.

CHAPTER SIX

THE OPTIMISATION OF HYDROCARBON DEGRADATION BY SOIL MICROORGANISMS

6.1 Introduction

Much of the work on biodegradation of hydrocarbons has been done *in vitro*. Laboratory systems such as `microcosms' are simply experimental designs which attempt to simulate some of the *in situ* conditions, or part of the environment under study. The information generated is a function of the design features of the microcosm used (some microcosms will enable biodegradative capacity to be assessed while others will be limited to revealing biodegradative potential).

Experiments with homogenised soil are often the least complex and the most traditional of all the methods used in soil microbiology and much of our fundamental knowledge has arisen from studies of this type. It must be remembered that, despite the seminal nature of this research, it is only one aspect of the microbial world, a component that is capable of responding to the thoroughly artificial conditions imposed upon it in laboratory experiments (Burns, 1988).

One advantage of microcosms is their replicability. Good agreement between microcosm and environment has been reported for xenobiotic biodegradation and bioaccumulation (Cole *et al.*, 1976; Bott *et al.*, 1977). However, although microcosms may be functionally similar to the ecosystem being mimicked, it should be emphasised that they are analytical tools and **not** reproductions of ecosystems.

The aim of this component of the research programme was to determine the rates of hydrocarbon degradation in soil over time, in small jars which

represented very simple microcosms, and to establish which of the nutrient supplement ratios, if any, would optimise those rates. Although the experiment was made in duplicate, this was not for statistical purposes; trends were simply to be determined so that the results of the experiment could be used in further investigations.

6.2 Aerobic Degradation

6.2.1 Materials and Methods

6.2.1.1 Soil Samples

Four 5 kg samples were collected from the experimental site with a hand-held auger. Soil was taken from a depth of between 10 and 50cm, at various locations on the site (sampling points **a**, **b** and **c**, **Figure 6.1**) where contamination was moderate to heavy (>20,000 mg C kg^{-1} soil). The samples were then mixed together thoroughly and used in the microcosm experiments. Four 1 kg subsamples (field-moist) were sterilised by γ-irradiation (Gamwave (Pty) Ltd) at a dosage of 2.5MRad. The sterile soil was handled aseptically when added to the jars i.e. it was dispensed on a laminar flow bench (LabAir), using sterile spatulas, into alcohol-sterilised jars.

6.2.1.2 Carbon and Hydrogen Analyses

Carbon and hydrogen analyses were made on six samples of soil taken from the three different areas on the experimental site (sampling points **a**, **b** and **c**, **Figure 6.1**).

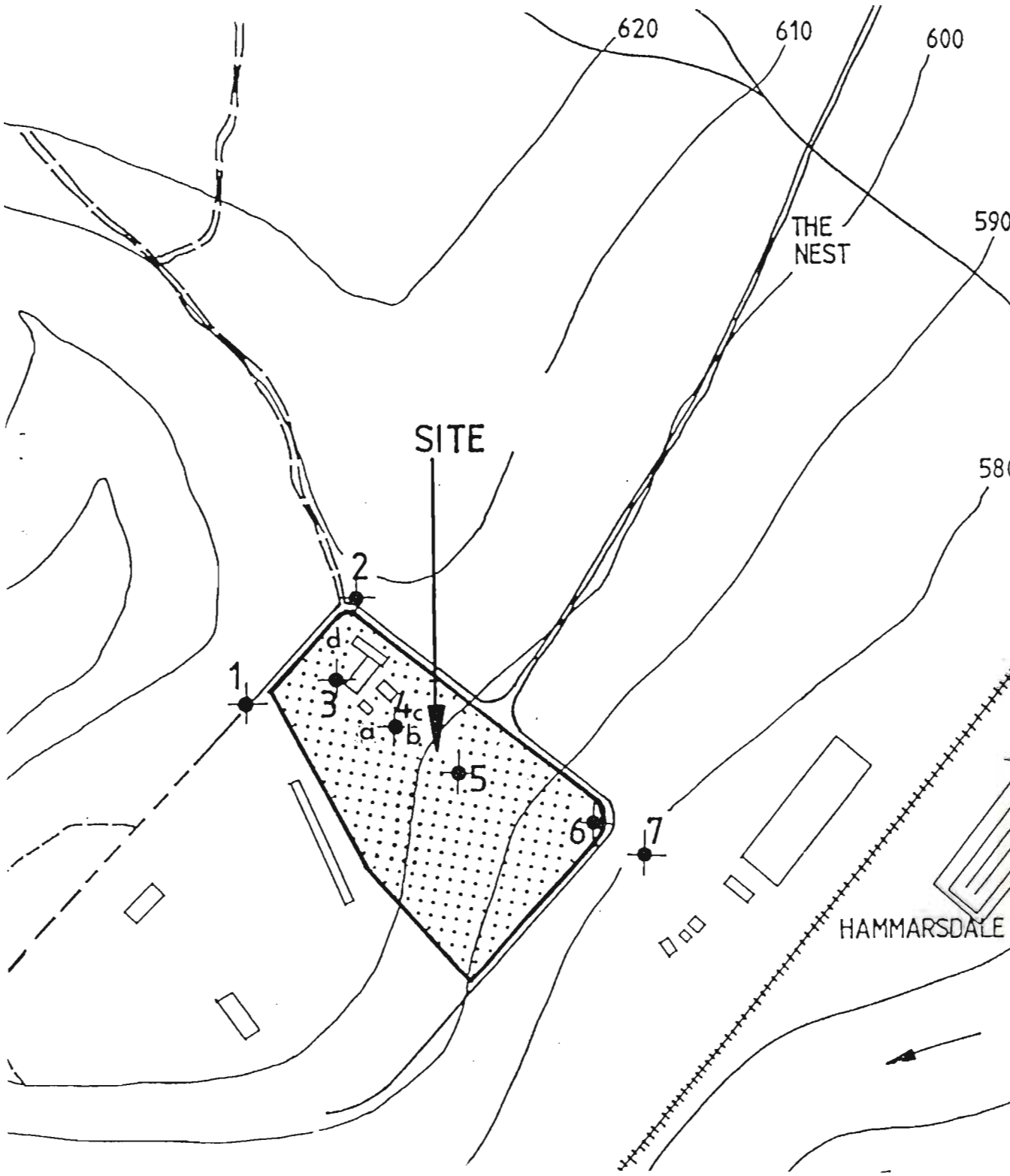


Figure 6.1: Aerial site map, showing sampling sites a, b, c and d.

6.2.1.3 Inorganic Nutrient Treatments

Six combinations of C, N and P were added to the different soils in the jars. These treatments were based on an analysis of the organic carbon present in the contaminated soil (**Table 6.1**). The supplements were initially applied to the jars in the correct ratios required for the microorganisms to catabolize the *total* (calculated) carbon in each sample of soil. The treatment combinations of C:N:P (added via NH_4NO_3 and K_2HPO_4) were: (A) 20:1:1; (B) 15:1:1; (C) 10:1:1; (D) 10:1:2; (E) 5:1:1; and (F) a single biological control which received water only. Abiotic (sterile) microcosm controls received identical treatments.

6.2.1.4 Soil Aeration

Since a limiting factor for hydrocarbon degradation is known to be oxygen when there is a depth dimension, the experiment was made in shallow glass jars (volume ~100 m*l*) to minimise this possible limitation.

6.2.1.5 Jar 'Microcosms'

Glass jars (100 m*l* capacity) were soaked in Extran™ for 2 hours, rinsed with a 1M solution of HNO_3 followed by deionised water (twice), dried overnight at 110°C and then autoclaved at 121°C for 15 minutes. Forty gram samples of contaminated soil (fresh weight) were placed into each jar. Six different C:N:P ratios (**Section 6.2.1.3**) were added to duplicate jars. In addition to these, sterile controls which received the corresponding C:N:P supplement, and a control with water only, were established in duplicate, for each sampling event. The added nutrients (Saarchem, Analytical Grade) were each dissolved in 3.5 m*l* of deionised water, which corresponded to 80% of the field capacity of the same volume of soil packed at a bulk density of 1370 kgm^{-3}. This ensured retention of the nutrients in the microcosms without water limitation or waterlogging. After supplementation, each soil sample

was thoroughly mixed, the jars were weighed, sealed with parafilm (to prevent water evaporation but allow oxygen diffusion), and incubated at 28-30°C in the dark. Each week, the jars were re-weighed and replenished with deionised water if necessary. Jars, in duplicate, were destructively sampled at 7-day intervals, and the soils placed into plastic bags, before heat-sealing and storing at -17°C until required for analyses. At the time of analysis, the soils were divided for Infrared (IR) analysis and pH measurement.

6.2.1.6 Media

(i) Soil extract agar

Method was the same as that used in **Section 5.2.2.2.**

(ii) Mineral salts agar (IMA)

Mineral salts medium for the plates was prepared as described in **Section 5.2.2.3**. Prior to use, 50 µl of oil were filter sterilised (0.4 µm), dispensed onto each plate and spread over the surface with a sterile glass spreader.

6.2.1.7 Soil pH

Soil samples from each jar were air-dried at room temperature (23-26°C) in the dark for 72 hours, ground and passed through a 2 mm sieve. Ten grams of each sample were individually placed in 50 ml beakers, to which 25 ml of a 1M KCl solution were added. Each slurry was stirred for 1 minute and allowed to stand for 15 minutes. The pH of the supernatants were then measured with a combined glass-calomel electrode (Crison 2002, GK2711 Radiometer).

6.2.1.8 Chemical Analyses

(i) Infrared Spectrophotometry (IRS)

5g of soil from each jar, and 5g of anhydrous Na_2SO_4 (Saarchem) were placed in 30 m*l* glass vials. Ten m*l* of carbon tetrachloride were added to each vial, prior to sealing. The sealed vials were shaken vigorously for 15 secs, placed in a sonicating bath (Whaledent Biosonic) at ambient temperature for 30 minutes and stored under a fume hood overnight. The solvent was decanted into another 30 m*l* vial which contained 1 g of Florisil (60-100 mesh, Polychem). The Florisil was prepared by heating to 650°C for two hours prior to cooling and the addition of 6% (w/w) distilled water. This preparation was stoppered tightly and left overnight (room temperature). The extract was shaken vigorously for 1 minute, after which it was filtered into an IRS cell (SMM Instruments). The sample cell was transferred to the spectrophotometer (FTIR-4300 Shiadzu Fourier Transform Infrared Specrophotometer) and scanned at wavelengths between 400 and 4000 cm^{-1}.

6.2.3 *Results and Discussion*

6.2.3.1 Carbon Contents in Contaminated Soil Samples

The mean carbon content (**Table 6.1**) of a range of samples analysed approximated to 8%(w/w), which corresponds to 80,000 mg C kg^{-1} soil. Although some of the samples contained small amounts of humus and root material, this was unlikely to account for more than 1% of the total.

The percentage carbon in the contaminated soil facilitated the development of a classification system, which was used throughout the study to distinguish between 'heavily', 'moderately' and 'lightly' contaminated soil (**Table 6.2**).

Table 6.1 : The percentage (w/w) carbon and hydrogen of contaminated soil samples collected from the experimental site at sample points a, b and c.

Site Sampled	Percent Carbon (w/w)	Percent Hydrogen (w/w)
Adjacent to brick reservoir (a)	2.09	0.82
Adjacent to brick reservoir (a)	2.29	0.34
Next to effluent dam (b)	3.05	0.53
Next to effluent dam (b)	3.51	0.54
Between reservoir and dam (c)	17.36	1.20
Between reservoir and dam (c)	20.79	1.32
MEAN	8.18	0.79

Table 6.2 : 'Heavy' (HC), 'moderate' (MC) and 'light' (LC) contamination concentrations.

Classification	mgC kg^{-1} Soil	Percent Carbon (w/w)
Heavy	> 20,000	>2.0
Moderate	20,000 - 5,000	0.5-2.0
Light	<5,000	<0.5

6.2.3.2 Soil Nutrient Ratios and their Effects on Hydrocarbon Degradation

The limitation of petroleum biodegradation due to a nutritional imbalance between the substrate carbon supplied by the oil and the nitrogen and phosphorus required for microbial growth has been extensively reviewed in relation to the marine environment (Atlas, 1977; Bartha and Atlas, 1977). As the nitrogen and phosphorus reserves of many soils are low, a similar situation can be expected to exist in this environment. Several previous reports on the stimulation of oil biodegradation by mineral fertiliser supplementation have been published (Jobson *et al.*, 1974; Verstraete *et al.*,

1975; Raymond *et al.*, 1976) but no systematic effort has been made to determine the optimal fertiliser concentrations.

The quantities of N and P required to convert 100% of the petroleum carbon to biomass can be calculated from the C:N and C:P ratios found in cellular material. In theory, approximately 150 mg of nitrogen and 30 mg of phosphorus are consumed in the conversion of 1 g of hydrocarbon to cellmaterial (Rosenberg *et al.*, 1983). In reality, a complete assimilation of petroleum carbon into biomass is not achievable under natural conditions. Some of the petroleum compounds are recalcitrant or are metabolised slowly over long periods and this component may well constitute the major part of the carbon load. From petroleum compounds which are labile, some carbon will be mineralised to carbon dioxide. Thus, the efficiency of conversion of substrate carbon (oil) to cellular material is less than 100%. Population turnover also facilitates the recycling of nutrients. In this study, the optimal C:N and C:P ratios for petroleum biodegradation were, thus, expected to be wider than the theoretical values of 10:1 and 100:1, respectively. Confirmation of this could only be found by experimentation. Determination of the total hydrocarbons confirmed that the highest percent biodegradation (63.52 - 66.7 %, **Table 6.3**) was reflected by 10:1:1 to 20:1:1 supplementations. With the highest level of fertilisation (5:1:1), biodegradation was only slightly greater than in the sterile control and was lower than the water control. This was possibly due to the development of highly saline conditions by the addition of higher concentrations of soluble N and P salts. No significant promotion of biodegradation was observed in response to higher phosphorus concentrations (10:1:2). In fact, the additional P appeared to slow biodegradation slightly. **Figures 6.2 A, B, D** and **E** show similar trends in the rates of hydrocarbon removal, indicating that biodegradation proceeded as well with C:N ratios of 20:1 as with 15:1 and 10:1. Beyond this salt concentration, microbial activity is inhibited. This effect is demonstrated by the comparison of **Figure 6.2** C (5:1:1) with **Figure 6.2** F (sterile control), where the rates of hydrocarbon removal are very

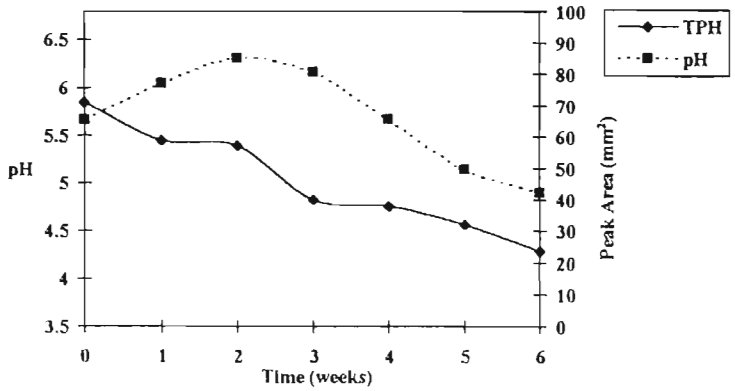
similar. The high salt concentrations seemed to exert a "sterilisation" effect. In both of the controls (**Figure 6.2** F and G) and in the soil with the highest supplement (**Figure 6.2** C), the pH did not decrease with time compared with the other treated soils, where a causal relationship between microbial activity and the pH change seemed evident. It was accepted that the losses which were calculated for **Figures 6.2** C and F were largely the result of abiotic processes.

The experimental design eliminated leaching and minimised denitrification losses. However, these factors would probably account for the loss of some of the applied nitrogen fertiliser in a full-scale operation. Thus, any management protocol should include re-supplementation to reinstate the optimal C:N:P ratio.

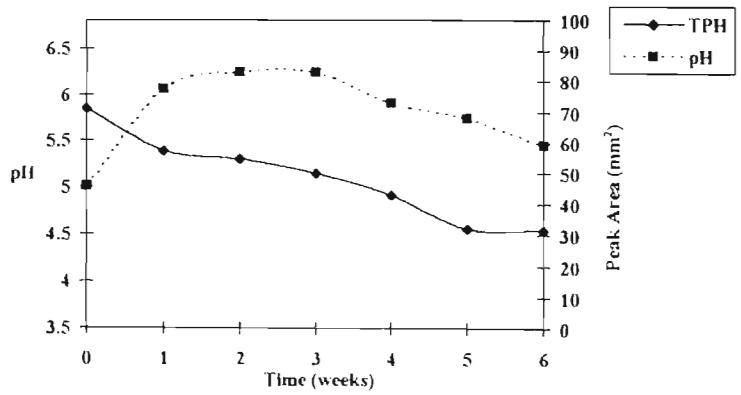
Table 6.3 : Total hydrocarbon (TPH) degradation of heavily contaminated soil, incubated with different nutrient ratios, over a six week period at 28-30°C (n=2)

TREATMENT	% TPH REMOVAL (mean)	MEAN % TPH REMOVAL RATE WEEK^{-1}
20:1:1	65.25	10.87
15:1:1	63.52	10.59
10:1:1	66.7	11.11
10:1:2	55.6	9.27
5:1:1	34.80	5.80
STERILE	29.4	4.90
WATER	42.84	7.14

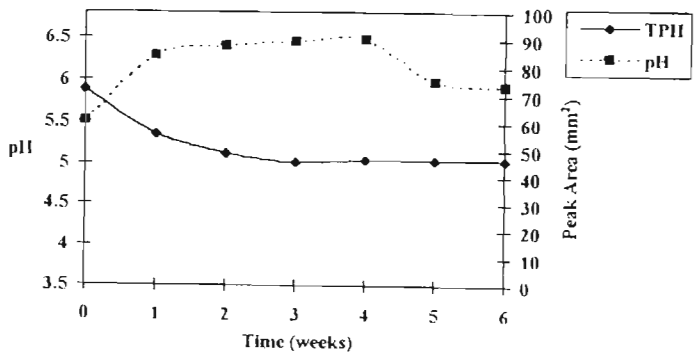
Potassium, a standard ingredient in agricultural fertiliser formulations, was found to be present in excess of its actual microbial requirement and, thus, no effort was made to determine the exact requirement concentration for this element.



(A) C:N:P = 10:1:1

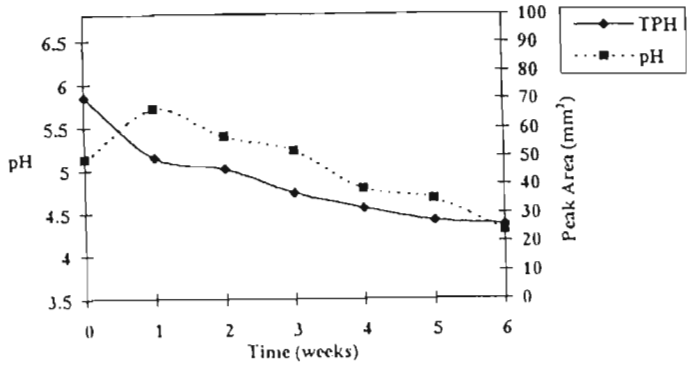


(B) C:N:P = 10:1:2

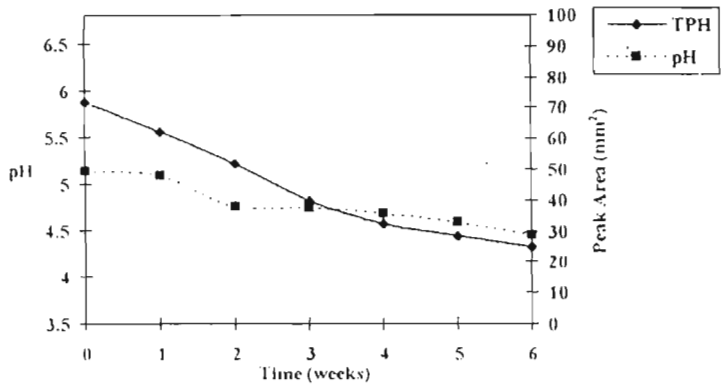


(C) C:N:P = 5:1:1

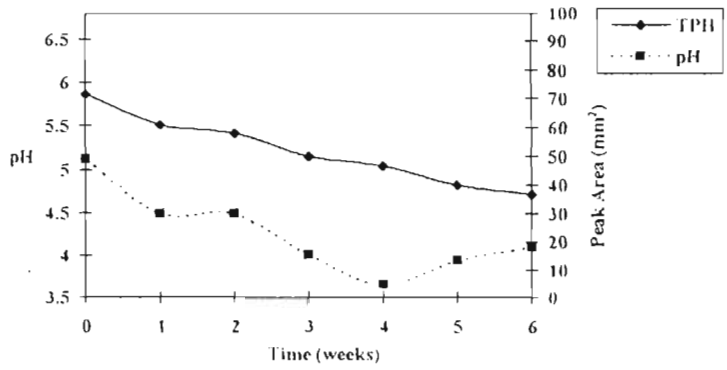
Figure 6.2: The pH change and corresponding change in peak area (Total Petroleum Hydrocarbons = TPH) in the batch cultures which contained various C:N:P ratios of : (A) 10:1:1; (B) 10:1:2 ; and (C) 5:1:1.



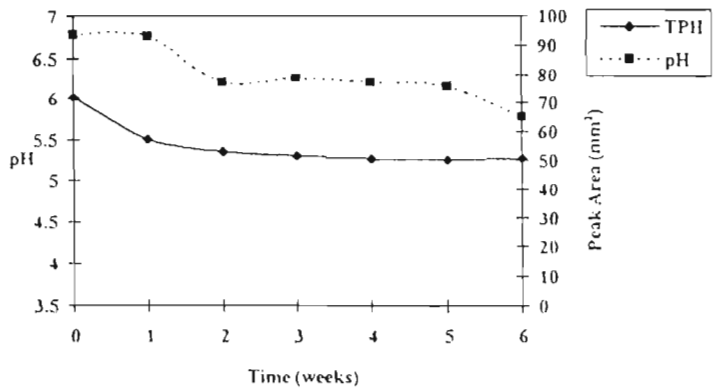
(D) C:N:P = 15:1:1



(E) C:N:P = 20:1:1



(F) Water only



(G) Sterile Control C:N:P = 5:1:1

Figure 6.2: The pH change and corresponding change in peak area (Total Petroleum Hydrocarbons = TPH) in the batch cultures which contained various C:N:P ratios of : (D) 15:1:1; (E) 20:1:1 ; (F) 5:1:1 (Sterile Control); and (G) Water only (control).

This preliminary investigation confirmed the existence of an actively-metabolising, indigenous microbial community, which was stimulated by the addition of N and P, in a C:N:P ratio of between 10:1:1 and 20:1:1, and whose component species were capable of hydrocarbon degradation at a maximum (calculated) carbon mineralisation rate of approximately 11% $week^{-1}$.

6.2.3.3 Soil Micronutrients

Trace element deficiency in soils is rarely encountered and research has shown that the application of a trace element solution to soils has only a marginal effect (Dibble and Bartha, 1979b). Micronutrients were not applied in this experiment although they may be more critical if phytoremediation is considered as an option. The dual elemental requirements of plants and microorganisms may cause some competitive deficiency.

6.2.3.4 Soil pH

The jar experiments indicated that, with ammonium nitrate as a nitrogen source, few significant pH changes resulted during the six week period. Microbial activity did not appear to be inhibited by the pH changes although there was a possible correlation between hydrocarbon disappearance and pH. The total hydrocarbon (TPH) concentration decreases were accompanied by slight pH decreases (**Figures 6.2 A-G**). There was no apparent relationship, however, between the actual C:N:P ratio and the drop in pH. Where water was added (only), more extreme pH fluctuations were recorded, suggesting that in this sandy soil the addition of ionic species slightly improved its buffering capacity. Extremes of pH are often inhibitory to the majority of microbial processes, while the pH of soils can have significant effects on accumulation, immobilisation and leaching of added elements (ElBagouri, ElNawawy, Abdal, Al-Daher and Khalafawi, 1994). Fortunately, the use of ammonium nitrate as a nitrogen source

prevented the dramatic pH fluctuations experienced in previous experiments (**Chapter 5**).

6.2.3.5 Soil Microorganisms

The plating experiments had two objectives: (1) to estimate the sizes of the heterotrophic, aerobic microbial populations in the soil after the addition of different nutrient supplements; and (2) to estimate the numbers and proportions of contaminant degraders in the total heterotrophic population.

Population counts (**Table 6.4**) indicated the presence of a substantial number of both general heterotrophs (approximately 3.4 x 10^7 ml^{-1}) and hydrocarbon degraders (approximately 4.42 x 10^6 ml^{-1}) in the treatments. The microbial counts were reduced in the jars which were supplemented with nutrients at the highest ratio (5:1:1), while the sterile controls showed minor (insignificant) contamination. The jars which received water only contained high numbers of microorganisms although these numbers were lower than in the nutrient-supplemented soil. There was a very close correlation between microbial numbers, rate of hydrocarbon disappearance and nutrient treatments. Thus, it can be deduced that the hydrocarbons in the contaminated soil were biodegraded by hydrocarbonoclastic microorganisms and that this process was facilitated by nutrient supplementation. Clearly, stimulation of the microorganisms by nutrient addition was successful.

Table 6.4 : Mean microbial counts, on solid media, of treated contaminated soil samples after 24 hours incubation at 30°C (n=3)

C:N:P Supplement	Nutrient Agar Mean	Soil Extract Agar Mean	IM Agar + Oil Mean
Sterile	15	11	8
Water	5.5×10^4	3.1×10^4	2.7×10^3
5:1:1	2×10^3	5.3×10^3	2.1×10^2
10:1:1	7.3×10^7	4.59×10^6	8.9×10^6
10:1:2	2.9×10^7	3.1×10^6	2×10^6
15:1:1	1.1×10^7	8.7×10^6	8.32×10^6
20:1:1	9.6×10^7	8.5×10^6	9.1×10^6

6.2.3.6 Soil Water Content

The aerobic biodegradation of simple or complex organic material in soil is commonly greatest in the presence of 50 to 70 % of the soil water-holding capacity (Pramer and Bartha, 1972). Inhibition at lower values is due to inadequate water activity, while higher values interfere with soil aeration. It is possible that hydrocarbons, by rendering some surfaces hydrophobic, reduce the water-holding capacity of the soil and thus increase the availability of the water (Dibble and Bartha, 1979). The soils in the jars and in subsequent experiments were, therefore, maintained at 80% of the field moisture capacity (~50% water-holding capacity).

The findings of this study were primarily for use in subsequent experiments as guidelines with respect to the nutrient-microorganism responses and degradation potential. The data were not subjected to thorough statistical scrutiny and were not meant to represent absolute values. Problems with representivity of samples due to the heterogeneous distribution of hydrocarbons on the site, and the absorption of oil to the glass preclude the assumption that the measured values were exact. Trends were, however,

consistent and the experimental methodology was carefully replicated to give the best possible comparative information.

6.3 Surfactant Screening

6.3.1 Introduction

Many xenobiotic compounds which represent important environmental pollutants are hydrophobic and persist in sediments and soils. In these environments, such compounds are frequently exclusively sorbed to organic matter and/or clay so that little are present in the water phase. Because of the persistence and sorption of many compounds, and the fact that the non-sorbed compounds are often readily biodegradable, it is possible that sorption results in a protection of such chemicals from microbial attack.

Attention has, therefore, been given to the possible use of surfactants for the removal of sorbed pollutants in contaminated aquifers (Lee *et al.*, 1988). Similarly, the effects of several non-ionic and anionic surfactants on the solubilisation of compounds (e.g. phenanthrene and anthracene) sorbed to soil have been tested with soil-water suspensions. If a surfactant solubilises the sorbed compound, the molecule(s) may become readily available for microbial utilisation and the consequent biodegradation might obviate the necessity of removing and then destroying the desorbed chemical (**Figure 6.3**).

The growth of microorganisms (*Acinetobacter* spp., *Corynebacterium* spp. and *Pseudomonas* spp., for example) on hydrocarbons in a culture medium is often accompanied by emulsification of the insoluble carbon source (Gutnik and Rosenberg, 1977). This is generally attributed to the production of an extracellular emulsifier during growth on the hydrocarbon (Finnerty and Singer, 1983). Hydrocarbon uptake is, thus, facilitated by hydrophobisation of the cell envelope or an increase of the interfacial area by hydrocarbon

emulsifying biosurfactants to improve the uptake of substrate (Zajic and Seffens, 1984). Industrial surfactants with minimal toxicity cannot replace them in all cases.

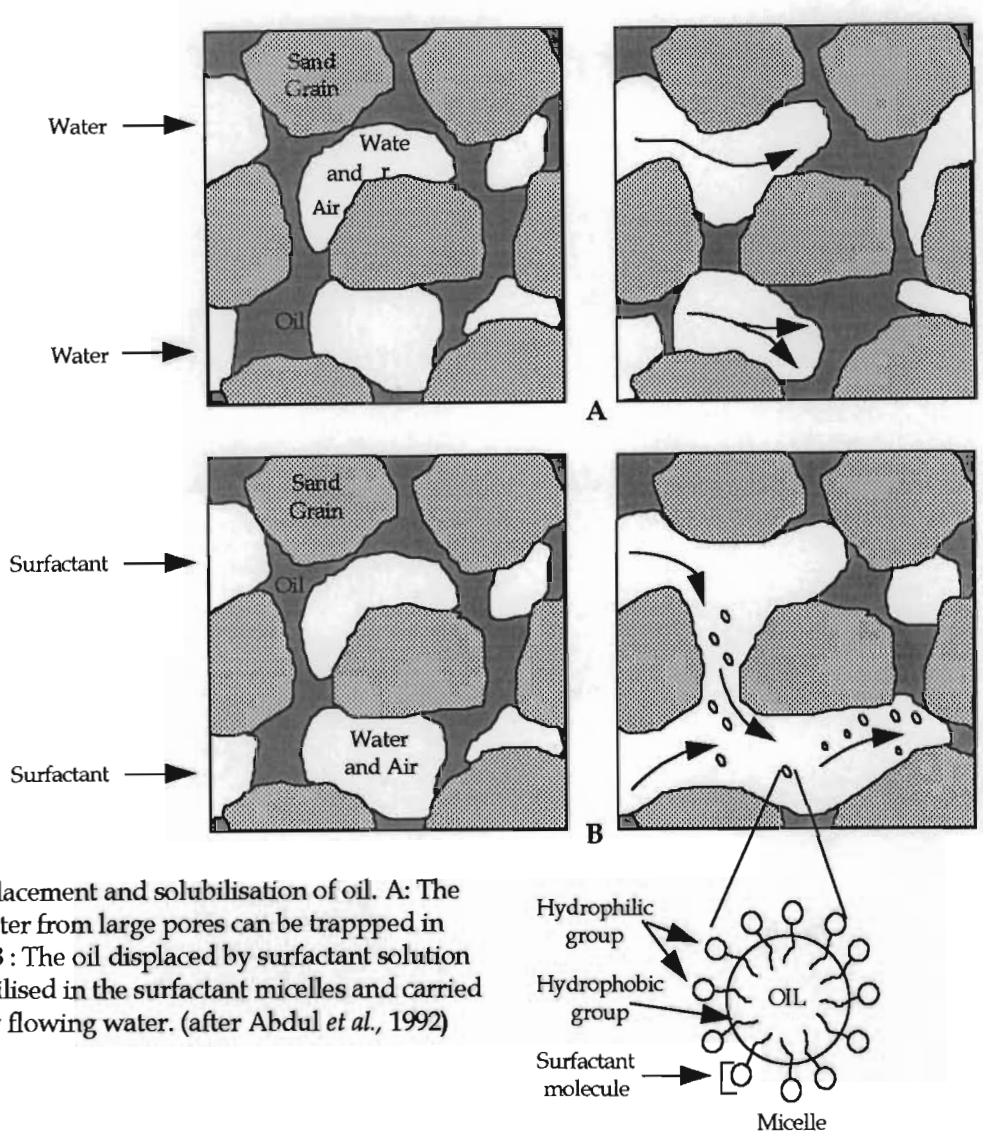


Figure 6.3 : The displacement and solubilisation of oil. A: The oil displaced by water from large pores can be trappped in smaller pore spaces. B : The oil displaced by surfactant solution is dispersed and solubilised in the surfactant micelles and carried through the pore by flowing water. (after Abdul *et al.*, 1992)

Some chemical dispersants may enhance hydrocarbon oxidation while others inhibit it (Cooney, 1984). In any event, the dissolution and emulsification of hydrocarbons appear to have a positive effect on degradation rates. If there are no adverse toxic effects, dispersion could accelarate microbial degradation (Atlas, 1981).

Three non-ionic, ethyloxalate surfactants were tested to determine whether they met three important criteria, namely, that they should be non-toxic to

microorganisms; they should adequately solubilise the oil adsorbed to the soil; and they should not be used preferentially as a carbon source in the presence of hydrocarbons.

6.3.2 Materials and Methods

6.3.2.1 Emulsification Capacity in Batch Culture

Mineral salts (IM) medium (100 m*l*) was dispensed into each of a series of Erlenmeyer flasks which contained 10 g of heavily contaminated soil (>20,000 mg C kg^{-1} soil). Three chemical dispersants (Arkopal N-050; Arkopal N-060 and Emulsifier 2491, Hoechst S.A.(Pty) Ltd) were individually added to give concentrations of 0.01%, 0.1%, 0.5% and 1.0% (v/v). The emulsifying capacity of the dispersants was estimated by measuring the turbidity of the solution by spectrophotometry at a wavelength of 545 nm (Mulkins-Phillips and Stewart, 1974) after thorough mixing and standing for 1 hour.

6.3.2.2 Batch Cultures

To examine whether the dispersants supported microbial growth, solutions were withdrawn aseptically from similarly prepared flasks which were incubated at 30°C in a rotary shaker (15 rpm) for three days. The solutions were filtered through 0.4µm filters, and the filters were subjected to critical point drying prior to SEM examination (as described in **Section 5.2.6**)

6.3.2.3 Media

(i) Double-Layer Agar Plates

Agar plates, made with a support layer of IMA which contained 0.1% (v/v) oil extracted by Soxhlet, were overlaid with a layer of molten agar which contained 2,500, 5,000, 7,500 or 10,000 m$g l^{-1}$ of dispersant. The plates were

inoculated with 50µl of suspension from either hydrocarbon or hydrocarbon+surfactant enrichment batch cultures which were prepared as described in **Section 5.2.** The plates were then incubated (28-30°C) for 4 weeks in the dark to determine possible carbon source utilisation.

(ii) Nutrient agar (NA)

Plates containing NA were also inoculated with the same batch enrichment cultures to determine whether the microorganisms were stimulated or inhibited by the presence of a surfactant.

6.3.3 *Results and Discussion*

6.3.3.1 Surfactants and their Emulsification Properties

Emulsifier 2491 is a non-ionic, polyglycol fatty acid ester. Its hydrophil-lipophil balance (HLB) value, which is an important parameter describing physical properties of surfactants, is 11.7 (the higher the HLB value, the higher the hydrophilicity). This value is rather low, and so the emulsifier could be described as slightly hydrophilic. It is dispersible in water, and soluble in hydrocarbons, wool fat and most vegetable and mineral oils (Hoechst S.A., 1992).

Arkopal-N grades are non-ionic surfactants which are used in the detergent and technochemical industries. The basic structure is a nonylphenolpolyglycoether, with the N-050 [$C_9H_{19}C_6H_4O(CH_2CH_2O)_5$] and N-060 [$C_9H_{19}C_6H_4O(CH_2CH_2O)_6$] indicating the number of ethylene oxide molecules. The physico-chemical and processing characteristics of the Arkopal N grades depend largely on the ratio of the hydrophobic molecules (nonylphenol) to the hydrophilic or water-solubilising chain (number of ethylene oxide molecules). When mixed with aliphatic hydrocarbons, the solubility quickly decreases with increasing polyglycolether chain length, while all the Arkopal grades are soluble with aromatic hydrocarbons. The Arkopal-N-050 and -N-060 types were described as slightly hydrophilic and

were, therefore, recommended by Hoechst (Germany) for mixed oil systems.

With a concentration of 0.5% (v/v), the Arkopal-N-050 and Arkopal-N-060 surfactants were more efficient emulsifiers of the oil on the soil than the Emulsifier 2491. In contrast, Emulsifier 2491 exhibited better emulsification properties in concentrations between 0.01% and 0.1% (v/v) than the Arkopal types (**Table 6.5**). The highest reduction in surface tension, as indicated by low % transmittance values, was achieved with the Arkopal-N-060 at 1% (10,000 mg l^{-1}).

Table 6.5 : Percent transmission at 545nm, after standing for one hour, of the supernatant of 0.1, 0.5 and 1.0 % (v/v) aqueous solutions which each contained a commercial emulsifier and 10g of heavily contaminated soil.

Surfactant	Concentration (Mgl^{-1})	% Transmission (545 Nm)
Arkopal-N-060	100	102.2
	1000	106
	5000	9.4
	10 000	4.7*
Arkopal-N-050	100	28.0
	1000	92.6
	5000	9.3*
	10 000	132.2
Emulsifier 2491	100	32.3
	1000	22.7
	5000	22.3
	10 000	14.3*

* = Optimal concentrations

If dispersion alone was the controlling factor in microbial degradation of spilled oils, the effectiveness of the dispersants tested would be rated, in decreasing order of effectiveness, as Arkopal-N-060 > Arkopal-N-050 > E2491. However, from experiments made by Mulkins-Phillips *et al.* (1974), the order in which the dispersants would enhance the biodegradation of the

n-alkane fraction would be E2491 > oil alone > Arkopal-N-050 and Arkopal-N-060.

6.3.3.2 Toxicity of Surfactants and Their Use as a Preferential Carbon Source

Only one of the three dispersants (Emulsifier 2491) supported growth **(Plate 6.1** C and D**).** Filters which were examined by scanning electron microscopy (SEM) revealed only clay particles and cellular debris with the Arkopal N-050 and N-060 types **(Plate 6.1 A** and **B),** for all three concentrations. The inhibitory effects may have been due to disruption of the cohesive effects of the bacteria or their adherence to the hydrocarbon (Rosenberg *et al.*, 1983). Inhibition may also have been due to a disruption in the cell membranes by dispersant interaction with the lipid structural components. The reaction of the surfactants with enzymes and other proteins essential to the proper functioning of the bacterial cell or the masking of subcellular structures may also have been causative factors (Foght *et al.*, 1989).

The filters prepared from the supernatant of the Emulsifier 2491 batch culture showed a population of large rod-shaped and yeast-like cells with thin fibrils (**Plate 6.1** C and D) which appeared to be responsible for the adhesion of cells to one another and, possibly, to the hydrophobic surfaces (Cooper, Zajik and Denis, 1981). It can be deduced that this emulsifier was non-toxic to the microorganisms and, indeed, may have had a stimulatory effect on growth. This observation was confirmed when E2491 was added to the enrichment cultures which contained heavy and moderate oil-contamination (**Table 6.6**).

The results presented in **Table 6.6** demonstrated that E2491 did not dramatically influence the growth of the microorganisms in the batch cultures. The control cultures which received only nutrient medium (IM) and 0.01 - 0.05% (v/v) emulsifier showed little or no growth after 4 weeks, which implied that the emulsifier was not readily utilised as a carbon

source. In all cases, except with the C:N:P ratios of 5:1:1, the microbial populations of the contaminated soils were stimulated by nutrient supplements and showed vigorous growth. This trend was identical in the cultures which also received the emulsifier, which suggests that growth was not retarded by its presence. However, it is possible that the concentrations of emulsifier used were below the toxicity threshold.

Table 6.6 : Growth on NA plates inoculated with supernatant of enrichment cultures with moderate (MC) and heavy (HC) oil contamination and with or without Emulsifier 2491 (S).

Inoculum Source	Final E2491 Concentration in Batch Culture	Growth
CONTROL	0.01% E2491	+/0
CONTROL	0.05% E2491	+/0
CONTROL	0.10% E2491	+
HC	-	+++
HC	-	+++
HC	-	+++
HC + S	0.01% E2491	+++
HC + S	0.05% E2491	+++
HC + S	0.10% E2491	+++
MC	-	++
MC	-	+++
MC	-	++
MC + S	0.01% E2491	+++
MC + S	0.05% E2491	++
MC +S	0.10% E2491	++

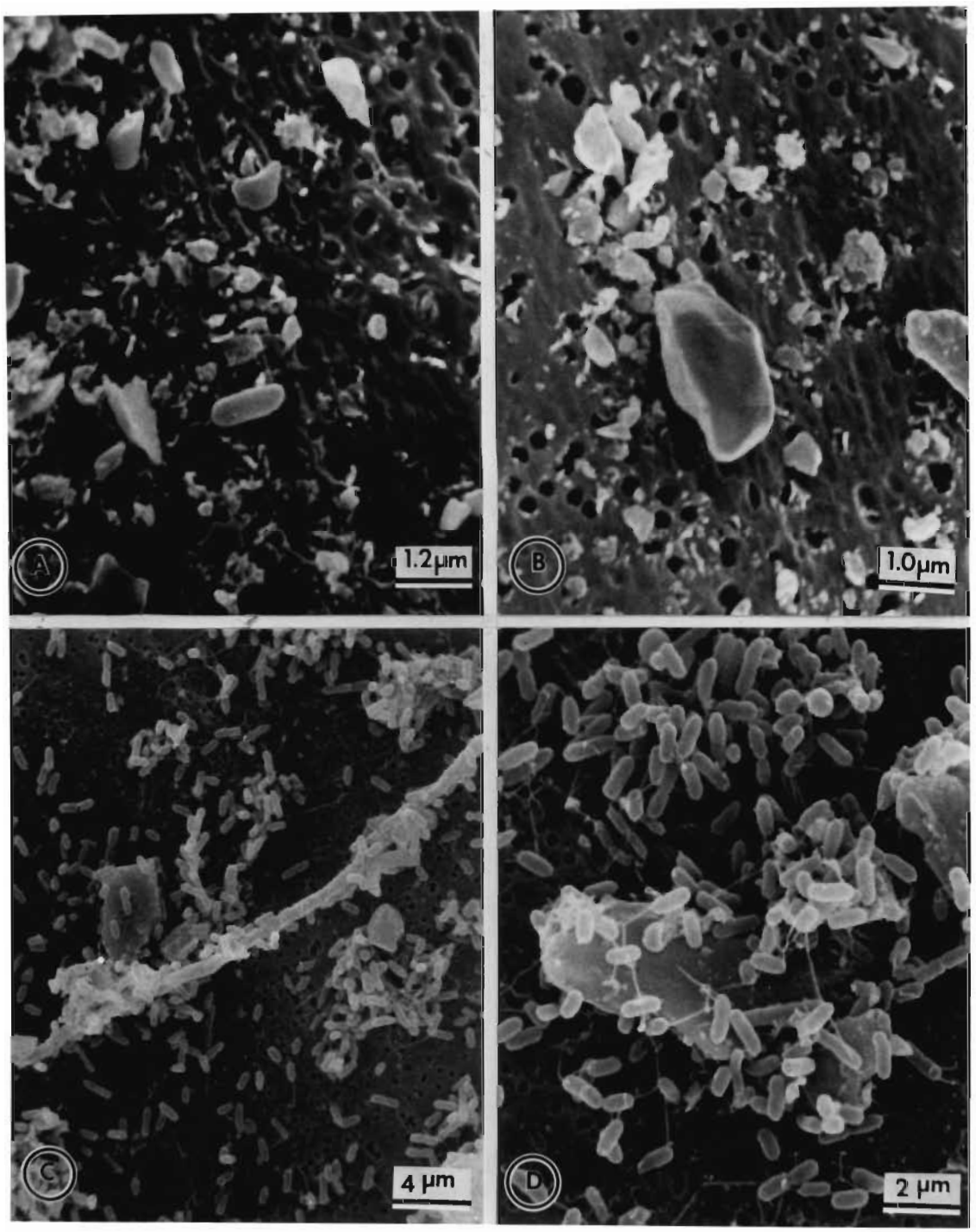
+ sparse growth ++ moderate growth +++ confluent growth

- no growth +/0 very sparse growth

PLATE 6.1 (A)-(D) :

Scanning electron micrographs of cellular debris and clay particles which were present in the supernatant of enrichment cultures inoculated with heavily contaminated soil and treated with (A) Arkopal-N-050 and (B) Arkopal-N-060.

The supernatant of an enrichment culture inoculated with heavily contaminated soil and treated with Emulsifier 2491 showed the presence of many rod-shaped bacterial cells, fungal hyphae (C) and large yeast cells (D), held in association with one another with fine extracellular fibrils.


A
1.2µm
B
1.0µm
C
4 µm
D
2 µm

To test whether the E2491 was bacteriostatic or bactericidal in concentrations ≤ 10 000 mgl^{-1}, IMA plates which contained 0.1% (v/v) oil (extracted from the soil) ,as a carbon source, and a double-layer of agar containing 2500, 5000, 7500 and 10,000 mgl^{-1} Emulsifier 2491 (E2491), were subsequently inoculated with supernatant from the hydrocarbon enrichment cultures. Growth was then recorded after 4 weeks (**Table 6.7**).

Table 6.7 : Growth of indigenous microorganisms from enrichment cultures inoculated with moderately (MC) and heavily (HC) contaminated soil on IMA support plates plus oil with an overlayer containing Emulsifier 2491

Support Layer	Inoculum Source	Culture Dilution	E2491 Concentration (Mgl^{-1})	Growth
IMA + OIL	MC	10^{-4}	2500	++
IMA + OIL	MC	10^{-6}	2500	++
IMA+ OIL	MC	10^{-8}	2500	-
IMA + OIL	MC	10^{-4}	5000	++
IMA + OIL	MC	10^{-6}	5000	++
IMA + OIL	MC	10^{-8}	5000	+
IMA + OIL	MC	10^{-4}	7500	+++
IMA + OIL	MC	10^{-6}	7500	++
IMA + OIL	MC	10^{-8}	7500	+
IMA + OIL	MC	10^{-4}	10 000	+++
IMA + OIL	MC	10^{-6}	10 000	+++
IMA + OIL	MC	10^{-8}	10 000	++
IMA + OIL	HC	10^{-4}	2500	+++
IMA + OIL	HC	10^{-6}	2500	+++
IMA + OIL	HC	10^{-8}	2500	+++
IMA + OIL	HC	10^{-4}	5000	+++
IMA + OIL	HC	10^{-6}	5000	+++
IMA + OIL	HC	10^{-8}	5000	+++
IMA + OIL	HC	10^{-4}	7500	+++
IMA + OIL	HC	10^{-6}	7500	+++
IMA + OIL	HC	10^{-8}	7500	++
IMA + OIL	HC	10^{-4}	10 000	+++
IMA + OIL	HC	10^{-6}	10 000	+++
IMA + OIL	HC	10^{-8}	10 000	++

+ sparse growth ++ moderate growth +++ confluent growth - no growth

The results presented in **Table 6.7** reveal two major trends. As the concentration of oil increased from moderate to heavy contamination, the microbial numbers also increased. This was expected, since a higher concentration of utilisable carbon was available to stimulate growth. This trend was consistent for both contamination concentrations throughout the concentration range of added emulsifier, and it was concluded that in these concentrations of E2491 [≤1% (v/v)] it was neither bacteriostatic nor bactericidal.

It has been postulated (Tiehm, 1994) that the degree of toxicity to hydrocarbon-degrading microorganisms is related to the lipophilicity of the surfactant. Non-ionic surfactants with hydrophobic portions similar to the surfactants used in this study were found to be most toxic with 6 to 13 ethylene groups per molecule (Cserhati, Szoegyi and Bordas, 1982; Cserhati, Illes and Nemes, 1991). In another investigation, the toxicity decreased with longer ethoxylate chains and was related to membrane-damaging effects (Cserhati, Soegyi, Bordas and Dobrovolsky, 1984). Thus, the more hydrophobic surfactants which seem to exhibit better solubilising properties are tolerated by few bacteria (Edwards, Luthy and Liu, 1991). In the present study, a similar trend was observed when plates were overlaid with dispersant as the sole carbon source. No growth was seen on the agar-set plates for the Arkopal types, while 8 different isolates were observed in the presence of E2491. Upon subculturing, a few colonies appeared to possibly be using the E2491 as a carbon source. They were, however, very slow growing, with visible colonies only apparent after 3.5 weeks incubation.

6.3.3.3 The Potential Use of Surfactants in the Degradation of Hydrocarbons in Soil

The process of hydrocarbon uptake by microbial cells occurs predominantly as solubilised or 'accommodated' substrate. The first step in hydrocarbon degradation is the introduction of molecular oxygen with the solubilised or

'accommodated' hydrocarbon via cell-associated enzymes (Reddy *et al.*, 1982), which implies that the rate of solubilisation or dissolution of the oil may control growth. This is consistent with the conclusions of Wodzinski and Coyle (1984) that the hydrocarbon mineralisation rate is limited by solubility and, hence, hydrocarbon availability.

Together with their bactericidal/bacteriostatic effects, the Arkopal type surfactants may have been unsuitable for this application since an *in situ* protocol would require high concentrations of electrolytes. Electrolytes apparently reduce their water solubility, and could lead to salting-out, especially at high concentrations and temperatures (Hoechst S.A. (Pty) Ltd.). Also, unless added to slightly warmed water, the viscosity of the solution is significantly increased which, for field purposes, is not acceptable.

These experiments demonstrated the potential effectiveness of surfactant solutions for soil washing. More particularly, they indicated that the surfactants must be carefully selected to prevent inhibitory effects and colloid dispersion. For *in situ* applications, the alkylethoxylates would be preferred to the alkylphenol ethoxylates due to the undesirable degradation products of the latter, which are suspected to be more toxic and refractory than the original chemicals. Additionally, the concentrations of surfactants in water must be optimised to control the size of the micelles, as both soil dispersion and formation of large surfactant micelles can lead to the clogging of soil pores and subsequent diversion of the washing solution from the contaminated zone (Nash and Traver, 1986).

Although Emulsifier 2491 did not have the highest emulsifying property of the three dispersants tested, since Arkopal N-060 displaced the most oil from the soil, it did show potential and was, therefore, selected for further study.

CHAPTER SEVEN

THE USE OF FUNGI IN BIOREMEDIATION

7.1 Introduction

Fungi are probably as important as bacteria in the decontamination of compromised terrestrial environments. The biomass of fungi in soils is estimated to be five to ten times higher than that of bacteria, but the fungal activity is about one order of magnitude lower, as indicated by the specific respiration rates (Fritsche, 1992). The activities of aerobic bacteria in metabolising xenobiotics are well documented, whereas similar information about fungi is limited.

Both bacteria and fungi are ubiquitous in soil and members of both groups contribute to the biodegradation of hydrocarbons. Historically, *Cladosporium resinae* (now named *Hormoconis resinae*) has dominated this field of study, as it was the fungus most frequently isolated from aviation fuels. Filamentous fungi contain members that utilise short-chain hydrocarbons for growth. Nyns *et al.* (1968) found three important genera, namely *Fusarium*, *Penicillium* and *Aspergillus*, with the ability to assimilate hydrocarbons. Cerniglia and Perry (1973) reported that several species of *Penicillium* and *Cunninghamella* exhibited higher levels of biodegradation than the bacterial species (*Flavobacterium*, *Brevibacterium* and *Arthrobacter* spp.) which were isolated from the same environment. At least 40 species of fungi have been found to use components of crude oil as growth substrates (Davies and Westlake, 1979).

Mycelial organisms are able to penetrate insoluble substances such as oil, and this is thought to increase the surface area available for bacterial attack. Fungi are also able to grow under more environmentally stressed conditions, such as low pH and poor nutrient status, where bacterial growth might be limited (Davies and Westlake, 1979). Their physiological and

metabolic characteristics, therefore, make them potentially well-suited for bioremediation.

White rot fungi, a particularly interesting group belonging to the Basidiomycetes, have been given much attention recently. They are unique in their ability to degrade lignin to simple end products by the action of oxidases and peroxidases. These characteristics are directly applicable in the treatment of xenobiotics which have complex structures which are often resistant to bacterial degradation. Most studies have focused on *Phanaerochaete chrysosporium*, although species such as *Funalia gallica, Coriolus versicolor, Poria cinerescens, Pleurotus ostreatus, Trametes versicolor, Chrysosporium lingorium* and *Bjerkandera* spp. have also been implicated in the degradation of polycyclic aromatic hydrocarbons (Baud-Grasset *et al.*, 1993). It appears that this degradation is possible due to the structural similarities of these pollutants to portions of the lignin substructure and the low level specificity of the complement of ligninases produced by these fungi.

In addition to the filamentous fungi, there is also considerable literature available on the alkane-oxidising yeasts. Yeasts of the genera *Candida, Torulopsis, Rhodotorula, Saccharomyces* and *Trichosporon* have been isolated and are capable of converting alkanes to the corresponding alcohols and aldehydes (Atlas, 1981). Much of this research on yeasts focuses on single-cell protein production with hydrocarbons as the substrate.

Despite the findings that filamentous fungi and yeasts are active in the degradation of a range of hydrocarbons, there have been no publications regarding the application of indigenous fungi (except specific monocultures of white rot fungi) in a full-scale bioremediation project. The component of the research programme described in this chapter had the specific objective of enriching, isolating and identifying hydrocarbon-degrading fungi which showed potential for use in bioremediation. The target group was indigenous species obtained directly from the soil at the experimental site.

7.2 Phase I : Enrichment and Isolation of Soil Fungi

7.2.1 Materials and Methods

7.2.1.1 Soil Samples

Soil samples were taken from the experimental site, between depths of approximately 0 and 50 cm, by use of a hand-held auger. The samples were divided into three categories i.e (i) control soil which was uncontaminated but of the same type as the other soil samples; (ii) moderately contaminated soil with an approximate oil concentration contamination between 5,000 and 20,000 mg oil kg^{-1} soil; and (iii) heavily contaminated soil with a concentration of >20,000 mg oil kg^{-1} soil. The samples were stored in heat-sealed plastic bags at 4°C until required.

7.2.1.2 Enrichment

Samples (10 g, field moist) of each contamination level were placed in sterile Erlenmeyer flasks which contained sterile nutrient broth (Oxoid). A series of air stones was connected via tubing to small aquarium pumps (Kilio Special V-2) and placed in the flasks to aerate the medium. The pH was lowered to 4.6 with conc. HCl to favour fungal growth. "Garden soil" (1000 g) was added to 1 *l* of tap water and autoclaved for 30 minutes at 121°C (15lb psi). After the addition of 3 g of $CaCO_3$, the supernatant was filtered through a double thickness of Whatman No.1 filter paper until the solution was clear. The soil extract was re-autoclaved, the pH lowered to 4.6 with conc. HCl and antibiotics added as per Czapeks medium described below. Three flasks, each containing 200 m*l* of soil extract diluted to 350 m*l* with deionised water, were individually inoculated with 10 g of each of the soil types and sparged for 72 hours, as per the nutrient broth cultures, at ambient temperature (26-32°C).

7.2.1.3 Isolation

(i) Nutrient medium

The solid medium (STD) for the isolation of fungi was a modified Czapeks medium, as described by Raymond *et al.* (1976) and contained the following (per litre deionised water): K_2HPO_4, 1 g; $NaNO_3$, 3 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; and agar, 20 g. After autoclaving for 15 minutes at 121°C (15lb psi), the following antibiotics were dissolved in 2.5 m*l* of distilled water, filter sterilised (0.4 μm) and added to the cooled (~40°C) medium : penicillin, 0.01 g; Chloramphenicol, 0.5 g and Streptomycin, 0.025 g. The pH was then adjusted to 4.6 with conc. HCl.

(ii) Hydrocarbons

Three hydrocarbons were initially chosen as representatives of, respectively, the saturates (n-hexadecane), *iso*prenoids (pristane), and a mixed hydrocarbon substrate, laboratory paraffin. Five grams of liquid hydrocarbon were added to 15 m*l* diethylether, before addition to 5 g fine colloidal silica. Each mixture was slurried in a mortar and placed in a fume cabinet until the solvent had evaporated to leave the hydrocarbon adsorbed to the silica. The hydrocarbon-adsorbed silica was then added to the nutrient medium and autoclaved for 15 minutes at 121°C (15lb psi).

(iii) Solid media

(1) Three segments were cut out of the STD Czapeks agar plates and molten agar, containing a different hydrocarbon, was poured into each of the sectors. Each plate, therefore, contained 3 radially extending arms of different hydrocarbons.

(2) STD Czapeks agar plates were individually overlaid with 0.1 m*l* of hexadecane, pristane or paraffin.

(3) Potato Dextrose Agar (PDA) plates were used as a general medium for the identification of the isolates.

Direct isolations were made by sonicating (Whaledent Biosonic) a 1:25 soil:distilled water slurry for 5 minutes (25°C) to release the fungal spores bound to the soil surfaces. The plates were inoculated with 0.1 m*l* of each of the enrichment cultures and incubated at 25°C for 48 hours. The colonies were then subcultured onto fresh plates.

7.2.2 *Results and Discussion*

7.2.2.1 Enrichments

Profuse fungal growth was observed in the nutrient broth flasks after 72 hours. However, due to the high nutrient concentrations in the medium, and the presence of an alternative carbon source, the medium was not as selective for the enrichment of hydrocarbon degraders as intended. Therefore, the growth of bacteria and other opportunistic microorganisms was high. A decision was made, consequently, to use a low-nutrient soil extract instead of the nutrient broth for further enrichments. In contrast, growth was observed around the air stones of the soil extract flasks after two weeks and the selection of eleven strains of hydrocarbon degraders resulted. Bacterial growth was not observed.

7.2.2.2 Isolation and Identification

Approximately 30 fungal strains were isolated from the enrichment cultures, of which eleven were able to degrade the hydrocarbons(McGugan and Lees, 1993). Most of the strains were identified as *Fusarium, Penicillium, Trichoderma* or *Aspergillus* spp. with colony morphology and spore shape and size as criteria (**Table 7.1**). The fungal isolates were maintained on Potato Dextrose Agar (PDA) at 25-28°C.

Table 7.1 : Growth of eleven fungal isolates on different solid media offering three carbon sources.

Isolate	Organism	Hydrocarbon Overlay			Sectored Plates	Growth on PDA
		Hexadecane	Pristane	Paraffin		
A	*Trichoderma* sp.	+ + +	+ + +	+ + +	+ + + Hexadecane	Powdery green flat colony
B	*Trichoderma* sp.	+ +	+	+ + +	As above	Powdery green colony
C	*Fusarium* sp.	+ + +	No growth	No growth	+ + Paraffin	Extensive sectoring. Pink, brown & white colony
D	*Trichoderma* sp.	+	No growth	No growth	No preference	Powdery dark green colony
E	*Fusarium* sp.	+ + +	No growth	No growth	+ + Hexadecane and + pristane	Extensive sectoring. Pink, brown and white colony
F	*Penicillium* sp.	+ + +	+ +	+	+ + + Hexadecane	Green with daughter colonies
G	*Fusarium* sp.	+ + +	No growth	No growth	No preference	Fluffy white and pink colony. No sectoring
H	*Trichoderma* sp.	+ + +	No growth	No growth	+ + + Hexadecane	Granular green colony. Yellowing of PDA
I	*Trichoderma* sp.	+	No growth	No growth	No preference	Brown culture with fluffy white mycelium
J	*Basidiomycete (Possibly white rot)*	+ + +	+ + +	+ + +	No preference	Fluffy white, slow growing mycelium
K	*Aspergillus niger*	+ + +	+	No growth	+ + + Hexadecane	Black sporangia with white mycelium

+ = sparse growth, + + = moderate growth, + + + =extensive growth

The isolated species were comparable with those isolates documented by other authors who reported these, and many other, fungal genera to be capable of hydrocarbon degradation (Zobell, 1946; Bossert and Bartha, 1984). It appears from **Table 7.1** that the preferential substrate for all strains was n-hexadecane. This was not surprising as the simple low molecular weight alkanes are generally more soluble than the heavier, more complex,

pristane molecules and there have been many reports of microbiological preference for alkanes (Mulkins-Phillips and Stewart, 1974a; Walker *et al.*, 1976; van der Linden, 1978; Atlas, 1981). Surprisingly, in this instance, no yeasts were isolated. This is unusual, as yeasts have been cultured as single-cell protein on hydrocarbon substrates in the past and members of the *Candida* and *Saccharomyces* genera are frequently referred to in the literature (Walker *et al.*, 1975; Atlas, 1981), and were found in batch cultures of previous experiments (**Section 5.3.1**).

7.3 Phase II : Fungal Species Degradative Capacity Evaluation

7.3.1 Preliminary Strain Selection

7.3.1.1 Materials and Methods

(i) Oil-Agar Plates

Soxhlet-extracted oil (2 m*l*) was added to 100 m*l* molten Czapeks agar in a 150 m*l* Erlenmeyer flask. The mixture was then sonicated for 5 minutes to facilitate the formation of an emulsion. Before the droplets could coalesce, the medium was applied as a 3 mm overlayer to STD Czapeks agar plates.

(ii) Strain Selection

Inoculation was made with 1 cm^2 of active mycelium, aseptically cut from 5-day old cultures The plates were sealed with masking tape and incubated at 25°C. Growth was monitored daily and an attempt was made to rate the growth by measuring the means of two diameters at right angles of the expanding colonies over a 10-day period.

7.3.1.2 Results and Discussion

The attempt to quantitatively estimate the growth of the colonies over 10 days was unsuccessful as the growth patterns of the strains were irregular.

Many of the colonies sporulated heavily and gave rise to numerous daughter colonies. The selection, thereafter, was based on a visual evaluation of the nature of their growth habit and denseness of the colonies, rather than their rates of colonisation of the STD Czapeks agar plates.

Fungal species which produced compact, circular colonies were selected for further study; strains that produced a sparse, spreading mycelium were discarded. The latter growth habit is often indicative of poor substrate affinity where the fungal mycelium scavenges the medium in search of a more labile substrate than the oil. These cultures also tended to sporulate extremely rapidly on the oil-agar. Expression of such survival mechanisms is also indicative of low substrate affinity (F.H.J. Rijkenberg, personal communication). Six strains (isolates **A**, **B**, **E**, **F**, **J** and **K**, **Table 7.1**) were selected for further study.

7.3.2 *Secondary Strain Selection*

7.3.2.1 Materials and Methods

Glass columns (200 m*l*) (**Plate 7.1**) were each fitted with a recycling loop to circulate a complex basal salts medium (IM)(Watson-Craik, 1990). One gram aliquots of oil extract (Soxhlet-extracted with dichloromethane) were each adsorbed onto seven high density foam structures (7 x 3 cm). The six fungal isolates were individually inoculated onto the foam rubber, which provided an attachment surface for growth, and placed into a glass column. A seventh uninoculated column acted as the control. Each of the glass vessels was filled with basal salts medium, which was recirculated through the system by a peristaltic pump (Ismatec IPN 24B) at a rate of 600 m*l* per day. Aeration was provided by sparging with an aquarium pump attached to a glass manifold with air stones. The cultures were incubated, in the dark, at room temperature for 21 days after which time the foam was removed and the residual oil extracted with dichloromethane for gravimetric analysis.

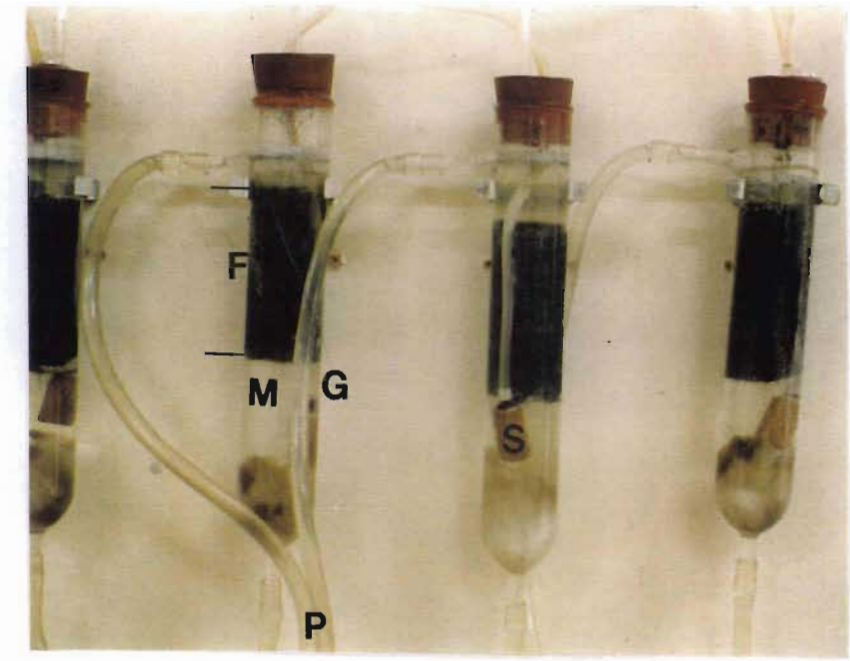


PLATE 7.1 : Glass columns (G) were each fitted with a loop to recycle a basal salts medium (M). Aliquots of oil were adsorbed onto foam (F), onto which fungal isolates were inoculated. Medium was recycled using a peristaltic pump (P), which was aerated with an aquarium pump fitted to a manifold with airstones (S).

7.3.2.2 Results and Discussion

Visible increases in fungal growth occurred in the columns for 14 days. However, extraction of the oil from all the columns after 21 days showed no significant reduction in the total concentrations when compared to the control. It is likely that the fungi initially metabolised the lighter oil fractions for growth. In the control, the light fractions were absent from the foam, and probably existed in the headspace as a result of volatilisation due to the vigorous bubbling of the spargers.

This method was selected because of the difficulty of extracting oil residues from the liquid medium. Solvent partitioning in separation funnels proved to be a fruitless exercise due to the formation of a stable emulsion which would not separate, even after repeated mixing and very long standing

times. The immobilisation of oil onto a matrix which could absorb the substrate while serving as a support for fungal growth seemed to be a more logical approach. The foam was tested for its chemical stability/solvent resistance and its absorptive capacity (for oil) in aqueous media.

Selection was not made on the basis of the gravimetric results since, although the columns were closed loop systems, volatile compounds appeared to have been lost when the foam was extracted. Visual observations were employed to assess the fungal reaction to the oil, which was not quantifiable but was accepted, for the purpose of simply choosing appropriate fungal strains, as a relatively dependable method. The fungal strains differed in their abilities to grow on the mixed oil substrate in the columns and strains were, thus, selected for 'soil box' studies on the basis of biomass production on the oil-impregnated foam matrix. The three strains with the highest visual biomass production after 21 days were a *Fusarium* sp. (FUZ 13), a *Penicillium* sp. (FUZ 16) and a basidiomycete (suspected white rot - possibly *Chrysosporium* sp.) (FUZ 9).

7.4 The Demonstration of Soil Bioremediation by Fungal Treatment

7.4.1 Introduction

To date, there have been no published reports of the sole use of fungi in a full-scale landfarming or *in situ* bioremediation scheme. While bacterial bioremediation has had a powerful, wide-ranging, impact on environmental cleanup, it has proven difficult, or impossible, to apply to certain contaminants. Fungal remediation is an innovative technology which uses naturally-occurring strains, including lignin degraders. The use of fungi is of particular interest in addressing the persistent organic compounds, such as the heavy fractions of oil, for which bacterial remediation methods have been slow or ineffective.

The major impediment to field application has been the production of appropriately large volumes of fungi which are required for site remediation. In this study, use was made of a unique method which was designed originally in the Department of Microbiology and Plant Pathology, UNP to bulk-up *Trichoderma*, a fungal biocontrol agent.

The following experiments were made to test the efficacy of the three selected strains of fungi in the remediation of oil contaminated soil. This preliminary study did not involve replicates, as trends were expected to emerge, the results of which could be used later in a more detailed experimental design. Infrared spectrophotometry (IRS) was used as a simple and rapid screening method to detect any changes in hydrocarbon concentration or a subsequent increase in degradation products.

7.4.2 Materials and Methods

7.4.2.1 Soil Boxes

Eleven glass boxes (30 x 30 x 25 cm) were each filled with 20 kg (15 cm depth) of heavily contaminated (>20,000mgC kg^{-1} soil, fresh weight) soil from the experimental site. Polystyrene boards (30 x 30 x 2 cm), with holes drilled at regular intervals, were placed at the bottom of each box before filling to facilitate the accumulation of leachate without the development of anoxic conditions by waterlogging. Each box was then covered with black plastic to prevent photo-oxidation of oil components. The soil was maintained at 70% of the maximum water holding capacity by distilled water additions.

7.4.2.2 Fungal Bulking

Barley grains were soaked in water for 48 hours, drained and 400 m*l* (approx. 290 g) portions placed in each of 28 polyethylene plastic bags (25 x 45 cm) before sealing with cotton wool stoppers to allow gaseous exchange.

The sealed bags were then sterilised by autoclaving at 121°C for 15 minutes (15 lb psi). One sterile bag per strain was initially inoculated by aseptically adding the contents of a completely colonised PDA plate, sliced into 5 mm cubes. Subsequent bags were then inoculated with 50 g of the colonised grain. The bags were incubated, in the dark, at 25°C until the grain was completely colonised (approx. 3 weeks). The colonised grain was then mixed into the contaminated soil at an inoculation rate of 6 g barley 100 g^{-1} soil (fresh weight).

7.4.2.3 Treatments

Each soil box which received nutrients was treated at the recommended C:N:P ratio for aerobic metabolism of 25:5:1 (Kennedy *et al.*, 1975) by adding NH_4NO_3 and K_2HPO_4 (Polychem). The nutrients were dissolved in 150 m*l* distilled water and mixed into the soil after each sampling event. All boxes received the same volume of water for the duration of the experiment. Tilling was achieved by turning the soil over with a small garden trowel every 10 days. Care was taken to ensure that the same tillage pattern and duration (5 minutes) was followed for every box.

Eleven soil boxes were treated as follows :

(1) Contaminated soil, barley + *Fusarium* sp. (FUZ 13), tilling, no nutrients;

(2) Contaminated soil, barley + *Penicillium* sp. (FUZ 16), tilling, no nutrients:

(3) Contaminated soil, barley + *Chrysosporium* sp. (FUZ 9), tilling, no nutrients;

(4) Contaminated soil, no barley, no nutrients, tilling (Control 1);

(5) Contaminated soil, barley + FUZ 9, 13 and 16, tilling, no nutrients;

(6) Contaminated soil, no barley, nutrients, tilling (Control 2);

(7) Contaminated soil, sterile barley, no nutrients, tilling (Control 3);

(8) Contaminated soil, barley + *Chrysosporium* sp. (FUZ 9), tilling, nutrients;

(9) Contaminated soil, barley + *Fusarium* sp. (FUZ 13), tilling, nutrients;

(10) Contaminated soil, barley + *Penicillium* sp. (FUZ 16), tilling, nutrients; and

(11) Contaminated soil, sterile barley, nutrients, tilling (Control 4).

7.4.2.4 Sampling

Every 10 days, four random soil samples were collected as cores from each box, with a glass tube (300 mm x 15 mm i.d.). The four samples were composited (100 g), placed into plastic bags and thermally sealed, before storing at -17°C until required. After tilling, and before additional nutrient treatments, a final soil core was extracted for soil pH determination.

7.4.2.5 Analyses

(i) pH

The method used was the same as that described in **Section 6.2.1.**

(ii) Infrared Spectrophotometry (IRS) of Total Petroleum Hydrocarbons (TPH)

Soil (5 g) and 5 g anhydrous Na_2SO_4 were placed in a 30 m*l* glass vial and 10 m*l* of CCl_4 were added prior to immediate sealing. The vial was shaken vigorously for 15 secs, placed in a sonicating bath (Whaledent Biosonic, ambient temperature) for 30 minutes and then stored under a fume hood overnight. The solvent was decanted into a second 30 m*l* vial which contained 1 g Florisil. (The Florisil was prepared by heating to 650°C for 2 hours, after which 6% (w/w) water was added. This preparation was tightly stoppered and also left overnight at room temperature). The extract was then shaken vigorously for 1 minute after which it was filtered into an IRS cell. The sample cell was transferred to the spectrophotometer (FTIR-4300 Shiadzu Fourier Transform Infrared Specrophotometer) and scanned at wavelengths from 400 to 4000cm^{-1}.

(iii) Scanning electron microscopy (SEM)

Soil samples, with visible fungal colonisation, were collected from various boxes, fixed to a metal stub, and frozen in liquid N_2. The specimens were then transferred to the sputter-cryo system where they were held under a vacuum at -180°C and coated with a gold-palladium layer, before viewing under the SEM (Hitachi S-570).

7.4.3 Results and Discussion

7.4.3.1 Visual Observations

The method employed for bulking-up the fungal strains was very successful. Although contamination occurred occasionally it was confined to single bags. Thus, the entire batch did not have to be discarded. After transfer to the soil boxes, the fungal response in the soil was favourable and visible growth was observed through the glass walls in all of the inoculated boxes within the first ten days (**Plate 7.2**). The importance of oxygen in the subsurface was demonstrated by the extent of growth which was seen in the pockets of trapped air at all depths. After 15 days, extensive fungal colonisation of the uninoculated barley was also observed (Boxes #7 and #12).

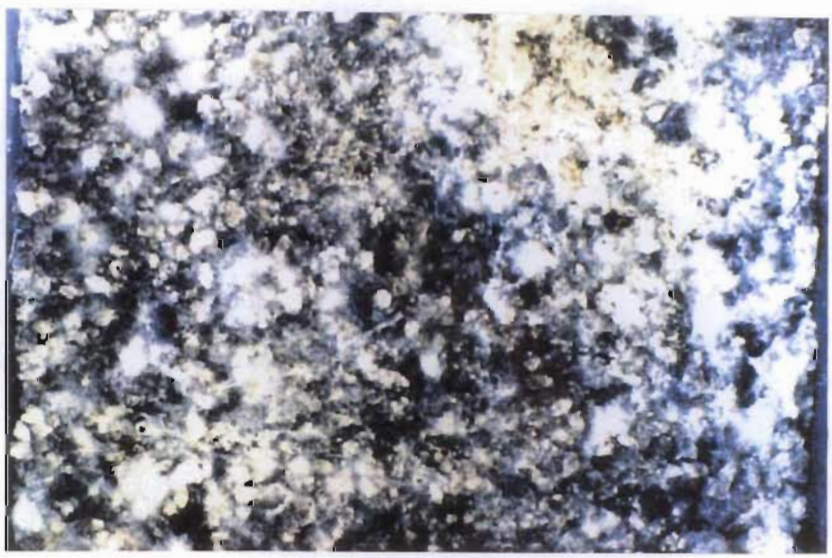


PLATE 7.2 : A photograph of one of the soil boxes (#5) in which fungal growth is clearly visible in "air pockets".

Surprisingly, the fungi in the boxes which received nutrient supplements (#8 - #10) took longer to show visible signs of colonisation than those without nutrients. However, they showed a greater biomass development after 20 days which persisted for a longer period than those receiving water only. The addition of sterile barley had a stimulatory effect on the indigenous microbial population at a rate that was marginally less than that of the other supplements. Colonisation was vigorous for the initial four weeks, after which the growth proceeded at a slower, steadier rate and was still visible a month after the experimental period was concluded (50 days).

The growth patterns of the different genera of fungi varied considerably. The *Penicillium* spp. (FUZ 16) had a short lifespan from spore germination to the formation of spores. Small pieces of substrate were densely colonised by the fungus. Spore production occurred heavily over the substrate and there was little extension of the mycelium into the surrounding soil. The basiodiomycetes (FUZ 9), by comparison, colonised the substrate with a long-lived, slower growing mycelium which subsequently migrated into the soil.

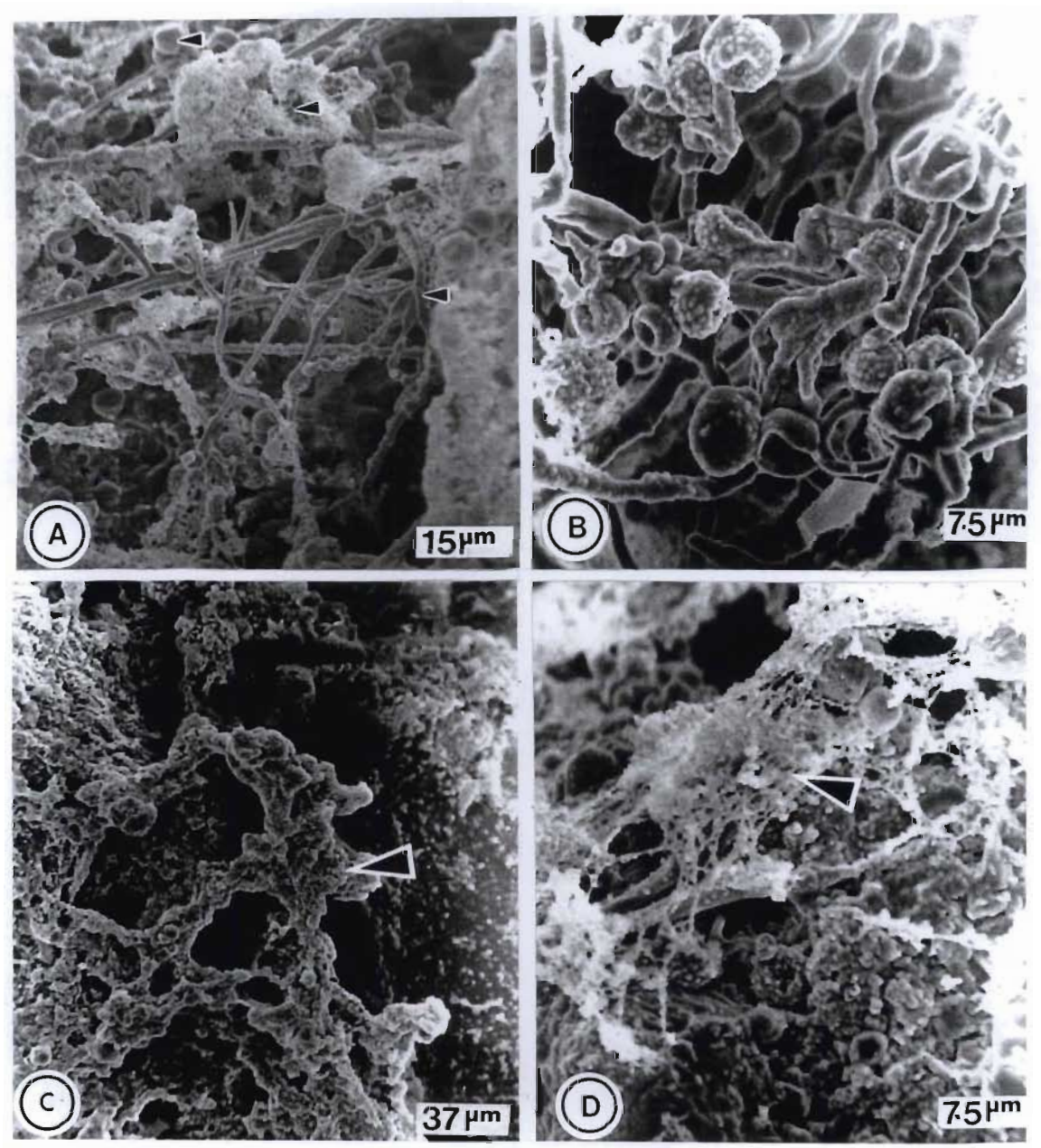
7.4.3.2 Scanning Electron Microscopy (SEM)

Electron microscopy revealed the extent of colonisation of the soil by the fungi (**Plate 7.3 A-C**). Interwoven mycelial mats consisting of fruiting bodies and fungal hyphae were closely associated with the contaminated soil and the substrate. Extracellular fibres that appeared to be of bacterial origin were also observed (**Plate 7.3 D**).

PLATE 7.3 (A)-(D) :

Extensive colonisation of the soil and barley is shown in the scanning electron micrographs where fungal hyphae (A) and fruiting bodies (B) form densely around the barley substrate, and the contaminated soil (C). Arrows indicate hyphae associated with extracellular fibres that may be of bacterial origin (D).

[Plate courtesy of B. R. McGugan, University of Natal, Pietermaritzburg, 1994]


A
15µm
B
7.5µm
C
37µm
D
7.5µm

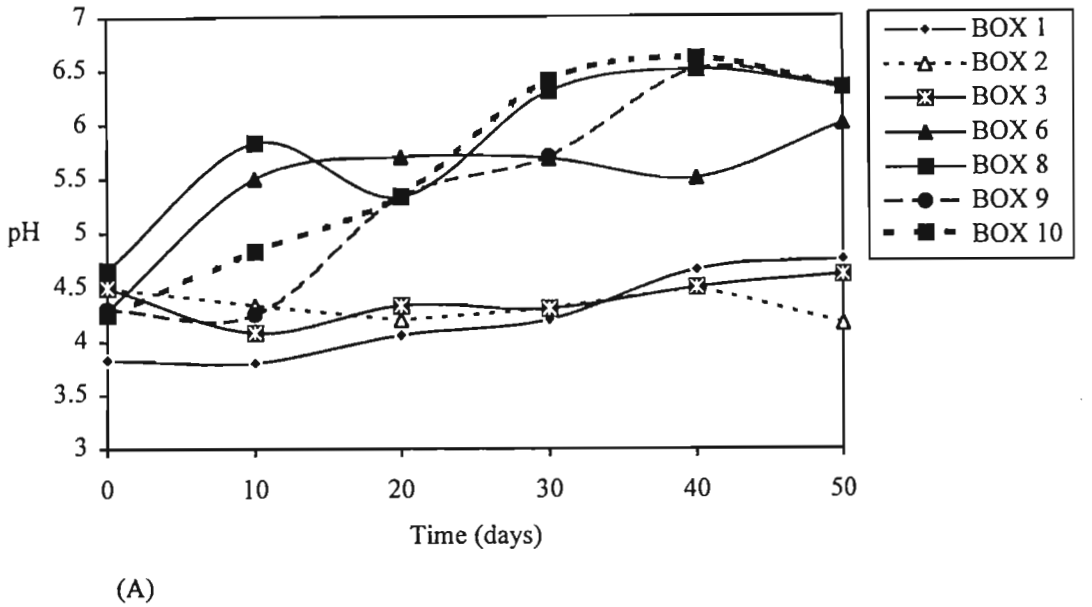
7.4.3.3 pH

The boxes that received nutrient supplements (#8 - #10) and a fungal inoculum showed a substantial increase in the soil pH, from approximately 4.5 to 6.5 (**Figure 7.1 A**). In contrast, the boxes which received an identical inoculum but without nutrient supplements (# 1 - #3) showed a relatively constant, low pH throughout the experiment. Box #6, which received nutrients, but no inoculum or barley also showed a marked increase in pH. This increase may have resulted from denitrification of the added ammonium nitrate. The nitrate may have been reduced which, with the accumulation of ammonium ions, could have effected an increase in pH.

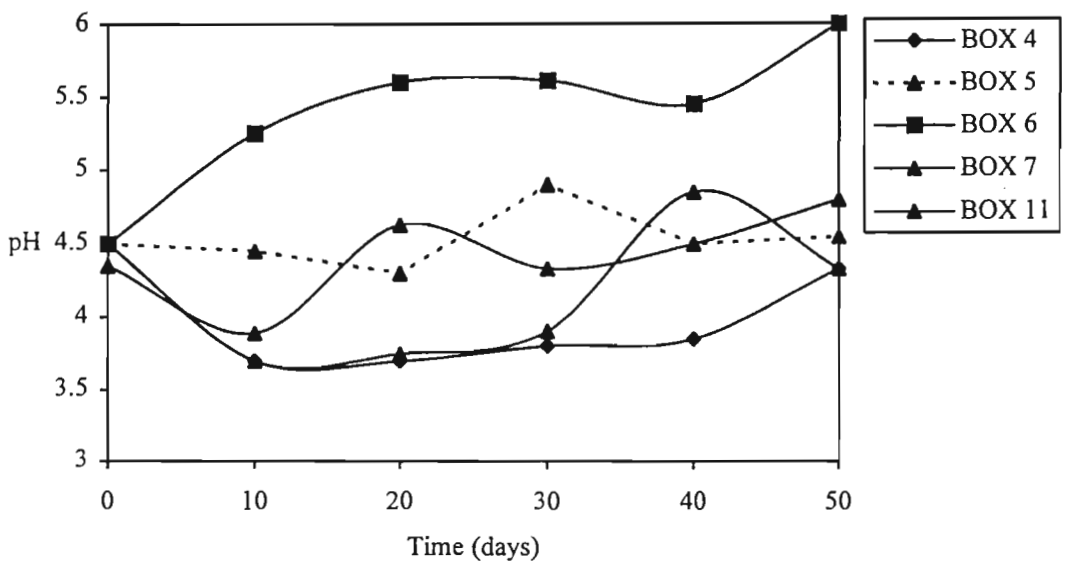
Figure 7.1 B shows the pH changes which occurred when neither barley nor nutrients were added (#4); all three strains were added at once, but without nutrients (#5); nutrients were added but no barley (#6); sterile barley was added (#7); and when the soil was merely watered and left undisturbed (#11). Again, the pH remained relatively constant throughout the 50-day experimental period when no nutrient supplements were added. It appeared that a rise in pH was unrelated to fungal biomass and/or activity as fungal growth was observed in all the soil boxes, particularly box #7.

7.4.3.4 Hydrocarbon Degradation

Infrared spectrophotometry was used as the analytical technique as it has advantages over gas chromatography in that sample preparation is relatively simple and inexpensive and a result can be obtained rapidly.



(A)



(B)

Figure 7.1 : The pH changes which occurred (A) in the soil boxes in the presence (Boxes 8-10) and absence (Boxes 1-3) of nutrients; and (B) in the control treatments (Boxes 4-7, 11). Box 5 was inoculated with all three fungal species.

It is useful for determining the types of bonds that are present in the molecules of the sample as the infrared laser is able to effect bond vibrations that are recorded on a spectrograph. Particular types of bonds, for example C-H bonds in hydrocarbons, stretch within a very narrow frequency range. Functional group bonds appear in the same region, regardless of the molecular structure. The peaks on the IRS trace represent the percentage transmission of the IR laser through the infrared cell. From these peaks, comparisons between samples can be made. There are three bands of importance on the IRS traces which represent the following bond groups : C-H bonds at 3000 cm^{-1}; C=O bonds at 1700 cm^{-1}; and the fingerprint region of 700-1500 cm^{-1} (bands in this region result from combined bending and stretching motions of atoms and are unique for every sample/compound) (Hart, 1987).

The nature of the soil contamination at the experimental site was so variable that appropriate standards were unavailable. Therefore, it was not possible to calculate the concentrations of hydrocarbons in the samples. However, by examining the peaks in the regions described, it was possible to determine whether or not degradation had taken place compared to a sample taken at the beginning of the experiment. Since samples taken at the ten day intervals gave very little comparative information, the samples taken at time 0 (T_0) and time 50 (T_{50}) (**Table 7.2**) will be discussed.

Hydrocarbon disappearance from the soil over the experimental period was estimated by measuring the heights of the hydrocarbon peaks at 3000 cm^{-1} of samples taken at T_0 and T_{50} for each treatment. Differences in the percentage transmission of the infrared laser were compared since the percentage transmission of the laser beam increases proportionately with hydrocarbon disappearance.

Table 7.2 : Mean reduction in total petroleum hydrocarbons, represented by peak heights in the 3000 cm^{-1} (peaks A a&b) and 700-1500 cm^{-1} (Peak B) region of the IRS scan, of 5 soil samples taken at T_0 and T_{50} (n=4)

Sample	Peak Aa Height, mm T_0	Peak Ab Height, mm T_0	Peak B Height, mm T_0	Peak Aa Height, mm T_{50}	Peak Ab Height, mm T_{50}	Peak B Height, mm T_{50}	% Reduction (mean)
Box #3 (HC,B,FUZ 13, T, no Nuts)	110	95	6	93	84	23	13.51
Box #10 (HC, B, FUZ 13, T, Nuts)	120	110	3	95	84	107	22.24
Box #11 (HC, SB, T, Nuts)	110	100	6	105	92	17	6.28
Box #8 (HC, B, FUZ 9, T, Nuts)	117	105	12	95	87	54	15.9
Box #6 (HC, No B, T, Nuts)	113	95	7	101	93	8	6.37

HC = Contaminated Soil B = Barley SB = Sterile Barley FUZ = Fungal inoculum T = tilling

Nuts = nutrient supplementation

The percentage reduction of hydrocarbons did not exceed 22.24 % over the experimental period although peaks in the 1700 cm^{-1} region, indicating the presence of carbonyl (C=O) groups, underwent some significant changes between the two sampling events. The changes in these peaks were particularly noticeable in samples taken from the boxes which received nutrient supplements (Boxes # 10 and 8). This indicated the accumulation of catabolic (oxygenated) intermediates, possibly as a result of fungal metabolism (or mixed microbial catabolism) via terminal-, subterminal- and/or β-oxidation (Klug and Markovetz, 1973).

The results indicated that degradation *was* taking place, albeit slowly. This could be attributed to the fact that the barley provided the microorganisms with a high proportion of labile carbohydrate which was rapidly depleted, initially in preference to the hydrocarbons, as a source of carbon and energy. Also, the production of water resulted in the development of anaerobic

conditions after about 30 days, despite the preventative measures taken, which may also have contributed to the slow degradation rates observed.

Although not conclusive at this stage, the basic trends revealed that the fungal component of the indigenous microbial population from the contaminated soil was readily stimulated by the addition of nutrient supplements, and that growth under aerobic conditions was vigorous in the presence of a hydrocarbon mixture. The importance of fungi in the degradative process justified additional exploratory work.

These experiments were, subsequently, extended with different treatment combinations. Plate and batch culture media formulations were revised, gas chromatographic analysis of selected samples was employed and microbial activity was measured by the flourescein diacetate bioassay (FDA). Problems which were experienced in this preliminary study, such as waterlogging, the development of anaerobiosis, and the preferential use of the bulking agent as a carbon source were reconsidered in the subsequent experimental design which examined the use of straw, poultry manure, maize cobs, composted pine bark and barley beer waste as alternative organic supplements. The fungal remediation of the oil-contaminated soil was subsequently undertaken as a separate study (McGugan, Lees and Senior, 1995).

CHAPTER EIGHT

PHYTOREMEDIATION : SOIL RECLAMATION USING SOYBEAN PLANTS IN CONJUNCTION WITH NUTRIENTS AND COMPOSTED PINEBARK

8.1 A Review of Phytoremediation

The remediation of contaminated soils in the United States is a multi-billion-dollar-a-year industry (Salt *et al.*, 1995). Sites are generally remediated through a wide variety of engineering-based methods that have evolved over the last three decades. These technologies can be grouped into two broad categories : a) isolation and containment techniques; and b) decontamination techniques (Cunningham and Berti, 1993b).

Isolation and containment techniques exploit physical, chemical and hydraulic barriers to isolate the pollutant and prevent its escape. This remediation strategy offers no actual reduction in the quantity of the pollutant on a particular site but the potential environmental risk is reduced. Examples of containment methods include vaults, caps and hydraulic isolation curtains, as well as physical adsorption or entrapment into a 'stable' matrix such as cement (Sims *et al.*, 1990). In contrast, decontamination methods reduce the total quantity of the contaminant. Site decontamination permits increased flexibility in terms of decisions about future land use. Examples of decontamination include soil washing, vapour extraction and microbial bioremediation, most of which were extensively discussed in **Chapter 1**. One common site decontamination technique used, particularly in South Africa, is the excavation of the contaminated material and disposal in a Class 1 landfill. This, despite its unsophistication and relatively high cost, is often the preferred remediation route for small sites. It is dependable, leaves a clean site, and has definitive start and end points. To its disadvantage, it represents a transfer of the pollutant to another location, and questions of residual liability linger. Furthermore, the siting of

new hazardous waste landfill operations is becoming increasingly more difficult.

The development of a site remediation strategy involves balancing legal, physical, chemical, biological and economic considerations. Philosophically, one would prefer that the site is left as pristine as possible after treatment, but this is not always technically feasible. The chemical and physical properties of certain hazardous wastes sometimes preclude all current site decontamination techniques except for excavation and reburial. Often seemingly plausible solutions, on closer examination, result simply in transferring the pollutant from one medium to another, with a resultant increase in complexity and perhaps even volume of the material to be treated.

Remediating a site with minimal disruption often has legal, economic and aesthetic appeal. With certain contaminant or site conditions, for example leaking underground storage tanks (LUSTs), *in situ* remediation is common (Charbeneau, Bedient and Loehr, 1992). However, soils with relatively immobile contaminants and compact soil structure pose technical challenges to all *in situ* techniques (Cunningham and Berti, 1993b). Engineering technologies are continuously being developed that may have potential, for example electro-osmosis, thermal decomposition and soil fracturing, but in most cases these options are extremely costly and difficult to implement. Vegetation-based solutions (phytoremediation) seem to be an interesting and cost-effective alternative which pose another exciting challenge to the research community.

Phytoremediation is defined as "the use of green plants to remove, contain, or render harmless, environmental contaminants" (Cunningham and Berti, 1993b). This definition applies to all plant-influenced biological, chemical and physical processes that aid in the remediation of contaminated substrates. The use of plants for wastewater treatment is over three hundred years old. Only very recently, however, have phytoremediation methods

been applied to contaminated soils, sludges and sediments (Sandermann,1992).

Although phytoremediation *per se* is a relatively new concept, relevant expertise and techniques from well-established fields are easily transferable (Cunningham and Berti, 1993b). The concept simply requires a new paradigm. Traditionally, we think of crops as productive and as a source of food. Plants as degradative or adsorptive entities requires rethinking their agronomy, biochemistry, microbiology and molecular biology (Cunningham and Berti, 1993a). For example, certain microbial and plant engineering strategies that have been considered commercial failures in the past due to yield decreases of as little as 5% may now have renewed promise. A yield decrease of 50% or more may be acceptable if it were to achieve maximum site decontamination.

The ability of plants to survive in soils declared "hazardous" has proven to be impressive. Most site personnel view vegetation as 'debris' because they are unfamiliar with the physics and chemistry of plants (Cunningham and Berti, 1993b). Yet, both the internal and external surface of many plant parts are the habitat of microbial communities that could be exploited (Cunningham and Berti, 1993a). Root surfaces, for example, maintain active microbial biofilms. These and a root's mycorrhizal extensions into the soil may significantly augment soil-root-surface contact and increase the plant's own metabolic capabilities. The exploitation of these rhizosphere communities to remediate soil contaminants is now an active area of research (Shimp *et al.*, 1993).

As mentioned, plants have received very little attention under the focus of *in situ* treatment methods. Yet, most terrestrial ecosystems subject to contamination have significant plant populations that have a vast range of microorganisms associated with their roots. Plants may stimulate the removal of hazardous organic substances by uptake and accumulation, by metabolism and/or by microbial biotransformation in the rhizosphere.

Combining the autotrophic nature of plants with the degradative capacity of microorganisms could prove to be an efficient treatment technique to remediate soil and/or groundwater, if effective planting and management strategies can be developed.

8.2 Soil Horizons and Microbial Distribution

Microbial distribution in soil is related to depth. At the surface, there exists an A horizon, followed by the B and C horizons underlain by the bedrock. The A horizon is usually characterized by an abundance of humified organic material, relatively high porosity and, therefore, good soil aeration. The B horizon is distinguished by the presence of colloidal particles and low amounts of humus that have been leached from the A horizon (Shimp *et al.*, 1993). In general, the deeper the soil horizon, the lower the organic matter concentration and the lower the porosity and soil aeration (Foth, 1990).

When contamination is confined to the A horizon, current bioremediation technologies may be satisfactory. However, by selecting appropriate plants, bioremediation may be enhanced in the A horizon and can be made possible in both the B and C horizons which is more agreeable economically. As a passive system, plants are much less costly to manage than other technologies (Shimp *et al.*, 1993).

8.2.1 The Rhizosphere and Associated Microbial Communities

The rhizosphere, the region immediately surrounding the root of a plant, serves as an enrichment zone for increased growth of certain bacteria and, as such, is a metabolically active zone which seems to be an ideal environment for pollutants to be degraded co-metabolically, due to the presence of exudates and root-associated microflora. Different species of plants support different bacterial flora via a complex interaction of growth enhancers and inhibitors. Any proposed scheme which includes plants in

bioremediation should consider not only the plant, but also the microbial communities that it supports at different soil depths and soil.

Plant-microorganism interactions have been known and studied for more than a century (Clarke, 1969; Newman, 1978; Lynch, 1982; Bowen and Theodorou, 1984; Lynch, 1990). Many studies have shown that the microbial communities in the root zone have positive effects on the growth of plants, such as by increasing the rate of mineral uptake by roots, improving nitrogen fixation, enhancing root elongation, etc., whereas other microoganisms are involved in plant pathology (Umali-Garcia *et al.*, 1980; Lin *et al.*, 1983).

Work describing microbial systems that can be acclimated to degrade complex mixtures of xenobiotics has been reported although few studies have described plant species that are capable of living in soil contaminated by chemicals and have the relevant rhizosphere microorganisms to degrade those compounds.

Some studies have identified rhizosphere microorganisms in isolation that are capable of degrading complex chemical compounds. For example, bacteria of the genus *Rhizobium* have been shown to be capable of degrading the herbicide glycophosphate (Liu *et al.*, 1983). The bacteria are specifically associated with the root nodules of legumes and their numbers in soil may be increased by the growth of legumes. In this way, legumes could enhance the degradation of glycophosphate indirectly. Rhizobia also degrade a wide range of aromatic compounds (Parke and Ornston, 1984). Walton and Anderson (1990) explored this phenomenon when they measured degradation of trichloroethylene in soil. Four dominant plant types were present on the contaminated waste site, namely a grass, *Paspalum notarum* var. *saurae* Parodi; a legume, *Lespedeza cuneata* [Dum.] G. Don.; a compositae, *Solidago* sp.; and a tree, Loblolly pine *Pinus taeda* L. The most active degradation occurred in the soil sampled from around the roots of *L. cuneata*. This was postulated to be due to the nitrogen-fixing bacteria

associated with the root nodules. Soil samples from areas without plant roots, either within a contaminated area or elsewhere, showed lower rates of degradation than samples from around the roots of each of the four dominant plant species that were examined.

Soil microorganisms are able to degrade a wide variety of xenobiotics, but the time for remediation varies. McFarlane *et al.* (1981) showed that soil planted with alfalfa (*Medicago sativa* L.) or Bermuda grass (*Cynodon dactylon* [L.] Pers.) could serve as a strong sink for benzene vapour at a concentration of 0.11 mg l^{-1} in air. Unplanted soil depleted the benzene at half the rate of planted soil. Removal of the above-ground portions of the plant immediately before testing had no effect on the rate. Thus, it was concluded that the root-associated microorganisms, or the roots of the plants, had enhanced the degradation process (Shimp *et al.*, 1993). Another possible cost-effective method for bioremediation was suggested by Licht (1990) and Schnoor and Licht (1991) which involved the use of deep-rooted poplar trees planted as a buffer zone at the edge of a contaminated site.

8.2.2 The Magnitude of the Rhizosphere

The linear extent and surface area of the root system are factors in the degradation of contaminants in soil. For example, crested wheat grass (*Agropyron cristatum* L. Gaertn.) has >200,000 m of roots per cubic metre of soil with 20,000,000 root tips (Shimp *et al.*, 1993). If each tip supported 1 mg of bacteria, it would provide 20 kg of bacterial mass to carry out metabolism. The crucial factor, of course, is the volumetric distribution of the root tips and the associated microorganisms.

Roots can be extensive in deep, unsaturated soils with *M. sativa* L. roots growing to a depth of at least 10 m (Shimp *et al.*, 1993). Even "shallow-rooted" maize (*Zea mays* L.) and sorghum (*Sorghum vulgare* Pers.) can penetrate to a depth of 2 m. Nitrogen-fixing bacteria have been recovered from root nodules of mesquite (*Prosopsis* spp.) trees at depths of 3.9-4.5 m

(Walden *et al.*, 1989) indicating that even such aerobic, plant-associating species may be present at great depths under suitable conditions of enrichment. Whether rhizobia live in the soil at these depths in the absence of the plant or whether they colonise the root as it grows is unknown.

A difficulty of bioremediation technologies is to deliver the limiting factor to microbial growth to the site of the contaminants. While 'pump-and-treat' technology brings contaminants to the surface where oxygen and nutrients are adequate, it leaves part of the contamination adsorbed to the soil particles in the subsurface. Injecting oxygen and nutrients down boreholes partially addresses this problem but *in situ* remediation is limited to the soil region that is supplied by the nutrients and often some of the contamination is unaffected because of non-uniform flow. An important effect of plant roots is that they may increase the oxygen availability as well as the solute transport by improving the hydraulic conductivity and diffusivity of the soil (Gish and Jury, 1983). Oxygen is essential for the growth of many microorganisms which live in the soil subsurface where oxygen availability is limited. Continuously dying root ends produce tiny channels and pores in the soil which facilitate the diffusion of oxygen from the surface and promotes the movement of nutrients through the soil, thereby enhancing microbial growth. By selecting species appropriate to the depth of the contamination, plant roots should be able to penetrate the entire area and supply and facilitate the distribution of limiting nutrients for microbial growth.

8.2.3 Root Exudates and Enhanced Microbial Activity

Rhizodeposition and root exudation are two means by which substrate is provided to the rhizosphere. Rhizodeposition, with the organic substances resulting from the decay of dead root hairs and fine roots, serves as an important carbon source for rhizosphere organisms (Bowen and Theodorou, 1973; Newman, 1978). Root exudates, which occur primarily in the area of the root tip, are classified as leakages, secretions, mucilages and lysates.

They consist of carbohydrates, amino acids, amides, organic acids and ions. Estimates of the annual amount of rhizodeposition and root exudates vary from 7 to 27% of the plant mass (Smith, 1990).

The addition of organic matter into humus-poor soil may be important to facilitate biodegradation of low concentrations of contaminants. With the large amount and wide variety of exuded compounds and depositions, roots should support a varied population of microorganisms, of which some might not be functional if they depended solely on the contaminant for substrate. That is, plants may provide essential co-substrates for the pollutant-degrading microorganisms (Shimp *et al.*, 1993).

8.2.4 Nutrient Accessibility

The aerobic degradation of organic chemicals, as previously described (**Sections 2.5.2** and **2.5.3**), primarily requires nitrogen and oxygen, followed by phosphorus and other inorganic nutrients (in a moist environment) which are essential for microbial growth and biodegradation. Usually, biodegradation is nutrient- or oxygen-limited, particularly under saturated soil conditions. Plant-aided, *in situ* biodegradation depends on (a) the composition of the rhizosphere microorganism populations; (b) root exudates that may act as supplemental substrates; (c) nitrogen present in the water, supplied by decaying roots, or fixed by symbiotic or free-living bacteria; (d) oxygen-transfer to the soil; and (e) the kinetics of microbial degradation, which are also dependent on temperature, nutrient concentrations, E_h, pH and the presence of water (Shimp *et al.*, 1993).

Phosphorus in soil is, generally, unavailable for plant uptake or microbial consumption (C.A. du Plessis, personal communication). The rhizosphere is able to solubilise phosphorus by the chemical activity of root exudates and the biological activity of rhizosphere bacteria and mycorrhizal fungi. Although the processes are not well understood, the presence of bacterial

species of genera such as *Bacillus, Pseudomonas* and *Agrobacterium* improves the uptake of phosphorus by plants, particularly those with mycorrhizal roots (Smith, 1990).

Roots may also be able to sustain a healthier microbial population by providing trace elements and nutrients (vitamins and growth substances) which may be important as the microorganisms can be stressed by the toxicity of the contaminant. Although microbial populations are sensitive to factors such as temperature, pH and concentrations of various ions, they are also adaptable both within species and through changes in the relative species population. It is likely that, in most situations, the plant partner is more sensitive than the associated microorganisms (Shimp *et al.*, 1993).

8.3 Xenobiotic Uptake by Plants

Experimental work relating to the uptake of organic xenobiotics by plants has been limited mostly to studies of herbicides and PCBs (Pal, Weber and Overcash, 1980; Fries and Marrow, 1981; Aprill and Sims, 1990; O'Connor, Kiehl, Eiceman and Ryan, 1990; Ficklea and Fontenot, 1993). Plants can adsorb both organic and inorganic contaminants from soils (**Figure 8.1**).

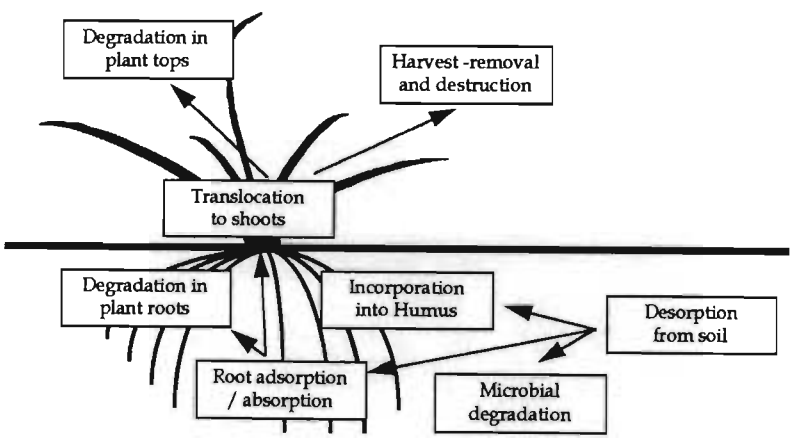


Figure 8.1: Phytoremediation : Possible pathways for the removal of a xenobiotic from soil (Salt *et al.*, 1995)

Absorption, sequestration and metabolic transformations of these pollutants are possible and potentially exploitable to remediate contaminated soil. Not all pollutants and mixtures are feasible. Due to perceived limitations of rooting depth and time requirements, most researchers currently target relatively non-leachable contaminants that pose little imminent risk to health or the environment (Cunningham and Berti, 1993b).

8.3.1 Plants and Organic Contaminants

Not all organic compounds are equally accessible to plant roots in the soil environment. Roots have an inherent ability to absorb organic pollutants, based on the relative lipophilicity of the compound (Bell, 1992). For optimal uptake, a chemical must have both water solubility and, to pass the membrane barriers of the root, a moderate level of lipid solubility.

Much of our knowledge about the behaviour of organic compounds in plant-soil systems is derived from the study, development and registration of soil-applied pesticides. The most common parameter used in the pesticide industry to predict plant uptake from the soil is the octanol-water partitioning coefficient (K_{ow}). Contaminants with a low log K_{ow}(<1) are considered water soluble and partition preferentially from a water phase into roots. These compounds would be predicted to cause groundwater contamination under most climatic conditions. Some sites might have hydrogeological conditions which could reduce the risk but plant roots do not generally accumulate these water-soluble compounds at a rate exceeding the passive influx in the transpiration stream. This suggests that these compounds are not appropriate targets for phytoremediation although such compounds can be accumulated in plants and many are considered mobile in both the xylem and phloem (Shimp *et al.*, 1993). Non-volatile pollutants with intermediate log K_{ow} values (1-4) are taken up by roots and are considered xylem-mobile, but generally phloem-immobile unless chemically altered by the plant. Compounds in this range would be considered suitable targets for phytoremediation, and the list of "priority"

pollutants that fall into this category is extensive (USEPA, 1993). Suitability for phytoremediation would clearly depend on soil type, rainfall etc. as the molecules could also be expected to cause groundwater contamination. Compounds with a high K_{ow}, such as polynuclear aromatic hydrocarbons, are those that are most strongly bioaccumulated through the food web because of their high lipophilicity (Briggs *et al.*, 1982).

The route by which organic compounds enter the environment is particularly important e.g. compounds that are denser than water, have lower K_{ow} values and come from point sources such as LUSTs would tend to have vertical concentration profiles in the soil. This would make them less amenable to phytoremediation without excavation. Compounds with K_{ow} values > 4 are likely to be adsorbed to roots but not substantially translocated to the shoot as they may be too tightly bound to soil and are not available for movement in the soil solution phase (Bell, 1992). These principles may also be applied to certain volatile compounds which can travel in the soil pores and become absorbed by roots.

Once the pollutant has been absorbed by the plant roots, it can have three fates. It may be (a) sequestered in the root tissue, often on forms not accessible to solvent extraction; (b) metabolised in the root; or (c) transported out of the root and into the shoot either as parent material or in a slightly modified form (Cunningham, Berti and Huang, 1995). Sequestration of many compounds is a common fate and is often associated with wall materials such as lignin (Briggs *et al.*, 1982).

For those compounds not transpired or biodegraded to non-toxic molecules, complete analysis of remediation efforts requires careful consideration of accumulation within the plant and possible accumulation in the food web (Paterson *et al.*, 1990).

Plants have significant metabolic activities both in the root and the shoot. Many of these metabolic capacities tend to be enzymatically and chemically

similar to those processes that occur in mammalian livers. Plants have been referred to as "green livers" (Sandermann, 1992) due to the similarities in detoxification processes, where hydroxylation followed by glycosylation activities are common ways to rid an organism of lipophilic toxins.

Thus, plants and trees are capable of assisting in the bioremediation of contaminated soil and groundwater. They provide a favourable subsurface environment by supplying oxygen and additional nutrients that promote microbial growth. Although much is still to be learned about microbial degradation in the rhizosphere and plant transport mechanisms, it may be possible to facilitate *in situ* bioremediation processes. By preventing the movement of contaminants beyond the compromised area by utilising vegetation to remove water, and by nurturing microorganisms in the rhizosphere that can degrade the contaminants, degradation could proceed at maximum rates.

8.3.2 The Phytotoxicity of Hydrocarbons in Soil

It has been observed, in petroleum-producing regions, that oil spills can "sterilize" soils and prevent crop growth for various periods of time (Schwendinger, 1968). Petroleum pollution of soil generally has strong adverse effects on the plant community. The manner in which petroleum acts on plants is complex and involves both contact toxicity and indirect effects, which are mediated by interactions of the petroleum with the abiotic and microbial components of the soil. The low-boiling point components of petroleum exhibit a high degree of contact toxicity to the sensitive portions of plant shoots and roots but they have little effect on the woody parts of trees and shrubs. Contact toxicity occurs primarily by the solvent effect of low-boiling point hydrocarbons on the lipid membrane structures of the cells. These volatile oil fractions apparently have a high "wetting" capacity and penetrating power. If they come into contact with plant seed they enter the seed coat readily and may kill the embryo (Ellis and Adams, 1961). The order of toxicity is positively correlated with polarity and inversely

correlated with molecular weight (McGill *et al.*, 1981). Thus, although large amounts of oil can be toxic, plant growth may also be inhibited due to the formation of anaerobic and hydrophobic conditions that interfere with soil-plant-water relationships.

Toxicity of the lower molecular weight hydrocarbons could be responsible for morphological effects, such as reverse geotropism, which may be observed with partially emerged seedlings and which appears to be caused by naphthenic acids affecting the plant growth regulatory systems (Fattah and Worth, 1970). The low-boiling point petroleum components are readily removed from the biologically active surface layer of moist, well drained soils through evaporation and leaching (Hunt *et al.*, 1973) and the effects of these components are, therefore, of short duration.

Although petroleum biodegradation is crucial in the rehabilitation of contaminated soil for plant growth, the process may temporarily increase petroleum toxicity not only by its oxygen and mineral nutrient demands but also by its metabolic intermediates. Products of incomplete microbial oil

degradation include fatty acids and terpenoid materials which possess phytotoxic properties (Stevenson, 1966). These, and other hydrocarbon metabolites, may amplify the phytotoxicity of polluted soils if not removed by leaching or humification processes. However, the application of oily wastes to soil in moderate amounts (1-5% v/w oil in the upper 15 cm soil layer) usually has less deleterious effects on the plant community than do large-scale accidental spills (Raymond *et al.*, 1976; Huddleston and Meyers, 1978; Kinako, 1981). Very low hydrocarbon concentrations (<1% v/v oil) may actually stimulate plant growth and crop yield (Pal and Overcash, 1978). This may be due, in part, to petroleum components acting as growth hormones (Fattah and Worth, 1970). Delayed growth enhancement may also occur in response to the greater humus content and water-holding capacity of soils that have recovered from oil contamination.

Plants have been integrated into engineering schemes in erosion control and are often used to maintain the integrity of ditches and berms (Cunningham and Berti, 1993b). Towards the end of a remediation strategy, a site erosion plan often specifies vegetative covers. Particularly robust grasses are considered for disturbed, polluted areas as an 'interim measure' (Oyler, 1988). Revegetation is always a visual confirmation that toxicity is declining, and thus remediation must be incidental.

Examination of the published literature thus reveals persuasive evidence that plant roots, in conjunction with their associated microbial communities, offer a potentially important treatment strategy for *in situ* biological remediation of chemically contaminated soils (Chaney, 1983; Aprill and Sims, 1990; Anderson and Walton, 1992; Sandermann, 1992; Banks and Schwab, 1993; Cunningham and Berti, 1993a,b; Shimp *et al.*, 1993). Under a variety of environmental conditions, vegetation has been shown to enhance microbial degradation rates of organic chemical residues in soils. These findings are important because vegetation may provide a low-cost alternative or supplement to expensive, capital-intensive technologies for soil cleanup (Salt *et al.*, 1995).

8.4 Seed Germination in Oil-Contaminated Soil

8.4.1 Introduction

From a knowledge of plant adaptation, it is reasonable to assume that certain crops should be more "oil tolerant" than others and thus better suited for reclamation (Schwendinger, 1968). Since revegetation of soils contaminated by oil is often a desirable goal, seed germination and plant growth bioassays with lucerne (*Medicago sativa*) and soybean (*Glycine max*) were made. These plant types were chosen, primarily, because they are legumes and are thus capable of fixing atmospheric nitrogen. Since the experimental soil was deficient in nitrogen, it would be both beneficial to the soil quality, and cost-effective, if this ability could be exploited.

Legumes are known to release residual nitrogen to the soil. This residual effect was judged, in one study, by the yield of a subsequent crop of wheat or rice. A maximum residual effect was apparent with soybean which increased the yield of the subsequent crop of wheat by 65.9% (Subba Rao, 1985). Furthermore, it was postulated that the mere presence of plant root material may have a stimulatory effect on the hydrocarbonoclastic microbial species in the soil.

8.4.2 Materials and Methods

One hundred commercially prepared seeds of both lucerne and soybean were each placed in a germination tray, consisting of a raised glass plate with moistened absorbent paper spread over it, and overlapping into a larger glass tray of water (wick). Concurrently, 100 each of lucerne and soybean seeds were planted at a depth of 1 cm in polystyrene seedling trays filled with moistened, heavily contaminated soil (>20,000 mgC kg^{-1} soil) and uncontaminated sand (control). The seeds were incubated at ambient temperature (25-45°C) for a week. The percentage germination was recorded at 3, 5 and 7 days.

8.4.3 Results and Discussion

The decrease in the percentage of seed germination (**Table 8.1**) (21% and 37% for soybean and lucerne, respectively) observed for seeds planted in oil-contaminated soil compared to the seeds planted in sand was in agreement with the results of Schwendinger (1968) and Dibble and Bartha (1979). Lucerne showed a 62% germination rate in oily soil, compared to 74% for soybean.

Table 8.1 : Percentage (mean) germination of lucerne and soybean seeds planted in sand (control) and in heavily contaminated soil (n=100).

Treatment	Soybean % Germination			Lucerne % Germination		
	Day 3	Day 5	Day 7	Day 3	Day 5	Day 7
Sand (Control)	95	95	95	97	99	99
Oil + Soil	32	68	74	49	62	62
Difference	59	27	21	57	37	37

The seed was shown to be 100% viable when germinated on trays. Thus, the reduction in germination may be attributed to the toxicity of petroleum compounds in the soil. Alternatively, the slower germination in the soil contaminated with oil could have been due to competition for oxygen between the germinating seeds and hydrocarbonoclastic microorganisms. Murphy (1929) reported a similar delay in wheat germination after the application of small amounts (1-2% w/w) of crude oil to the growth medium.

Temperatures in the glasshouse exceeded 43°C for four consecutive days during the trial. The seedlings were scorched and, subsequently, died. The observation was made during this time that the soybean seedlings appeared to be able to tolerate the heat better than the lucerne seedlings. Since the area requiring reclamation becomes very hot (30-45°C) during the summer months, a decision was made to pursue the use of soybean in a larger trial and to eliminate the lucerne.

8.5 Survival of Different Soybean Cultivars in Contaminated Soil

8.5.1 Introduction

Cultivars vary in their susceptibility to herbicides (Birch, Duxbury, Parsons and Greenfield, 1990) and their sensitivity to the herbicide application is

influenced by environmental conditions (Birch, Duxbury, Greenfield, Smit and Chapman, 1990). Similarly, of the 30 soybean cultivars available in Natal, some may also be more sensitive to oil contamination than others. An investigation of this possibility was undertaken to choose a cultivar that was most tolerant of the site conditions.

8.5.2 *Materials and Methods*

8.5.2.1 Seed

Soybean seeds of 30 cultivars were obtained from Ukulinga Farm (University of Natal, Pietermaritzburg) where a national trial was being made to test the relative performance of the cultivars under dryland conditions. The seeds were imbibed for two hours in a 2mM $CaCl_2$ solution before planting.

8.5.2.2 Inoculation

The seeds were inoculated with *Bradyrhizobium japonicum* (5×10^8 live cells g^{-1}, Stimuplant cc.). The inoculant was mixed thoroughly with 500 m*l* distilled water in which 5g of methyl cellulose were dissolved. The slurry was stirred thoroughly until all the seeds were coated. They were then spread in a cool shaded area to dry.

8.5.2.3 Seedling Growth

The seeds of each cultivar were planted in polystyrene seedling trays (2 x 12 cells) filled with heavily contaminated soil (>20,000 mgC kg^{-1} soil) or uncontaminated sandy soil collected from an area adjacent to the experimental site (control medium). The trays were placed in a greenhouse and subjected to a complete nutrient spray twice a day (Nutrimix™, MacDonalds Seeds). Seeds of each cultivar were also placed onto glass germination trays (**Section 8.2.2**) to test their viability. Germination was

recorded after 3 days. Emergence of the seedlings grown in the greenhouse was recorded photographically after two weeks and visual ratings of the seedlings were made. After six weeks, the seedlings were photographed, cut at the soil surface, dried for 48 hours at 60°C and weighed. Comparisons were made visually and gravimetrically and statistical analyses (Genstat) were used to identify the best three cultivars.

8.5.3 Results and Discussion

The heavily contaminated soil appeared, from the start, not to be a favourable medium for the germination of the selected seeds. As soon as the surface of the soil had dried, it formed a hard, hydrophobic crust over the seeds so that water was unable to infiltrate the soil. It seemed unlikely that the seeds would have received enough moisture to survive. Surprisingly, the seeds of all the cultivars germinated, albeit slightly slower (~2 day lag) than those of the control seeds, and a survival rate of between 37.5% and 100% was recorded (**Table 8.2** and **Plate 8.1**). A similar reduction in germination rates and survival of wheat, maize, rye grass, barley and oats was also noted by Murphy (1929), Udo and Fayemi (1975) and ElNawawy *et al.* (1994).

The cultivars grew more vigorously than was expected, given the oily conditions and extreme summer temperatures, although some showed a definite sensitivity to the presence of the hydrocarbons. Cultivars A5409, Dumela, Zebra, Knap, Pan 581, Bamboes, Highveld Top, Prima, Hennops, SCS1 and Columbus emerged first, while cultivars Nyala, Impala, Pan 790 and Pan 812 were observed to emerge at least 2 days later. After emergence, Impala, Pan 790, Pan 717, Wilger and Bakgat developed slowly relative to the control plants and exhibited symptoms that, without analytical confirmation, appeared to be the result of toxicity or inhibition by the oil. The symptoms (**Plate 8.1** C, D, E, F and G) that were most obvious were stunting, smaller leaves, thin stems and root-growth abnormalities. The rest of the cultivars exhibited symptoms that were also different from the control

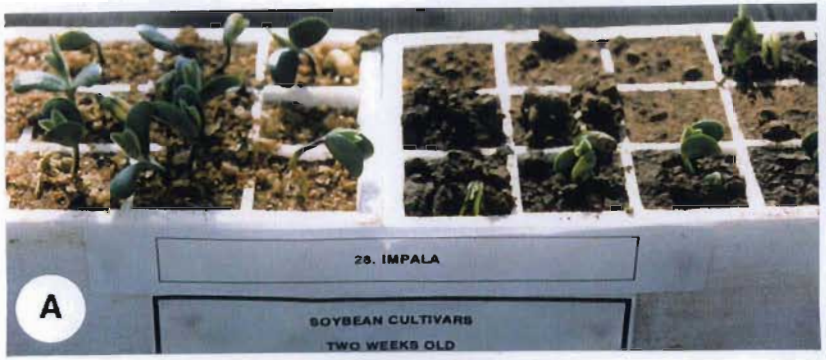
plants in that they were etiolated, a symptom regarded by plant pathologists to be the result of "unusual stress" (B. Garman, personal communication).

Every cultivar grown in contaminated soil showed the disruption of the positive and negative geotropic orientation of the root and shoot tips, respectively (**Plate 8.1E** and G). The root tips grew horizontally or, more often, upward, while the shoot tips and cotyledons were frequently oriented sideways or downward (**Plate 8.1 A** and **B**). This could have been the consequence of a number of conditions such as the roots did not favour the medium in which they were growing and chose to grow away from the inimical situation. Alternatively, it could be that the hydrocarbon-degrading microorganisms in the soil were competing with the plant roots for oxygen. In response, the roots sought a more oxygenated environment, developing what may have been a crude 'aerial root' similar to the adaptation found in mangrove plants. Another explanation is that a biochemical disruption in the root hormones caused the roots to grow in a different direction. The control seedlings in the sandy soil did not show this root behaviour, therefore it can be deduced that this was a reaction which could probably be described as a 'toxicity' response to some of the oil components.

Reverse geotropism, noted in soybean seedlings by Bossert and Bartha (1985) and Wang and Bartha (1990), was ascribed to the growth hormone-like action of some of the polycyclic aromatic hydrocarbon components of the oil. Some components of oils or their biodegradation products are known to resemble the structure, and exhibit the effects, of plant growth hormones (Gudin and Harada, 1974a; b). In their study, the severity of the decrease in seed germination and increase in plant growth inhibition were also consistent with Microtox™ measurements (Wang and Bartha, 1990).

Table 8.2 : Percentage germination of 30 soybean cultivars on trays, in sand and contaminated soil, and their mean dry mass (g) after 6 weeks (n=12).

Cultivar	Tray	Sand	Contaminated Soil	Contaminated Soil	Sand
	Percent Germination			Dry mass (g)	
Columbus	93.4	83.3	91.7	0.41	0.53
Bakgat	84.5	79.2	83.3	0.50	0.57
Prima	87.7	83.3	91.7	0.52	0.31
CRN 2233	92.1	95.8	75.0	0.42	0.61
A5308	91.1	87.5	70.8	0.46	0.69
A5409	92.1	100	79.2	0.54	0.80
PAN 494	91.0	91.7	83.3	0.34	0.57
Hutcheson	94.0	83.3	79.2	0.38	0.50
PAN 581	89.4	87.5	79.2	0.43	0.62
Highveld Top	90.1	95.8	95.8	0.38	0.53
Knap	92.9	87.5	83.3	0.46	0.70
PAN 717	93.9	91.7	87.5	0.39	0.49
Talana	90.7	62.5	79.2	0.32	0.50
Wilger	95.9	95.8	95.8	0.39	0.53
Hennops	100	87.5	87.5	0.43	0.47
Dumela	95.7	91.7	91.7	0.48	0.60
A7119	93.7	87.5	79.2	0.40	0.53
PAN 790	93.3	66.7	62.5	0.39	0.43
PAN 812	80.0	87.5	62.5	0.33	0.59
Forrest	85.9	87.5	91.7	0.44	0.61
Bamboes	78.3	79.2	87.5	0.35	0.49
SNK 60	93.5	79.2	87.5	0.52	0.63
PAN 723	92.5	83.3	87.5	0.49	0.50
Hutton	100	95.8	100	0.54	0.68
Zebra	94.6	95.8	100	0.52	0.62
Ibis	88.4	100	87.5	0.35	0.62
Nyala	80.0	83.3	37.5	0.33	0.72
Impala	96.9	79.2	50.0	0.26	0.57
Gazelle	98.9	79.2	95.8	0.50	0.55
SCS 1	100	91.7	100	0.56	0.73


28. IMPALA
SOYBEAN CULTIVARS
TWO WEEKS OLD
A


SOYBEAN CULTIVARS
TWO WEEKS OLD
B


28. IMPALA
C


D


27. NYALA
E


F


G

PLATE 8.1 (A)-(G) :

The soybean seedlings in the cultivar screen were germinated in heavily contaminated soil and sand (control). Some cultivar seedlings, such as Impala (A), showed poor germination in the contaminated soil, whilst others, such as A5409 (B), germinated slightly later (~2 days) than those grown in the sand. Later, differences were more clearly observed and toxicity or inhibition of growth by the oil was observed as symptoms such as : stunting (C), small leaves and thin stems, and etiolation (D); or root-growth abnormalities (E and G). A general view of the trays (F), with the seedlings grown in oily soil on the left and the control seedlings on the right, shows the general effect of stunting.

Statistical comparison was made between the dry mass of cultivar shoots within each treatment, as well as between treatments (oily soil vs sand). Three cultivars were found to be most tolerant of the conditions, namely A5409, SCS1 and Hutton. Although the cultivar A5409 was ranked as number 1 (LSD=0.1, $P<0.05$), it was not as close in yield to its control sample as SCS1. SCS1 was, therefore, chosen as the most likely one to grow "normally" under hostile conditions. This cultivar was, therefore, used in all subsequent experiments. (Strangely, Prima seedlings in contaminated soil grew better than in sand, but this was considered to be an errant case which was probably not reproducible).

8.6 The Reclamation of Contaminated Soil Using Soybeans, Nutrients and an Ameliorant

8.6.1 Introduction

Soils contaminated with hydrocarbons or oily waste, typically, have high concentrations of organic carbon and, therefore, substantial amounts of mineral nutrients (especially N and P) are required to enhance biodegradation rates (Westlake *et al.*, 1978). The C:N ratios reported in the literature which have effected maximum oil degradation have varied from 17 (Kincannon, 1972) to 18 (Rasiah *et al.*, 1991) and 114 (Toogood and McGill, 1977) to 161 (Franke and Clarke, 1974). Huntjens *et al.* (1986) reported that ammonium nitrate applied at 40 kg N ha^{-1} enhanced oil biodegradation, but at 80 kg N ha^{-1} degradation almost ceased. Although the importance of supplementary N in enhancing oil decomposition is recognised, the appropriate form of applied N is not. However, this information is necessary to develop appropriate N management practices for remediating contaminated soil.

Although nitrogen is abundant in the atmosphere, it is generally a limiting factor for plant and microbial growth. There are several potential sources of nitrogen to overcome the shortfall, namely :

(a) the mineralization of soil organic matter;
(b) artificial fertilizers;
(c) biological nitrogen fixation in legumes; and
(d) organisms associated with tropical grasses (Gutteridge and Shelton, 1994).

Of these, N from soil is often insufficient for maximum plant growth especially in most tropical soils which are low in organic matter. Nitrogen from organisms associated with grasses is a minor source (Gutteridge and Shelton, 1994). Fertilizer N and that fixed by legumes are the largest potential sources with the latter being the cheaper (Gutteridge and Shelton, 1994). Biologically fixed N is transformed into leguminous protein, which may be returned directly to the soil as an organic mulch.

Legumes provide an environment for symbiotic nitrogen fixation with the heterotrophic bacteria of the genus *Rhizobium* (Sprent *et al.*, 1990). When a root hair of a legume encounters a rhizobial cell, a 'recognition' event occurs whereby the bacterial cell is able to penetrate the root. Ultimately, a root nodule containing bacteroides is formed. The plant provides carbon, nutrients and, perhaps, growth compounds for the modified bacteria, whereas the bacteria supply the crop with nitrogen. Forage legumes, when grown as cover crops, can provide 100-600 kg N ha^{-1} $year^{-1}$ (Sprent *et al.*, 1990). The tropical tree *Leucaena leucocephala* can fix nitrogen to the extent that it is planted with crops such as wheat to increase yields (Foth, 1990). Legume associations are, therefore, vital to sustain soil nitrogen fertility over long periods. A possible use of plants supporting nitrogen-fixing bacteria could, therefore, be in the remediation of oil spills. Partial degradation of petroleum products has been accomplished by the addition of urea and phosphate fertilizer, which increased microbial numbers. The use of nitrogen-fixing species may further enhance cleanup of oil-contaminated soils without the need for the addition of fertilizers (Sims, 1990) provided that the degree of phytotoxicity is not too high.

The aims of this experiment were, therefore :

(a) to determine whether the soybeans would fix atmospheric nitrogen in the absence of any other nitrogen source and in the presence of oil;

(b) to determine if nodules would form on plants in contaminated soil after inoculation with *Bradyrhizobium japonicum*;

(c) to determine whether a nitrogen residue or plant exudates would facilitate the biodegradation of hydrocarbons by the associated microorganisms without the necessity for other nutrient supplementation;

(d) to evaluate the effects of the hydrocarbons on the growth and survival of the soybean plants; and finally

(e) to determine whether the addition of composted pine-bark tilled into the soil would have a positive effect on hydrocarbon biodegradation and/or soybean growth.

8.6.2 Materials and Methods

8.6.2.1 Soil

All soil was collected, using a garden spade, from the top 50 cm of each location at the experimental site. Heavily contaminated soil (>20,000 mgC kg^{-1} soil) was collected from the region between the brick reservoir and the effluent dam; moderately contaminated soil (<20,000 mgC kg^{-1} soil) was taken from the region to the southwest of the reservoir; and uncontaminated soil was taken from an area directly opposite the latter site, to the northeast of the reservoir (to ensure that the soil type was the same for each sample) (Points **a, c** and **d; Figure 6.1**). Soil was transported to the laboratory and used immediately.

8.6.2.2 Pots : Trial 1

Twenty four non-draining pots were filled with approximately 3 kg of heavily contaminated soil (field moist). Similarly, 24 pots were filled with moderately contaminated soil and another 24 pots with uncontaminated

soil. Twelve pots from each soil category were used for soybean trials, while the remaining twelve pots of each were selected for nutrient trials. In each of the 36 plant trial pots three soybean seeds were planted at a depth of approximately 1 cm. Liquid nutrients were added in a C:N:P ratio of 10:1:1, using NH_4NO_3 and $NaHPO_4$ (Sigma), assuming a mineralization rate of 80%. Aliquots (30 m*l*) of nutrient solutions, corresponding to the treatments (N; N+P; P; or water), were added to the appropriate pot every 24 hours.

The pots were arranged in three randomised blocks of 24 under shadecloth. After seed germination, the seedlings were thinned to one plant per pot and allowed to grow for three weeks. The conditions of all plants were noted visually throughout the growing period.

8.6.2.3 Nutrients : Trial 1

Nutrients (N and P) were added as solutions of laboratory grade salts (NH_4NO_3 and $NaHPO_4$, Sigma) l^{-1} deionised water as follows :

Solution 1 : (HC) 18.75 g NH_4NO_3
Solution 2 : (MC) 11.07 g NH_4NO_3
Solution 3 : (LC) 2.16 g NH_4NO_3
Solution 4 : (HC) 18.75 g NH_4NO_3 and 25.20 g $NaHPO_4$
Solution 5 : (MC) 11.07 g NH_4NO_3 and 14.88 g $NaHPO_4$
Solution 6 : (LC) 2.16 g NH_4NO_3 and 2.88 g $NaHPO_4$
Solution 7 : (HC) 25.20 g $NaHPO_4$
Solution 8 : (MC) 14.88 g $NaHPO_4$
Solution 9 : (LC) 2.88 g $NaHPO_4$
Solution 10 : Water only.

8.6.2.4 Pots : Trial 2

Non-draining pots (36) were filled with approximately 3 kg of soil of different contamination levels (12 pots per contamination level) and were arranged in three randomised blocks of 12 under shadecloth. Six pots of

each category contained soil only while the other 6 contained soil and composted pine-bark (20% v/v). All of the pots were planted with soybean (SCS 1) seedling plugs grown in the greenhouse for 2 weeks in heavily contaminated soil. The pots were fertilized at the start of the study with the full complement of nutrients. Each pot was watered by hand at a rate of 50 m*l* day^{-1} for 4 weeks.

8.6.2.5 Nutrients : Trial 2

Nutrients were added as commercially prepared limestone-ammonium nitrate (28% N) (LAN), superphosphate (12% P) (Grovida) and muriate of potash (50% K) (McDonalds Seeds) in a N:P:K ratio of 3:2:1 (C:N = 10:1), calculated on an 80% carbon mineralisation rate. Heavily contaminated soil received 37.29 g LAN and/or 186.3 g superphosphate; moderately contaminated soil received 22.08 g LAN and/or 163.5 g superphosphate. All the pots received potassium as KCl, 24.84 g for heavily contaminated soil; and 14.7 g for moderately contaminated soil. The nutrients (total amounts) were mixed into the soil before filling the pots.

8.6.2.6 Pots: Trial 3

Draining pots (144) were filled with approximately 3 kg of soil of different contamination levels, corresponding to the experimental design in **Table 8.3**, and were arranged in two randomised blocks of 72 in a glasshouse with natural day length. Three soybean seeds (SCS1), after imbibing in a 2mM $CaCl_2$ solution for 1-2 hours, were planted into each of 72 pots. The seeds were inoculated with *Bradyrhizobium japonicum* (Stimuplant cc) after planting, by suspending the bacteria in water and adding 150 m*l* of the suspension to each pot. The plants were monitored daily for 10 weeks and observations were recorded photographically. Water was added to all pots, initially at a rate of 75 m*l* day^{-1} but this was gradually increased to 125 m*l* day^{-1} for the pots containing plants. The control pots without plants received 75 m*l* day^{-1}. Although the pots were allowed to drain, the volume of water

added was calculated from the pore volume of 3 kg soil (24.5m*l* kg^{-1}) and it was anticipated that little or no leaching would occur.

Table 8.3 : The nutrient supplements, pine-bark and lecithin which were added to the three levels of contaminated soil during Pot Trial 3.

Contamination Level	Nutrients	Pine-bark	Lecithin (g)
High (>20,000 mg C kg^{-1} soil)	Nitrogen 4.67g LAN*	20% (v/v)	5
	Phosphorus 4.18g SupP*	20% (v/v)	5
	Nitrogen & Phosphorus	20% (v/v)	5
Control	Water only (150 m*l*)	20% (v/v)	5
Moderate (10,000-5000 mgC kg^{-1} soil)	Nitrogen 2.76g LAN*	20% (v/v)	2.9
	Phosphorus 0.691g SupP*	20% (v/v)	2.9
	Nitrogen & Phosphorus	20% (v/v)	2.9
Control	Water only (150 m*l*)	20% (v/v)	2.9
No Contamination	Nitrogen 0.54g LAN*	20% (v/v)	-
	Phosphorus 0.048g SupP*	20% (v/v)	-
	Nitrogen & Phosphorus	20% (v/v)	-
Control	Water only (150 m*l*)	20% (v/v)	-

* SupP= Superphosphate LAN=Limestone ammonium nitrate

8.6.2.7 Nutrients : Trial 3

The nutrients (N and P) were added as polymer-coated slow-release LAN and superphosphate (Grovida) to prevent the development of saline conditions and to minimise the leaching of nutrients. Potassium was not added as the initial soil tests showed that sufficient potassium was present for soybean growth. The total quantities of nitrogen and phosphorus (C:N ratio was reduced to 20:1; N:P = 3 :2) were calculated based on a 50% carbon mineralization rate and to prevent overfertilization they were divided into three applications (at the start of the experiment, at 4 weeks and at 8 weeks).

8.6.2.8 Micronutrients

Micronutrients were constituted as three solutions. Ten m*l* of each solution kg^{-1} soil were added to each pot twice during the 12-week growing period

(at the start, and at 6 weeks). Solution 1 contained (g l^{-1} deionised water): KH_2PO_4, 265.5; Solution 2 contained (g l^{-1} deionised water): $ZnCl_2$, 1.051; $MgSO_4$, 29.91; $CuSO_4.5H_2O$, 1.186; $MnSO_4.H_2O$, 0.929; $FeSO_4.7H_2O$, 2.507; and Solution 3 contained (g l^{-1} deionised water): $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.0464; boric acid, 0.2937. Each solution was added separately, and mixed thoroughly into the soil, to avoid precipitation.

8.6.2.9 Soybean Lecithin

To a separate block of 24 pots (12 pots containing heavily contaminated soil and 12 containing moderately contaminated soil), nutrients similar to those added to the pots which received N, NP, P and water were added. With the first and second nutrient applications (every 3 weeks), 5 g and 2.9 g of commercially prepared lecithin granules (18% P) were added, respectively, and mixed into the soil. The pots were watered, sampled and analysed as before, for a period of 8 weeks.

8.6.2.10 Liming

Lime was added at 6 weeks and was based on a liming curve. This was made by adding various predetermined weights of $CaCO_3$ to 100 g of contaminated soil and mixing thoroughly. Soil sub-samples were taken (25g) and the pH measured (**Section 6.2.1.7**) after each lime supplement. The results were plotted and the lowest mass of calcium carbonate that was sufficient to raise the pH to 6.5 was calculated.

8.6.2.11 Soil Sampling

Soil (approximately 30 g) was sampled fortnightly from each pot, with an open glass tube (8 cm x 2 cm i.d.). The samples were each placed in a flip-top vial and sealed. The samples were taken directly to the laboratory where they were immediately analysed for fluorescein diacetate activity (FDA), pH and total petoleum hydrocarbons (TPH). The soil samples were then frozen

(-17°C) until gas chromatograph (GC) determinations were made on selected samples.

8.6.2.12 Analyses

(i) Fluorescein diacetate (FDA) bioassay (after Schnürer and Rosswall, 1982). *A* stock solution was prepared from FDA (20 mg ml^{-1}) dissolved in acetone and stored in the freezer (-17°C). Two grams of each of the soil samples were weighed and place in a 100 m*l* Erlenmeyer flask. A phosphate buffer was prepared (8.7g K_2HPO_4 and 1.3g KH_2PO_4 l^{-1} distilled water) and adjusted to pH 7.6 (Crison 2001). Twenty m*l* of sterile phosphate buffer were added to each flask and swirled to mix. The reaction was initiated by adding 0.2 m*l* of FDA from the stock solution to each flask labelled "a-d". Each treatment consisted of 3 replicates and one blank, which had soil and acetone, but no FDA (all flasks labelled "e"). The flasks were then shaken for 60 minutes on a rotary shaker (Labotec) at 26°C. The reactions were stopped by adding 20 m*l* of acetone to each flask. Each sample was filtered through No.1 Whatman filter paper to remove the soil residue, the filtrate collected in a test-tube, covered with parafilm and placed into an ice-bucket to reduce the volatilization of the acetone. The fluorescein concentration was determined by reading the optical density at 490 nm with a spectrophotometer (Beckman). Background absorbance for each treatment was corrected with the blank (each corresponding "e" extract). The results were recorded and the mg FDA g^{-1} soil were calculated by extrapolation from the standard curve (A_{490} x 0.000352-0.0046/0.000352).

(ii) FDA Standard/Calibration Curve

Standard curves were prepared by adding various amounts of FDA, ranging from 0 to 800 μg in 50 μg increments from the stock solution, in triplicate, to 5 m*l* of phosphate buffer in screw-cap vials. The tubes were capped tightly and maintained for 60 minutes in boiling water to hydrolyze the FDA. The hydrolyzed FDA was then added to the Erlenmeyer flasks containing 2 g of soil at natural moisture content. Another 15 m*l* of buffer

were used to wash the fluorescien from the tubes into the samples. The flasks were then shaken for 20 minutes on a rotary shaker at 25°C, after which 20 m*l* of acetone were added. The samples were filtered (Whatman No.1) and processed as described above.

(iii) Infra-red Spectrophotometry (IRS) for Total Hydrocarbon (TPH) Determination

Five grams of each soil sample were added to 5 g anhydrous Na_2SO_4 in a 30 m*l* glass vial. Ten m*l* carbon tetrachloride were added under a fume cupboard to each glass vial, which was then immediately sealed. The sealed vials were shaken vigorously for 15 secs, placed in a sonicating bath for 30 minutes, and stored under a fume hood overnight. The activated florisil was prepared the night before by desiccating in an oven, and adding 6% (v/w) distilled water to the florisil. The florisil was shaken vigorously in an air-tight bottle, and left overnight (ambient temperature). Each of the extractions was decanted into another 30 m*l* vial containing 1 g florisil, shaken vigorously for approximately a minute, and filtered into an IRS cell. Each sample was scanned from 400-4000 cm^{-1}.

(iv) Gas Chromatography (GC)

The oil concentrations of samples were determined with 40 g samples with 20 g Na_2SO_4 in a classical 8-hour Soxhlet extraction procedure with equal volumes of acetone and hexane. Each sample was added to a separating funnel with 300 m*l* of distilled water and shaken for 30 secs. The contents were then allowed to stand for at least 5 minutes before decanting and discarding the lower aqueous layer. The washing process was repeated twice. To individual 500 m*l* flasks, 5 g of anhydrous Na_2SO_4 were added, and to this the organic phase of each of the samples. The extract was reduced to 25 m*l* with a rotary evaporator (Büchi). The contents of the flasks were transferred to small glass vials and evaporated to dryness under a stream of compressed air. Using standard solutions of aliphatics and aromatics (Sigma), the compositions of the extracts were determined by GC [SPI: 80°C, 100°C min^{-1} to 320°C, hold; Column: 30 m x 0.2 µm DB 5

(Chromtech), 80°C for 5 min, 5°C min^{-1} to 300°C, hold for 15 mins; Detector: FID at 320°C, range 10^{-12}, attenuation 256; carrier gas nitrogen at 41 m*l* sec^{-1}]. Before injection (0.5 μ*l*), the samples were filtered through a C_{18} Sepak column (Millipore Waters). Peak areas of components in the samples were compared to those of the standard solutions and quantified. Separated components were not identified as this was a qualitative analysis.

(v) Saturated Soil Paste Extractions

Approximately 200 g of each air-dried soil sample, of known water content, were placed into a 500 m*l* Consol™ jar. The mass of the container and its contents were recorded. Distilled water was added to each jar and the contents stirred with a wooden spatula until a condition of saturation was reached i.e. the soil paste glistened as it reflected the light, flowed slightly when the container was tipped, and slid freely and cleanly off a spatula. The mixtures were consolidated from time to time during the mixing process by tapping the container on the workbench. After mixing was completed, the containers were covered and allowed to stand overnight. The criteria for saturation were rechecked. If free water had collected on the surface of the paste, an additional known quantity of soil was added, mixed and the container and contents reweighed. The saturation water percentage was calculated from the mass of oven-dried soil and the sum of weights of water added and that originally present in the air-dried sample. The preparations were remixed, and allowed to stand for a further 4 hours. Each was then transferred to a Büchner filter funnel fitted with a low-ash, highly retentive filter paper (Whatman 42). A vacuum (> 73kPa or 557 mm Hg) was applied and the filtrate of each sample collected in a clean bottle. The electrical conductivity (EC) was measured with an electrical conductivity probe (Radiometer) to determine the total concentration of ionised constituents (dissolved salts), after standardization of the probe with a 0.01*N* KCl solution (1.41 mmhos cm^{-1} @ 25°C). All extracts were diluted before the addition of the suppressant solutions to an EC<150 dSm^{-1}.

(vi) Atomic Absorption Spectrophotometry (AA)

Calibration Standards. Commercially available standards were used in the following concentrations and were stored in polyethylene containers : Sodium stock (300 ppm, or 100 me l^{-1});

Calcium stock (2000 ppm, or 100 me l^{-1});

Magnesium stock (1000 ppm, or 82.26 me l^{-1}); and

Potassium stock (3900 ppm, or 100 me l^{-1}). The standard solutions were diluted to 5, 10, 50 and 100 ppm with *dd* water.

Suppressants. Extracts (2 m*l*) in which Na, Ca and Mg were measured were further diluted by the addition of 8 m*l* 2500 ppm K solution. Extracts (2 *ml*) in which K was measured were diluted with 8 m*l* 1250 ppm Cs solution.

(vii) High Pressure Liquid Chromatography (HPLC) Anion Analyses

Soil extracts from the pot trial were diluted by a factor of 250, with *dd* water. Each sample (2 m*l*) was cleaned up with a C-18 and an Accell Sep-Pak cartridge (assembled in series, Millipore) after passing through a 0.45 µm filter paper (Waters). The samples were placed in glass autosampler vials and analysed for chloride, nitrate, nitrite and sulphate ions with an IC Pak A column (Waters 590 Programmable HPLC; Wisp 712; 430 Conductivity Detector; 745 Data Module). Commercially prepared standards (Waters) were used for quantification of the ions and were injected after every three samples to ensure consistency.

(viii) Foliar and Soil Analyses

At the end of the 12-week period, the plants were photographed, cut off at the base and then dried (60°C for 48 hours) to constant mass. After their mass was recorded, leaf samples were sent to Cedara Soil Laboratory (Pietermaritzburg) for foliar analyses of N, Ca, Mg, K, Na, P, Zn, Cu, Mn, protein and fat. Soil samples were also analysed at the Cedara Soil Laboratory and recommendations were given for future soybean planting by the Fertiliser Advisory Service, Cedara.

(ix) Scanning Electron Microscopy (SEM)

Pieces of soybean root material were taken from all plants after examination for root nodules and root density. The tissue was prepared as described in **Section 5.2.6.** Photographic observations were recorded.

(x) Statistical Analyses

Statistical analyses were made on all of the raw data from the Pot Trial III (Appendix 1 and 2), using the Statistical Analysis Software (SAS®) System. All interactions were evaluated and tested using the General Linear Models procedure. The Bonferroni (Dunn) and Dunnett's T tests were used at the 95% confidence level to determine significant differences between treatments and variables. Thereafter, Manova was used to compare high-order (overall) interactions and test the hypotheses with the Wilks' Lambda, Pillai's Trace, Hotelling-Lawley's Trace and Roy's Greatest Root to confirm the significance of the interactions.

8.6.3 Results and Discussion

8.6.3.1 Pot Trial 1 - Liquid Feed

Trial 1 was set up as a small-scale version of Trial 2 to observe the reactions of the plants to the contaminant and to ensure that the watering and nutrient supplementation regimes were adequate. The nutrients (C:N:P = 10:1:1) were added as laboratory salt solutions in daily increments; firstly, to prevent waterlogging as the pots were non-draining and, secondly, to prevent over-fertilization. The plants survived for 3 weeks after emergence (the emergence time was approximately 1 week) but gradually showed symptoms that appeared to be caused by manganese toxicity (M.P.W. Farina, personal communication). This was apparently caused by the wet soil conditions and the acidic pH of the soil (pH 4.2 - 3.9). Because there was no visible improvement in the appearance of the soil during the growing period, a decision was made to alter the form of the fertilizer to be added, as the laboratory chemicals had to be dissolved, and the amount of water added for this purpose was causing the waterlogging (non-draining pots

were used to prevent any losses of nutrients or soluble oil components). However, any *less* water would have resulted in extremely saline conditions.

8.6.3.2 Pot Trial 2 - Single Fertilizer (Solid) Treatment

Trial 2 was similar to Trial 1, except that it had been established that the very acidic conditions were unfavourable for growth. At low pH values many essential nutrients become unavailable to plants and their associated microorganisms while trace elements such as manganese, zinc and aluminium become soluble. These elements are extremely toxic to living organisms in high concentrations. Waterlogging exacerbates this condition because hydrogen sulphide and other by-products are formed, as a result of anaerobic microbial activity, which further acidifies the soil. Furthermore, it was realised that despite the ease with which one could formulate any nutrient combination with laboratory salts, this approach was both unrealistic and uneconomical in a field situation. The use of commercial fertilizers in 'prills' (granules) was, therefore, investigated and the nutrients finally added as commercially prepared compounds (Grovida) at a C:N ratio of 10:1 and an N:P:K ratio of 3:2:1. Draining pots were used in this experiment to prevent waterlogging.

In this experiment the plants, instead of being seeded directly into the contaminated soil, were given a slight advantage at their most vulnerable stage and were grown initially in seedling trays in the greenhouse where they received a dilute nutrient solution (Nutrimix™) twice daily. The plugs were then transplanted into the pots directly. Again, within three weeks of transplanting, all of the plants in the trial died. The symptoms, which were observed within two days of transplanting (an extremely rapid plant response), did not resemble those of any documented deficiency. The veins of the leaves became chlorotic (**Plate 8.2A**), curled and the primary leaves gradually became necrotic from the tips (**Plate 8.2 B** and C) until they abscised. The plants wilted and eventually died (**Plate 8.2D**). To investigate why the plants died so suddenly, analyses of the soil cations were made,

after it was discovered that the EC of the leachate from the pots was extremely high. Not only would this be detrimental to the plants but also to the indigenous microflora whose activity was, probably, retarded rather than enhanced. As before, no visible improvement in the soil was observed. Instead, a white crusting appeared on the surface of most of the pots. To investigate the source of these cations, samples of soil were taken from various points on the experimental site and analysed by similar techniques. Total cation and EC results are presented in **Table 8.4** which, when analysed further, showed extremely high readings for chloride, potassium and sodium (data not shown). The added KCl appeared to be the source of the problem, since the treatments did not include supplementation with Na.

'Soluble salts' in soils refers to the inorganic constituents that are soluble in water and are, therefore, immediately available for plant uptake through the soil solution. The salt concentration in the soil fluctuates constantly due to processes of diffusion, osmosis, adsorption to particles and leaching, and directly influences both plant and microbial growth. It is, therefore, important in terms of biological development to be able to measure the concentrations of ionic species in solution in the soil. This analysis facilitates soil classification into media which are poor or lacking in nutrients, or which have certain ions in excess of the plant or microbiological requirement, in which case the concentrations may be toxic or limiting to growth as in saline soils.

Electrical conductivity (EC) is universally used as a measure of the total electrolyte concentration of water. Soils which have high salinity levels (i.e. high EC values of saturated paste solutions) affect plant and microbial growth through an increase in the osmotic potential of the soil solution. A plant growing in a saline soil environment will, therefore, tend to suffer abnormal moisture stress and, under extreme conditions, may die from desiccation.

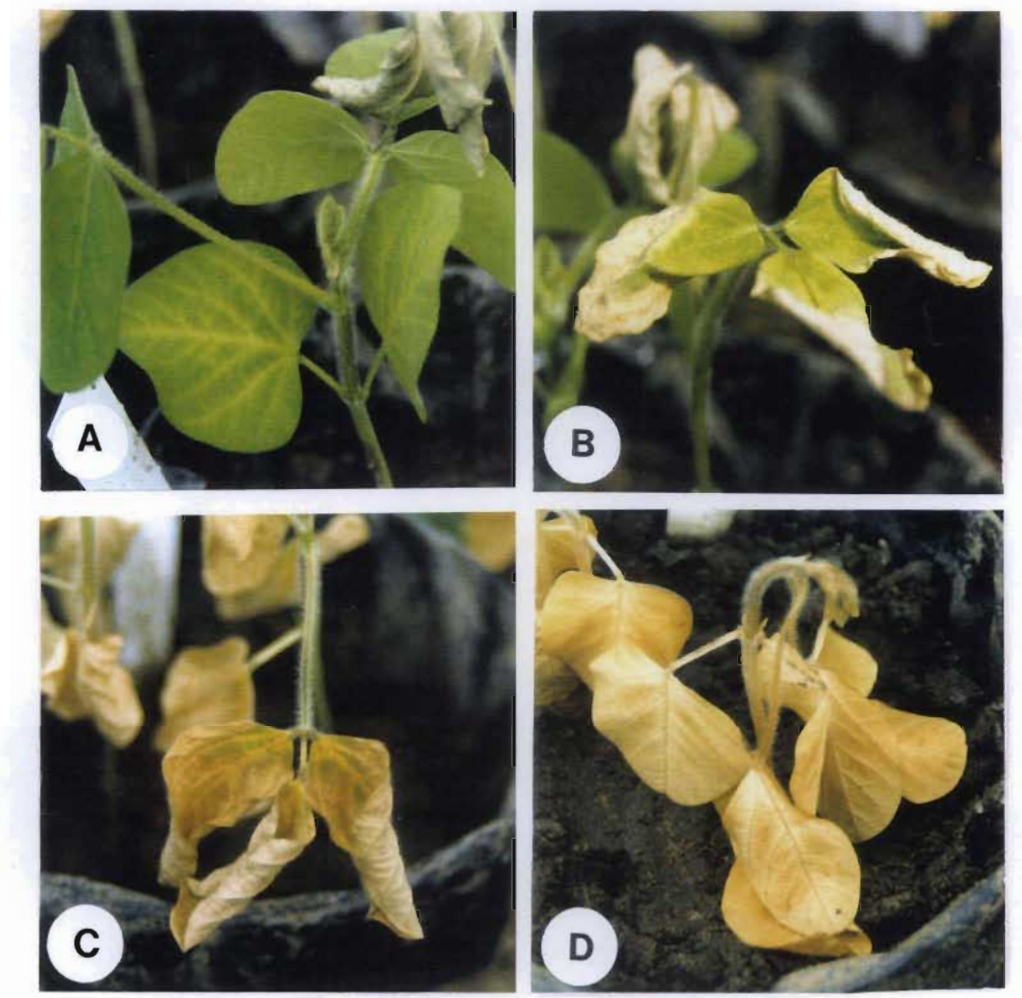


PLATE 8.2 (A)-(D) : Within two weeks of seedling transplantation into contaminated soil, during Pot Trial 2, symptoms began to be observed in which the whole plant wilted and veins in the leaves became chlorotic (A). The leaves gradually curled and became necrotic from the tips (B). The leaves finally became completely necrotic (C) and eventually died (D).

Although soybeans are moderately tolerant of soil salinity (Birch, Duxbury, Farina and Chapman, 1990), the concentration of soluble salts in all of the pots, except Pot 21* (**Table 8.4**), approached the salinity of seawater, which is damaging to all but a few plant and algal species. Comparison of the results of **Table 8.4** and **Table 8.5** shows, therefore, that the EC values were excessively high. It was established that the plants had died through desiccation due to a very high soluble salt content.

Table 8.4 : Soil extract results (saturated paste) for Pot Trial 2 (n=3).

TREATMENT	pH (extract)	EC (mS m^{-1})	% (w/w) Water Content	Total Salts (mg l^{-1})	Total Cations (me l^{-1})
MC NK	5.9	77.7	18.24	497.3	77 700
HC NK	6.29	63.3	15.66	405.1	63 200
MC PK	4.47	42.6	2.89	272.6	43 500
HC PK	4.04	59.6	15.99	381.4	59 600
MC NPK	4.83	86.9	16.38	556.2	86 900
HC NPK	4.91	98.1	20.34	627.8	98 100
MC NK PB	5.47	51.1	28.05	327.0	51 100
HC NK PB	5.17	87.7	28.17	561.3	8 800
MC PK PB	4.68	31.3	29.28	200.3	3 130
HC PK PB	4.28	33.5	29.31	214.4	3 350
MC NPK PB	5.05	41.0	30.19	262.4	4 100
HC NPK PB	4.77	68.3	23.68	437.1	6 830
MC NK	5.93	70.1	17.45	448.6	7 000
HC NK	5.77	102.7	21.28	652.8	10 200
MC PK	4.41	39.5	19.9	252.8	3 950
HC PK	4.4	51.6	19.32	330.2	5 150
MC NPK	5.02	72.5	20.16	464.0	7 250
HC NPK	5.23	94.7	20.84	606.1	9 500
MC NK PB	5.68	54.8	28.44	350.7	5 470
HC NK PB	5.34	72.6	27.28	464.6	7 260
MC PK PB*	4.41	25.4	30.99	163.2	2 550
HC PK PB	4.33	34.1	30.58	218.2	3 410
MC NPK PB	4.95	49.3	29.8	315.5	4 930
HC NPK PB	4.77	82.7	21.42	529.3	8 270
Control MC	3.81	5.33	17.67	34.1	533
Control HC	4.64	4.20	20.29	26.9	420

HC = Heavy contamination MC = Moderate contamination LC = Low contamination

N = Nitrogen P = Phosphorus K = Potassium

PB = Composted pine-bark *=Pot 21 (see text for detail)

Table 8.5 : Different EC levels and their salinity rating, and corresponding effect on plant growth (Richards, 1954).

EC (mSm^{-1})	Salinity Rating	Plant Response	Effect
0 - 20	Non-saline	Effects negligible	None
20 - 40	Slightly saline	Yield of very salt sensitive crops restricted	Slight
40 - 80	Moderately saline	Yield of salt sensitive crops restricted	Serious
80 - 160	Highly saline	Only salt-tolerant crops yield satisfactorily	Very Serious
> 160	Extremely saline	Only a very few salt-tolerant crops will survive	Critical

It was suspected that a contribution to the high concentrations of chloride and sodium ions resulted from the soil itself, which was sampled from the usual location at the experimental site. During this time, the tenants of the site were using the leaking brick reservoir for their wastewater and this could have promoted the salinity problem. Samples were thus taken from across the site to evaluate whether the inherent nutrients were the direct cause of plant death. Unfortunately, two days prior to taking the samples the area received very heavy rains and the results of the ion analyses indicated that the concentrations of salts were comparatively low (**Table 8.6**) perhaps due to leaching from the permeable sandy soil. Calculations, however, suggested that the supplements could not have been the sole source of salt.

Table 8.6 : Soil extract results (saturated paste) for field samples, showing the electrical conductivity (EC), total cations, pH and water contents for each sample area (n=3).

Sample	Sampling Source	pH (extract)	EC (mSm^{-1})	% (w/w) Water Content	Total Salts ($mg\ l^{-1}$)	Total Cations ($mmol_c\ l^{-1}$)
1F	HC, P4	6.94	15.40	19.03	9.90	141
2F	MC, P1	5.46	17.90	22.17	11.5	179
3F	HC, Small pool	8.00	10.80	17.45	6.90	108
4F	MC,P4	4.55	4.89	17.97	3.12	48.8
5F	LC,P1	3.66	9.14	17.71	5.80	90.6
6F	Leak at reservoir	7.00	4.12	16.08	2.64	41.3
7F	LC,P3	4.48	2.87	17.60	1.84	388
8F	HC, Large pool	8.15	14.20	23.59	9.10	142
9F	HC,P3	5.67	9 45	24.72	6.10	95.3
10F	MC,P3	5.05	43.20	18.35	2.77	43.3
11F	MC,P2	7.15	6.60	20.30	4.20	65.6
12F	Dam, at stream	7.19	7.52	17.50	4.80	75.0
13F	MC,Sampling pit	5.26	2.74	15.75	1.75	27.0
14F	6m S of reservoir	5.17	3.53	18.63	2.25	35.0
15F	LC,P2	4.23	5.09	17.20	3.26	50.9
16F	Dam, L of tree	5.84	4.37	24.75	2.79	43.0
17F	HC,P1	5.61	8.65	23.45	5.54	86.0
18F	HC,P2	6.84	11.42	19.70	7.30	114

HC = heavily contaminated MC = moderately contaminated

LC = low contamination F = Field sample P = Plot No.

8.6.3.3 Pot Trial 3 - Many Small Slow-Release Fertilizer Applications

(i) The Choice of a Suitable Nitrogen Source

In previous experiments (**Chapters 5 and 6**), mention was made of the often rapid decrease in pH that occurred as a result of the addition of nutrients. Phosphates do not generally effect a change in pH and so the effect was either caused by an increase in microbiological activity, which caused an increase in the concentration of soluble organic acids (the by-products of hydrocarbon degradation) and/or the acidification of the soil solution by the addition of ammonium. It is well known that the addition of ammonia or urea-based fertilisers causes acidification, while the addition of nitrate often has the opposite effect (M.P.W. Farina, personal communication). The choice of nitrogen source is of great importance in the reclamation of contaminated soil and the reactions of three different sources were tested by

a simple experimental procedure. Nitrate, ammonium and ammonium nitrate were added individually to separate contaminated soil:water slurries (1:1) as sole nitrogen sources. The effects were as predicted i.e. ammonium effected a rapid drop in pH after 24 hours of incubation, while nitrate caused a small increase in pH. The nitrate plus ammonium supplementation did not show any significant pH changes over the 72 hour period (**Figure 8.2**). Since the degradation of hydrocarbons does produce organic acids, it was desirable to choose a N source that would not exacerbate the pH fluctuations, particularly in a naturally acidic sandy soil which has little buffering capacity. This could be alleviated by the addition of dolomitic lime, which was also investigated. It was found that, if necessary, 10 g dolomitic lime kg^{-1} of soil could be added to increase the soil pH to approximately 6.5 (**Figure 8.3**).

In addition to the pH implications, it was necessary to also consider the form of nitrogen that was preferred by both the microorganisms and the plants. The ionic source of nitrogen that is preferred by microorganisms for protein synthesis is NH_4^+ rather than NO_3^- (Brock and Madigan, 1991). Nitrogen in the reduced form can be incorporated readily into amino acids, whereas the oxidised form must first undergo reduction. In soils, the NH_4^+ ion is held on the exchange complex and its movement into and through the soil water is thus restricted. In contrast, the NO_3^- ion can move to the plant root either by diffusion or by mass flow with water. Rapid nitrification of NH_4^+ can also produce NO_3^-, the ion source for the plant (Ladd, Butler and Amato, 1986).

Ease of movement into the root and ease of synthesis into amino acids and amides are not the only factors involved in uptake. Absorption of NH_4^+ depresses uptake of other cations, especially K^+, whereas NO_3^- depresses the uptake of Cl^- and SO_4^{2-} (C.A.du Plessis, personal communication). Thus, it seemed best to, simultaneously, supply the treatments with both sources of nitrogen and limestone (LAN) to satisfy the needs of the plant and the microorganisms, as well as to counteract any losses due to volatilisation or

leaching and pH fluctuations that may be detrimental to the degradative process.

The final consideration was the delivery of the nutrient to the organisms without the complications that were experienced in the first two pot trials.

(ii) *Controlled-Release Fertilizers*

Most commercial nitrogen fertilizers are readily soluble in water. Consequently, they are susceptible to denitrification, leaching, volatilisation or fixation in soil. A persistent problem with fertilizer use is how to control the release of soluble nutrients in such a way that an adequate concentration is maintained in the root zone to support plant growth while minimising potential nitrogen losses to the environment. Encapsulation of granular nitrogen fertilizers with membranes is a recent technique called 'controlled release technology'. This technology has been widely used in pharmaceutical science (Zhang *et al.*, 1993). It has great potential in agriculture as well as in the area of bioremediation.

The salinity problems that were experienced in Pot Trial 2, and the difficulties experienced in Pot Trial 1 in administering the 'appropriate' concentrations of N, P and K to the soil in response to the carbon load, led to the consideration of slow-release fertilizers. The benefits of having a soil environment in which nutrients would be released according to the demands of both the microorganisms and plants seemed to outweigh the possible economic disadvantage. Furthermore, on a site where a perched water table ensures that any soluble substances rapidly enter the groundwater, the prospect of being able to limit nutrient release so that it would be used before it was leached is also attractive.

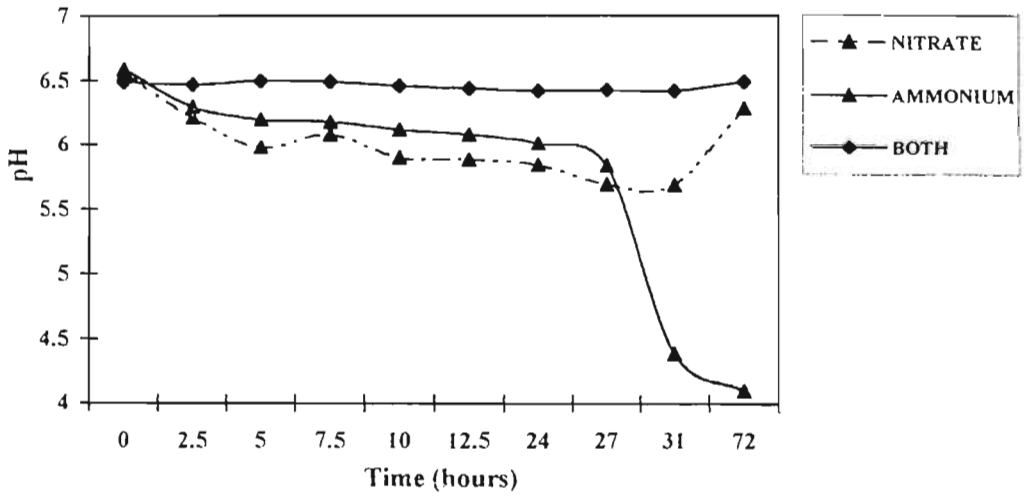


Figure 8.2: Changes in soil pH that occurred over 72 hours in a 1:1 contaminated soil:nutrient medium slurry after the addition of different nitrogen sources (nitrate, ammonium and ammonium nitrate) (n=3).

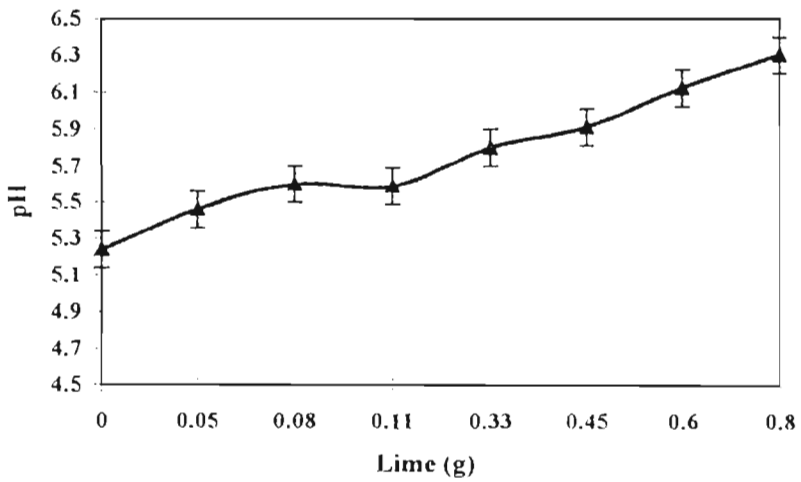


Figure 8.3: The change in soil pH as dolomitic lime was added to 100g of contaminated soil (n=3).

Coated nitrogen fertilizers with controlled release properties are prepared by physically coating with semi-permeable membranes or inert materials onto, otherwise, soluble fertilizer granules such as the Limestone-Ammonium-Nitrate (LAN) which was used in this study. The coating either dissolves or swells in water, releasing the fertilizer at a rate determined by the soil temperature, the percentage polymer surrounding the granules, and the water content of the soil (Bishop, 1993).

The membranes, apart from their encapsulating functions, have also been shown to improve the physical properties of soil (Terry and Nelson, 1986); increase shoot growth in radishes (Magalhaes *et al.*, 1987); aid in seed germination and emergence (Azzam, 1983); increase seedling survival (Gray, 1981); reduce the irrigation requirement for plants (Flannery and Busscher, 1982); and increase the nutrient recovery from applied fertilizers (Bishop, 1993).

The estimation of fertilizer in Trial 3 was very conservative, and soil and foliar analyses showed that many of the plants were deficient in nitrogen, although they had been inoculated with *Bradyrhizobium japonicum*. This exemplified one of the greatest difficulties facing bioremediation practitioners - how much nutrient is appropriate? Each situation is different with respect to carbon loading, soil type and other environmental variables which can affect nutrient availability. Ammonium concentrations exceeding 300 mg l^{-1} are toxic to microorganisms (Alexander, 1991), while groundwater concentrations of nitrates exceeding 10 mg l^{-1} are harmful to human life (World Health Organisation Standards). Maintaining the balance of nutrients and oxygen is the most critical step in *in situ* bioremediation, and the information gained by these experiments has shown that small amounts of fertilizer (preferably controlled-release) applied on a regular basis are favourable, compared to one large dose that corresponds to the nutrient required to mineralize the total amount of degradable organic material in the soil. On a large scale, financial constraints may detract from the actual benefits derived from these forms of nutrient supplementation

although it has been shown that considerably less fertilizer is required for the same vegetative yield than is used presently in agriculture (Bishop, 1993). Successful mineralization of oil using slow-release fertilizers, instead of soluble fertilizers, was reported by Atlas and Bartha (1976) and LaDousse *et al.* (1987).

(iii) Visual Symptoms of Toxicity or Deficiency

Soybean (*Glycine Max.* (L.) Merr.) plants were visually assessed for symptoms of mineral deficiencies and/or toxicities. The dried tissue and soil from each pot were analysed. The accurate diagnosis of disorders and/or mineral requirements depended on soil tests and analysis of plant tissues in addition to recognition of visual symptoms on the plant. Soil tests by themselves were considered inadequate as a basis for diagnosis of nutrient deficiencies or toxicities. The plants showed symptoms which could have been interpreted as nutrient deficiency symptoms or hydrocarbon toxicity symptoms. Some were only observed in the contaminated pot plants while other symptoms were observed in most of the plants.

Analysis of the soil before the trials (**Table 8.7**) showed an expected deficiency in N and P, although there appeared to be sufficient potassium in the soil to support a soybean crop. The percentage acid saturation was considered to be too high and lime was recommended.

Table 8.7 : Analytical results of soil taken from the experimental site before the start of the Pot Trials (n=2).

Sample	P ($mg.l^{-1}$)	K ($mg.l^{-1}$)	Ca ($mg.l^{-1}$)	Mg ($mg.l^{-1}$)	Zn ($mg.l^{-1}$)	Acidity Al + H $cmol_c.l^{-1}$	Total Cations $cmol_c.l^{-1}$	Acid Saturation %	pH
1	5	88	190	55	1.6	0.53	2.16	25	4.15
2	5	91	193	52	2.0	0.57	2.16	26	4.15

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After 8 weeks of growth, it was apparent that the plants growing in the moderately contaminated soil were the slowest to progress and 25% of the

plants had, for no discernible reason, wilted and died. Often, replicate plants in the same group reacted differently to the same treatments. It is possible that the soil which was sampled from the same area was not homogeneous with respect to oil concentration and the current site tenant's process waste. Strangely, the plants grown in the heavily contaminated soil responded better to the conditions than the latter and there may be a number of explanations for this. The soil may have been taken from an area that was heavily contaminated with oil and other by-products of the recycling processes that had compromised the site previously. These compounds contained sulphur, an element that is required in relatively large amounts for soybeans. Thus, the plants may have responded favourably. An alternative explanation may be that the heavily contaminated soil could have contained a higher concentration of naphthenic molecules that are known to mimic the action of plant growth hormones (Pal and Overcash, 1980), which can cause a stimulation in growth. These are different from the molecules which were suspected to have caused the changes in root morphology (**Section 8.4.3**). Seeds were inoculated with the *Bradyrhizobium japonicum* commercial inoculum (Stimuplant cc) but the plants grown in soil with no added nitrogen became pale green and spindly, although some small nodules did develop on these plants. It is probable that the bacteria responsible for nitrogen fixation were inhibited later in the experiment by the very low soil pH values.

Without exception, the plants grown in the uncontaminated soil grew faster and were more vigorous than the plants grown in the contaminated soil. They did not, however, initially grow as fast as expected and from the foliar analysis it was apparent that they may have been suffering from a copper and potassium deficiency. Later (8 weeks) in the trial, all of the plants were watered with a dilute micronutrient solution after pH adjustment of the soil to ~6.0 with 0.2mM $CaSO_4$ and a mixture of K_2HPO_4 and KH_2PO_4 was added. The plants showed a 'growth spurt' after this supplementation, although by the end of the trial (12 weeks) the control plants still did not reach the size that was expected for the growing period. The control plants

grown in pine-bark were more vigorous than those grown in soil only. Therefore, the effects recorded appeared to be due to a physical rather than a nutritional effect. Uncontaminated soil without pine-bark showed a tendency to compact upon drying, thereby preventing root expansion. This was alleviated by the addition of pine-bark.

This compaction would also have affected the microbial population, and could be one of the reasons why the microbial activity was so low.

The elemental analyses of the foliage (**Table 8.8**) showed that some of the deficiencies that occurred in the plants could have been caused by the stimulation of microbial activity and the subsequent competition for nutrients with the plant. This was concluded because the elements added were in sufficient amounts for successful soybean growth (**Table 8.9**). Competition, particularly in soils containing higher amounts of labile organic compounds, would deplete the soil of elements at a faster rate.

Table 8.8 : Foliar analysis of 10-week old soybean plants grown with different nutrient supplements (N, NP, P, or W) and in different soil [High (H), Medium (M) and Uncontaminated (L) soil, with or without Pinebark (PB)].

SAMPLE CODE	% (w/w) N	% (w/w) Ca	% (w/w) Mg	% (w/w) K	% (w/w) Na	% (w/w) P	% (w/w) Zn	% (w/w) Cu	% (w/w) Mn
H N	4.22	1.00	0.47	0.83	0.18	0.09	104*	4	87
H NP	2.84	1.47	0.47	1.05	0.14	0.15	75*	4	61
H P	nd	0.27	0.14	0.34	0.06	0.10	54	2	38
H W	nd	0.46	0.29	0.43	0.08	0.05	126*	2	43
M N	nd	0.48	0.27	0.54	0.11	0.24	205*	4	91
M NP	nd	0.59	0.28	0.47	0.17	0.22	158*	4	91
M P	nd	0.42	0.16	0.55	0.05	0.17	140*	2	56
M W	nd	0.12	0.09	0.65	0.03	0.06	0	2	29
L N	3.37	0.88	0.73	1.29	0.25	0.10	62	6	87
L NP	2.92	1.05	0.80	1.29	0.30	0.14	66	4	68
L P	2.18	0.97	0.74	1.70	0.24	0.13	50	4	70
L W	2.46	1.05	0.75	1.05	0.12	0.13	56	6	72
H N PB	nd	1.05	0.53	1.50	0.17	0.11	69	4	134
H NP PB	4.05	1.23	0.59	1.82	0.13	0.25	129*	4	167
H P PB	1.81	1.17	0.55	1.50	0.13	0.74	89*	4	85
H W PB	1.51	0.87	0.53	0.74	0.12	0.14	108*	4	110
M N PB	5.43	0.51	0.32	0.77	0.23	0.12	82*	2	82
M NP PB	4.01	0.71	0.40	1.02	0.29	0.25	72	2	99
M P PB	1.19	0.74	0.40	1.49	0.12	0.55	103*	4	65
M W PB	1.98	0.74	0.47	1.47	0.24	0.15	130*	4	86
L N PB	3.54	1.62	0.79	1.98	0.11	0.18	51	8	253*
L NP PB	3.59	1.51	0.74	2.04	0.21	0.19	58	9	226
L P PB	3.93	1.66	0.77	1.73	0.30	0.22	63	8	258*
L W PB	4.39	1.63	0.80	2.03	0.19	0.19	53	6	219

* = Toxic levels Shaded areas = Deficiencies nd = not determined

Table 8.9 : Soybean nutrient sufficiency ranges for upper fully developed trifoliate leaves sampled prior to pod set (adapted from Mengel *et al.*, 1987 and Sinclair, 1993), and excessive (toxicity) concentrations (Ohlrogge and Kamprath, 1968).

Element	Sufficiency Range	Excessive Concentrations (>)
N (g kg^{-1})	4.25 - 5.50	7.0
P (g kg^{-1})	0.26 - 0.50	0.8
K (g kg^{-1})	1.40 - 2.50	2.7
Ca (g kg^{-1})	0.36 - 2.00	3.0
Mg (g kg^{-1})	0.22 - 1.00	1.5
S (g kg^{-1})	0.20 - 0.60	-
Mn (mg kg^{-1})	21 - 100	250
Fe (mg kg^{-1})	51 - 350	500
B (mg kg^{-1})	21 - 55	80
Cu (mg kg^{-1})	10 - 30	50
Zn (mg kg^{-1})	21 - 50	75
Mo (mg kg^{-1})	1 - 5	10

- = not found

After examining the results of the foliar analyses and the soil analyses, and evaluating them in relation to the sufficiency values and visible symptoms, several trends were apparent :

- Where nitrogen was not added to the pots (heavy and moderate contamination), or where it was perhaps not added in sufficient amounts, as in the uncontaminated pots, a nitrogen deficiency was recorded (**Table 8.8**). This could have been due either to unsuccessful *Bradyrhizobium* proliferation, or the pH of the soil had decreased to a point where the environment became unacceptable to the nitrogen-fixing bacteria. Nodules were observed on the roots of all of the plants grown in contaminated soil which did not receive nitrogen, and on all of the control plants (uncontaminated soil). Therefore, it may be deduced that inoculation was initially successful and the pH of the soil was the probable cause of this deficiency.
- Most of the plants showed a phosphorus deficiency (**Table 8.8**). This may also have been a pH effect as under very acidic conditions aluminium toxicity can prevent phosphorus uptake. Also, the P was in a

form which was relatively unavailable to plants at low pH values. It was, however, more likely to have been an Al toxicity effect, as the soil analyses showed that the P concentrations were sufficient for growth (**Tables 8.10-8.13**). The only exception was where P was deliberately excluded.

- There was a notable deficiency in Cu and K in most plants grown in contaminated soil and this corresponded to Zn toxicity levels (**Table 8.8**). This effect was also reported by ElNawaway *et al.* (1994) who found that in barley straw and rye grass, the Zn concentrations increased in plants cultivated in soil treated with oily sludge, whereas concentrations of other elements such as Cu and K were reduced. It appeared that the conditions in the soil promoted Zn uptake, as the amounts of Zn found in the soils were not excessive (**Tables 8.9-8.13**). There was, thus, a definite correlation between contamination and Zn toxicity as the plants grown in uncontaminated soil showed normal Zn concentrations in their tissue (**Table 8.8**).
- The percentage acid saturation of the soil in all cases except the uncontaminated pots was considerably reduced. This was probably due to the oil occupying the exchange sites on the soil, therefore only a very low percentage of these sites were occupied by hydrogen or Al ions. Strangely, the pH values of these soils were low which seemed to be a contradictory result. The general conclusion that can be made is that ions cannot be held by the soil on exchange sites due to the oil and it is thus critical that the nutrients applied to the soil are of the slow-release type.
- A possible explanation for the more severe effect on the growth of the plants that were grown in the moderately contaminated soil, when compared to the other soils, was that, generally, the soil pH was lower in the former. This was a result of the heterogeneity of the soil and was presumably related to the history of storage and disposal of waste products on the site.

Table 8.10 : Soil test results (extractable ions) from pots containing heavily (H), moderately (M) and uncontaminated (L) soil which received different nutrient treatments (N, P, NP or Water) over a period of 10 weeks (Pot Trial 3).

Sample Code	P ($mg l^{-1}$)	K ($mg l^{-1}$)	Ca ($mg l^{-1}$)	Mg ($mg l^{-1}$)	Zn ($mg l^{-1}$)	Acidity (Al + H) $cmol_c l^{-1}$	Total Cations $cmol_c l^{-1}$	Acid Saturation %	pH (KCl)
H N	3	38	116	20	17.3	0.01	0.86	2	4.87
H NP	27	33	109	14	12.9	0.03	0.77	4	4.63
H P	30	23	308	18	9.1	0.50	2.25	22*	3.62
H W	6	28	29	3	10.9	0.09	0.33	27*	3.66
M N	5	30	23	4	8.8	0.05	0.28	19	3.91
M NP	22	27	22	2	8.8	0.02	0.22	8	3.91
M P	22	23	53	4	7.3	0.05	0.41	13	3.87
M W	5	31	25	3	7.2	0.2	0.43	47*	3.90
L N	3	63	367	233	1.9	0.03	3.95	1	4.51
L NP	15	80	384	182	2.4	0.03	3.65	1	4.63
L P	9	54	460	234	1.2	0.01	4.38	0	4.93
L W	3	73	327	245	3.1	0.03	3.86	1	4.94

Bulk density of samples = 1.31 g ml^{-1}

Table 8.11 : Soil test results (extractable ions) from pots containing heavily (H), moderately (M) and uncontaminated (L) soil and composted pine-bark (PB) (20% v/v) which received different nutrient treatments (N, P, NP or Water) over a period of 10 weeks (Pot Trial 3).

Sample Code	P ($mg l^{-1}$)	K ($mg l^{-1}$)	Ca ($mg l^{-1}$)	Mg ($mg l^{-1}$)	Zn ($mg l^{-1}$)	Acidity (Al + H) $cmol_c l^{-1}$	Total Cations $cmol_c l^{-1}$	Acid Saturation %	pH (KCl)
HN PB	3	57	88	21	10.6	0.03	0.79	4	4.71
HNP PB	47	76	117	20	8.4	0.03	0.97	3	4.56
HP PB	47	56	90	13	4.4	0.02	0.72	3	4.09
HW PB	3	58	101	24	5.0	0.02	0.87	3	4.29
MN PB	3	67	104	25	8.6	0.03	0.92	3	4.57
MNP PB	16	49	151	28	5.6	0.02	1.12	2	4.80
MP PB	22	79	123	24	5.9	0.02	1.03	2	4.98
MW PB	3	57	103	26	8.3	0.02	0.89	2	4.36
LN PB	12	160	684	252	1.8	0.16	6.06	3	4.01
LNP PB	18	179	648	245	1.9	0.13	5.84	2	4.04
LP PB	12	123	532	266	1.4	0.05	5.20	1	4.34
LW PB	5	121	571	264	1.9	0.05	5.38	1	4.33

Bulk density of samples = 1.10 g ml^{-1}

Table 8.12 : Soil test results (extractable ions) from pots containing heavily (H), moderately (M) and uncontaminated (L) soil which were planted with soybeans (PL) (SCS1) and which received different nutrient treatments (N, P, NP or Water) over a period of 10 weeks (Pot Trial 3).

Sample Code	P ($mg.l^{-1}$)	K ($mg.l^{-1}$)	Ca ($mg.l^{-1}$)	Mg ($mg.l^{-1}$)	Zn ($mg.l^{-1}$)	Acidity (Al + H) $cmol_c.l^{-1}$	Total Cations $cmol_c.l^{-1}$	Acid Saturation %	pH (KCl)
HN PL	5	73	101	19	27.9	0.02	0.86	2	5.33
HNP PL	87	79	620	79	34.6	0.05	3.99	1	5.38
HP PL	54	60	153	21	18.1	0.01	1.10	1	4.20
HW PL	6	54	87	17	12.7	0.09	0.79	11	4.20
MN PL	20	68	13	4	19.2	0.05	0.32	16	4.04
MNP PL	38	69	31	6	16.3	0.04	0.41	8	4.03
MP PL	52	66	53	7	16.3	0.05	0.53	8	3.99
MW PL	16	52	27	7	20.2	0.09	0.41	21	3.90
LN PL	5	63	203	211	1.7	0.09	3.01	3	4.44
LNP PL	11	49	229	170	3.8	0.04	2.71	2	4.70
LP PL	11	46	218	203	3.6	0.02	2.90	1	4.81
LW PL	3	42	245	231	1.9	0.01	3.25	0	4.66

Shaded areas = values too low for successful yields * values too high for successful yields Bulk density of samples = 1.36 g ml^{-1}

Table 8.13 : Soil test results (extractable ions) from pots containing heavily (H), moderately (M) and un-contaminated (L) soil and composted pine-bark (PB) (20% v/v) which were planted with soybeans (PL) (SCS1) and which received different nutrient treatments (N, P, NP or Water) over a period of 10 weeks (Pot Trial 3).

Sample Code	P ($mg.l^{-1}$)	K ($mg.l^{-1}$)	Ca ($mg.l^{-1}$)	Mg ($mg.l^{-1}$)	Zn ($mg.l^{-1}$)	Acidity (Al + H) $cmol_c.l^{-1}$	Total Cations $cmol_c.l^{-1}$	Acid Saturation %	pH (KCl)
HN PBPL	7	88	136	34	12.0	0.01	1.19	1	4.38
HNP PBPL	65	51	237	43	13.4	0.07	1.74	4	4.30
HP PBPL	59	82	181	32	7.5	0.03	1.41	2	4.72
HW PBPL	6	89	119	26	8.1	0.03	1.07	3	4.28
MN PBPL	7	86	85	20	6.1	0.03	6.84	3	4.01
MNP PBPL	59	104	462	74	9.0	0.13	3.32	4	4.13
MP PBPL	71	96	112	15	12.3	0.02	0.95	2	4.13
MW PBPL	9	92	90	17	8.6	0.02	0.85	2	4.21
LN PBPL	6	11	488	173	1.9	0.05	4.19	1	4.19
LNP PBPL	7	83	465	175	1.3	0.10	4.08	3	4.26
LP PBPL	10	105	518	175	1.7	0.06	4.36	1	4.24
LW PBPL	5	114	515	237	1.9	0.10	4.92	2	4.27

Bulk density of samples = 1.13 g ml^{-1}yields

(iv) Uptake of Elements by Soybean, and Root Health

Soybeans require relatively large amounts of phosphorus and potassium and, generally, respond well to added fertiliser. However, depending on the conditions, deficiency or toxicity symptoms may emerge. There are few published reports on the toxicity effects of oil or their components. The ones which have been made relate to stunting and reverse geotropism (Dibble and Bartha, 1979; Wang and Bartha, 1990).

The oil naturally caused the soil to become extremely hydrophobic. A direct result of this was dehydration as the water simply ran off, or was left as droplets on top of the dry soil. Strangely, once the soil was wetted to >80% of field capacity, the evaporation of water appeared to be considerably reduced. The water appeared 'bound' to the contaminated soil which had a tendency to stay very moist. The displacement of oxygen from the soil pores by the oil and water could have facilitated the accumulation of hydrogen sulphide, ethylene and soluble Mn and Fe which have the potential to decrease the numbers of organisms which are beneficial to the plant roots.

The drastic effect of the oil on plant growth was, therefore, probably not entirely due to its chemical properties, but also to the physical (hydrophobic) action of oil which inhibited nutrient solubility and water uptake by the plants. The sensitivity of soybeans to water stress varies at different growth stages and the plant leaf movements can be used to estimate the water status of the crop (P. Greenfield, personal communication). The leaves move as a result of a decrease in their water content and indicate the onset of water stress. In normal unstressed conditions, the soybean leaf blade is usually held at 40° below the horizontal. With the onset of stress the leaflets tend to invert, with the leaf blade moving to a vertical position before wilting completely under conditions of extreme water stress. These plant movements were very easy to recognise in the plants which were grown in contaminated soil. They were particularly evident in the early morning, even when the soil was very

moist. This was a good indication that the oil had affected the soil:plant:water relations.

Schwendinger (1968) noted that the symptoms of oil toxicity are typical of extreme nutrient deficiency of plants, and that since nutrient deficiency symptoms are indirectly proportional to water uptake, plant damage was most probably due to a derangement of the plant:water relationships of the roots within the soil.

The first visible symptom observed in the soybean plants was the wilting of leaves and the downward bending of leaf petioles. Older leaves became pale and later developed a mottled appearance (**Plate 8.3 A-D**). With time, the oldest leaves became yellow and died. As the air temperatures rose to >30°C (despite the cooling effect of the wet wall in the glasshouse), the symptoms appeared to become more severe. When examined, the root tips of plants grown in uncontaminated soil were white with a profusion of root hairs. In contrast, the roots from the cores of contaminated soil pots appeared brown and stunted. Electron microscopy revealed few or no root hairs.

Oxygen limitation seemed the most plausible explanation for the observations because the number of hydrocarbon-degrading microorganisms would have increased in response to the nutrient supplementation and would have very rapidly depleted the soil of oxygen. Under these conditions, root growth would be slowed, the uptake of nutrients (especially potassium and phosphorus) would be almost halted and the ability of water to move into the roots reduced. The foliar analyses confirmed that most of the plants grown in contaminated soil were deficient in both phosphorus and potassium. Symptoms resembling those described for potassium deficiency were observed in the plants which were grown in the contaminated soil, when the older leaves exhibited yellowing around their margins which later coalesced to form a continuous yellow border (**Plate 8.3 A** and **D**).

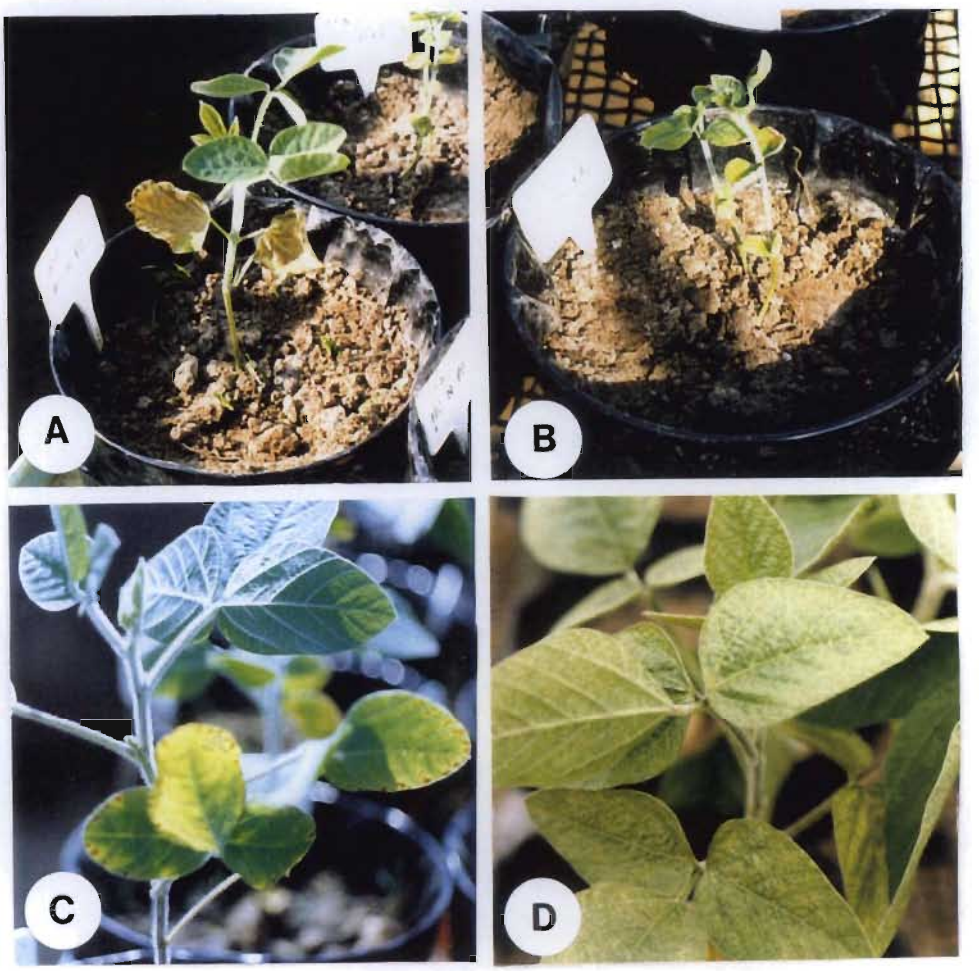


PLATE 8.3 (A)-(D) :

During Pot Trial 3, the soybean leaves exhibited symptoms which appeared to resemble nutrient deficiencies. The first symptom appeared in the older leaves which became pale and later developed a mottled appearance (D). With time, the leaves became yellow and died (A). Most leaves of plants growing in the contaminated soil exhibited yellowing around their margins which later coalesced to form a continuous yellow border which progresssed to patches of necrosis (B) and (C).

This yellowing progressed to necrosis. Although the foliar analysis of the plants grown in pine-bark and soil showed no potassium deficiency, some did show symptoms similar to those described. It is possible that toxicity of oil components may have contributed or intensified the response.

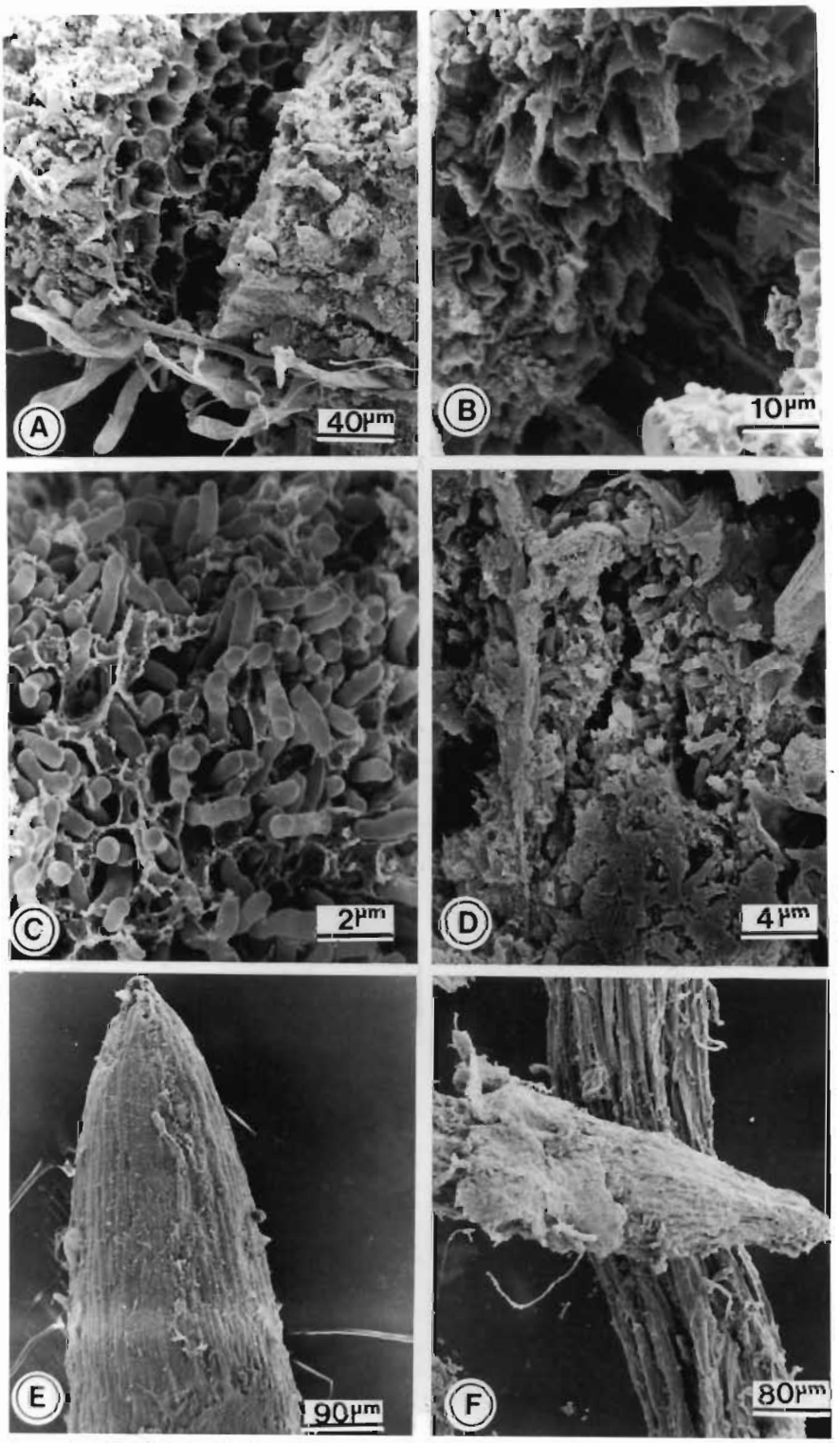
Other symptoms observed in the soybean plants were Mn, Al and Zn toxicities, all of which are known to occur in acidic soils. The toxicities of Al and Mn, in particular, are experienced first by the root caps of the plant. Since the root cap and the root hair zone are the most critical parts of the plant, damage to these areas will ultimately cause the death of the plant due to the inhibition of root growth and a change in the uptake of soil elements (R. Bennett, personal communication).

Scanning electron microscopy revealed, in fact, that there were considerable differences between the root tips of plants that had been grown in contaminated soil and those which had been grown in uncontaminated soil (**Plates 8.4** and **8.5**). The root cap of the root from a plant grown in uncontaminated soil showed cells lying side-by-side in a neat, orderly fashion which is characteristic of a healthy root (**Plate 8.4 E**). An abundance of root hairs, and possibly mycorrhizal fungi, was also evident (**Plate 8.6 A,** B and C). In the root taken from the contaminated soil, the entire root cap was in the process of sloughing off and there was a noticeable absence of root hairs (**Plate 8.4 F**). Furthermore, the root epidermis was absent and the internal structure of the root also appeared to be withered compared to the uncontaminated control (**Plate 8.4 A** and B).

PLATE 8.4 (A)-(F) :

Scanning electron micrographs showing the differences in appearance of the xylem and phloem tissues in a soybean root taken from (A) uncontaminated soil and (B) contaminated soil. Similarly, the inside of a nodule, taken from a root growing in (C) uncontaminated soil and (D) contaminated soil. The tissues in both taken from roots grown in contaminated soil appeared to have been disrupted and were less distinct than the tissues of the control plants.

The root cap of the root from a plant grown in uncontaminated soil (E) showed cells which were characteristic of a healthy root. In contrast, the root taken from contaminated soil (F) showed the entire root cap to be in the process of sloughing off and the absence of an epidermal layer.


A
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B
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C
2µm
D
4µm
E
90µm
F
80µm

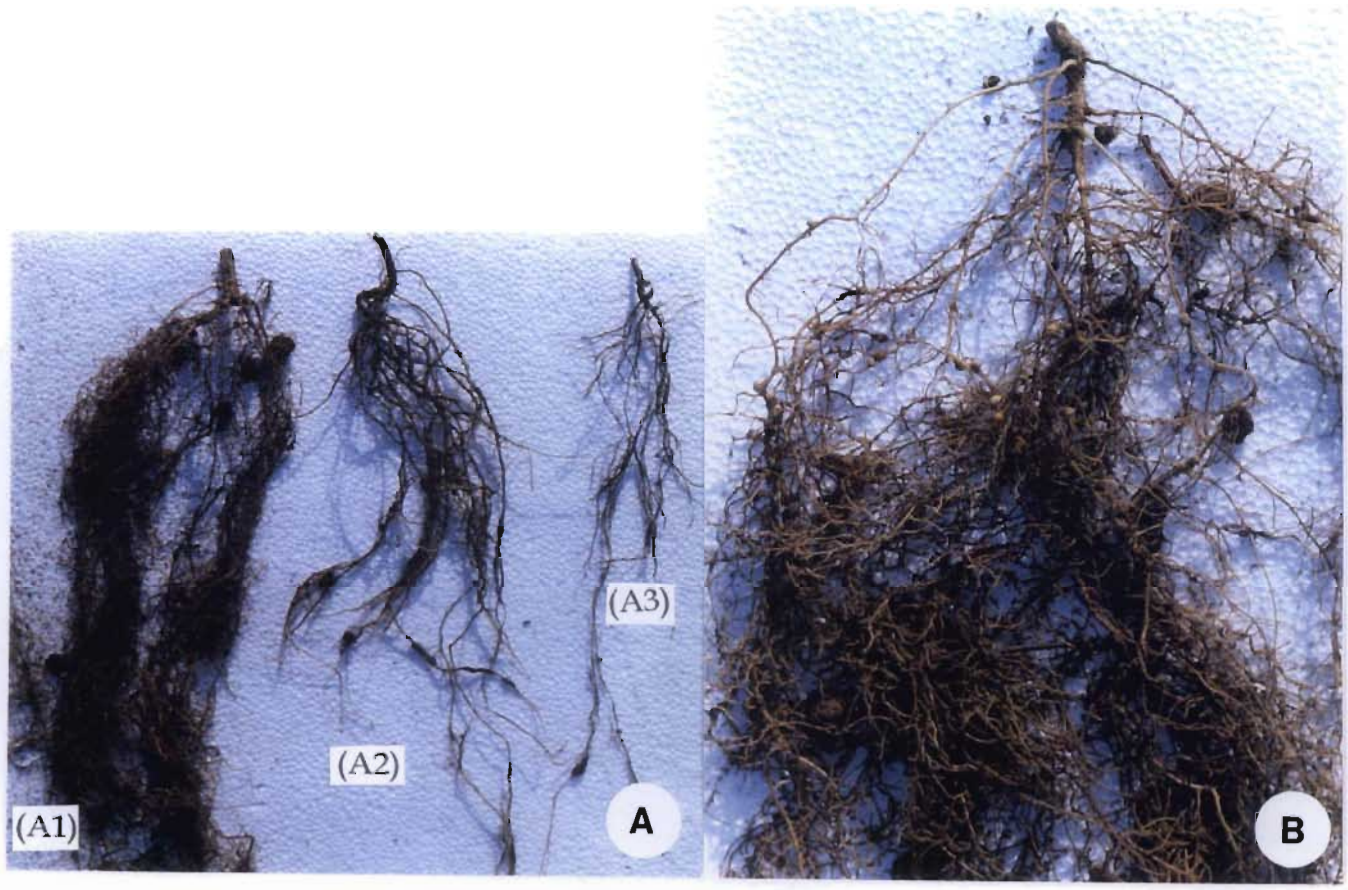
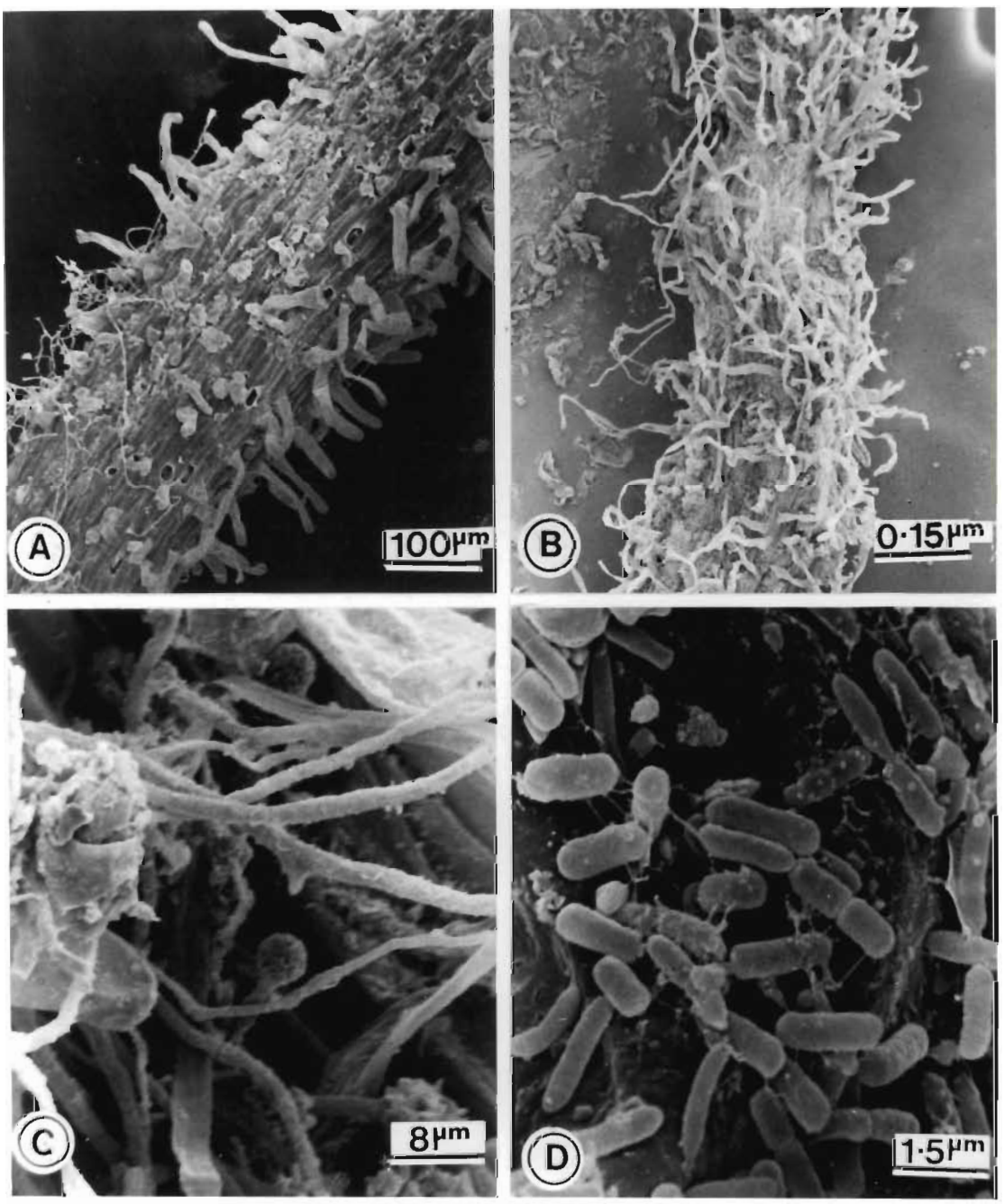


PLATE 8.5 : (A) : A photograph of the root structure of soybean plants grown in : (A1) uncontaminated soil + P; (A2) heavily contaminated soil + P+PB; and (A3) moderately contaminated soil + P+PB. (A1) had an extensive root system, while the roots of (A2) and (A3) were noticeably sparse and poorly established with few visible nodules.

(B) : Upon closer examination of (A1), one can clearly observe the root nodules formed by *Bradyrhizobium japonicum*.

Bacteria were observed inside black root tissue (**Plate 8.4** D) taken from contaminated soil. It is possible that they were hydrocarbon degraders and probably entered the root via the same route as the oil, particularly if the natural root barrier was missing. More evidence for toxicity was observed in the root nodules, where the bacteroides in the contaminated root were underdeveloped and tissue appeared dehydrated in comparison to the control (**Plate 8.6** C and D).

Corresponding root symptoms in response to Al toxicity have been recorded in wheat roots (de Lima and Copeland, 1994). In roots of untreated seedlings, the surfaces of the root apexes had an ordered appearance, with the epidermal cells clearly visible. The loosely-held peripheral cells which protect the root caps were also evident. Most of the cells on the surfaces of the root tips were clearly distinguished from one another. In the roots of treated seedlings, there was a decrease in the organisation of the peripheral cells of the root caps. Also, the epidermal cells of the meristems and the zones of elongation appeared to have disintegrated and individual cells could no longer be distinguished from one another. Some of the peripheral cells of the root caps also appeared to have been lost. With extended exposure to the toxic molecules, the epidermal and cortical cells lost all signs of organization, and most of the peripheral cells had either disintegrated or been sloughed off.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B
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C
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D
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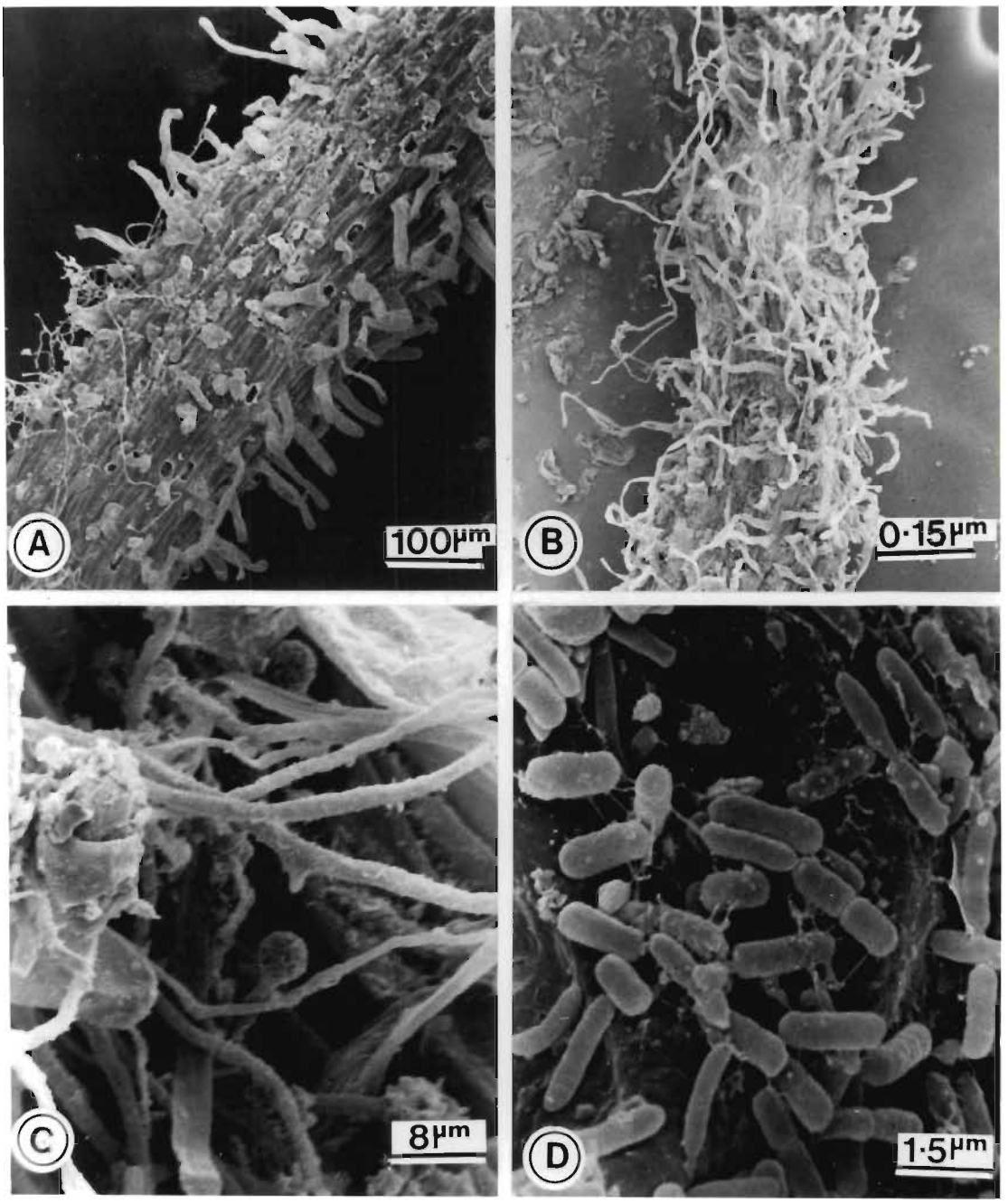
A
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D
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Wagatsuma *et al.* (1987) showed, in SEM studies with maize and pea, that the epidermis and cortex of the root apex were destroyed after exposure to 3 mg Al l^{-1} for 48 hours. These trends were so similar to the observations made in this study that one of two conclusions can be made : either the plants exposed to oil contamination were, as a result of low pH, also exposed to Al toxicity; or the reaction of the plant root was a phytotoxic response to the oil, which was a similar indication of the extent of toxicity as that recorded after root exposure to Al. Other toxic compounds would presumably cause the same response. Both explanations in this case could be valid considering the pH of the soil and the possible solvent effect that some of the hydrocarbons could have had on contact with the root.

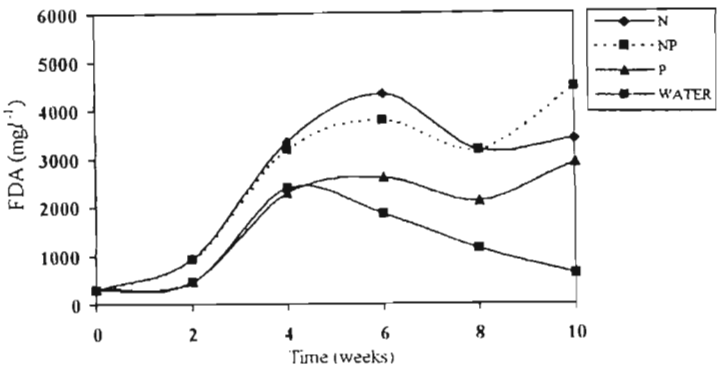
In either case, the result is the same - the outermost cell layers of the root would be the first to contact the hydrocarbons and, if the response recorded here was representative, then it would result in the death of the root tip. This would encourage lateral root development, with the tips undergoing the same trauma. **Plate 8.5** shows the difference in lateral root formation in contaminated and uncontaminated soil, which appears to support this theory. Nutrient uptake would be significantly affected, and deficiency or toxicity symptoms would be quickly seen in the foliage of the plant, which was observed in this experiment. In addition, a lack of root hairs would inhibit the formation of nodules as recognition between the nitrogen-fixing bacteria and the root hair could not occur, leading to a nitrogen deficiency. This was also observed in all plants grown in contaminated soil. In contrast, roots grown in uncontaminated soil showed a healthy response to pine-bark, nutrients and inoculation as well as a possible mycorrhizal relationship (**Plate 8.6 B** and C), and the development of many healthy root nodules.

It follows then that toxicity may be observed in the root and could be used as an early indicator for determining the suitability of vegetation for remediation purposes.

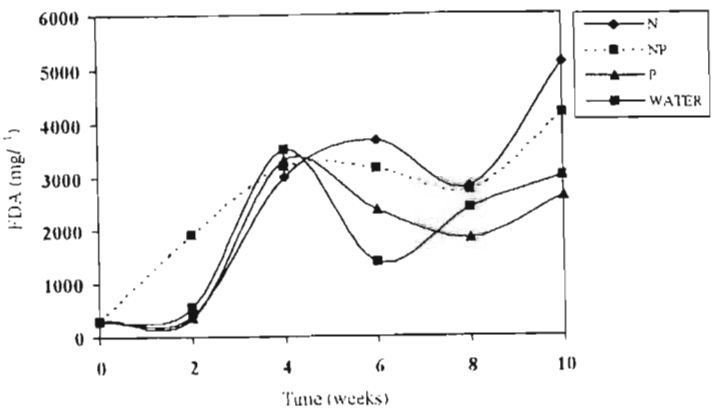
(v) Microbial Activity in the Soil

In laboratory-scale experiments, it is often possible to differentiate between biodegradation and evaporation or other physico-chemical losses by the use of sterile controls (Pramer and Bartha, 1972). Since such close scrutiny is difficult in a greenhouse or field experiment without expensive equipment, it was of special interest to measure the activity of the soil microbial community in response to the treatments. Constant or depressed microbial activity would signify little or no microbial involvement while strong positive responses would indicate a large microbial contribution.

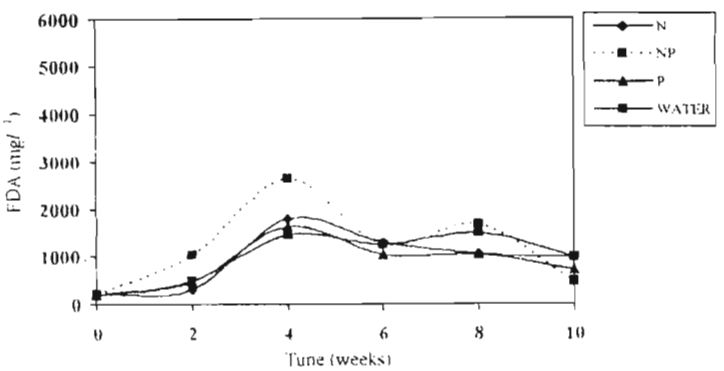
Figures 8.4 A and **B** showed strong positive changes in FDA hydrolysis activity in response to the various treatments. The largest activity responses were evident in the soils which received complete bioremediation treatment i.e. the pots which received nitrogen and phosphorus. The smallest changes in activity were recorded with the contaminated but untreated soil (water only). Although microbial activity in the uncontaminated control soil (**Figure 8.4 C**) showed only very slight fluctuations, it increased at 4 weeks in synchrony with the other treatments. The overall response in moderately contaminated soil was marginally lower than that measured in heavily contaminated soil and they were not found to be significantly different from one another ($P>0.05\%$). This minor difference was possibly caused by the difference in labile carbon content and, hence, catabolic biomass. The soils which received N or N and P were similar in their activity responses in all pots, while the activities recorded in soils which received P or water only were noticeably lower.



(A)



(B)



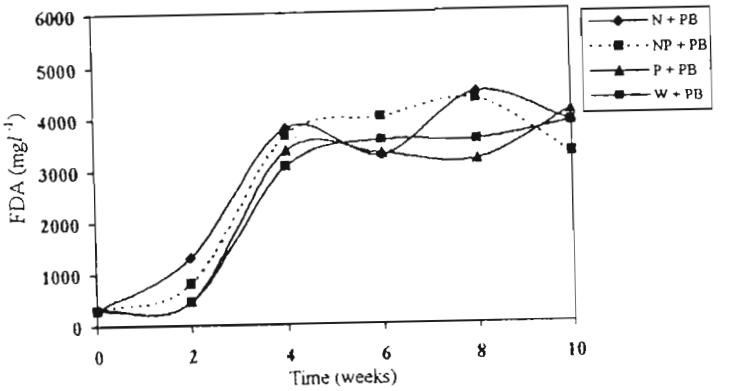
(C)

Figure 8.4: (A) - (C) : Changes in FDA activity (an estimate of total microbial biomass) of (A) heavily contaminated, (B) moderately contaminated and (C) uncontaminated soil extracts over 10 weeks, after supplementation with different nutrient treatments (N=Nitrogen, NP=Nitrogen + Phosphorus, P=Phosphorus, W=Water only) and in the presence of a soybean plant (n=3).

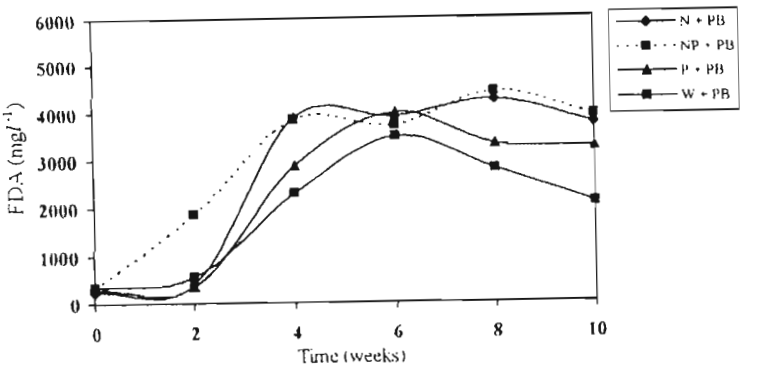
The addition of composted pine-bark to the soils (**Figures 8.4 D - F**), however, did not increase the microbial response significantly compared to the equivalent controls ($P>0.05\%$). However, these ameliorated soils did sustain microbial activities at maximum levels for all the treatments, including those which did not receive nutrients, for the 6 weeks following the first activity peak (4 weeks).

The improved porosity of the soil enabled the hydrocarbon-degrading biomass to increase with time, without oxygen becoming limiting. The addition of a substantial amount of lignin (and other elements) in the pine-bark was probably contributory to promoting the microbial activity which was demonstrated by the positive response in the otherwise untreated soil. The trends observed for heavily and moderately contaminated soil (**Figures 8.4 D and E**) were very similar, and corresponded closely with the responses measured in the soils without pine-bark (**Figures 8.4 A-C**).

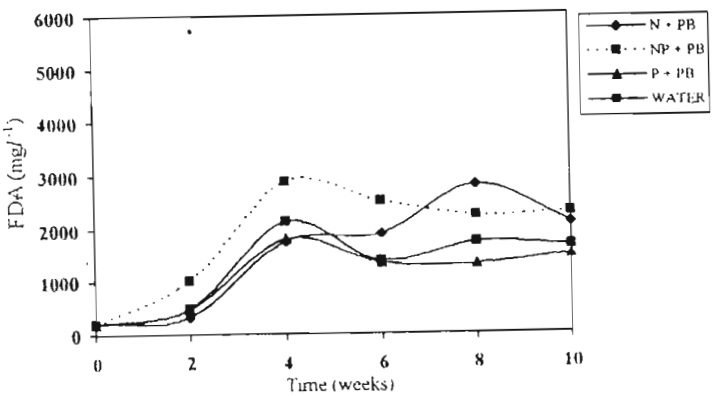
With the introduction of a plant to each system, the trend in FDA hydrolysis activity altered slightly (**Figures 8.4 G - I**). After increasing dramatically at 4 weeks (in agreement with the other treatments), the activities in the heavily contaminated soil then fell sharply to comparatively low levels for 3 weeks, before gradually recovering at 10 weeks. This was the likely result of a competitive nutrient demand between the plants and microorganisms. An adaptation period was possibly required by the microorganisms which was perceived as an activity "lag" but which may have provided the means for the observed recovery. In the moderately contaminated soil, the trends observed after the addition of pine-bark were identical to those trends observed after the introduction of soybean plants, except that in this case the soil which received water only did not respond as vigorously as the nitrogen treatments. This implied that the previously added composted pine-bark may have supplied the catabolic microorganisms with low concentrations of N and other nutrients, such as potassium, while the plant *per se* did not have the same enhancing effect.



(D)



(E)

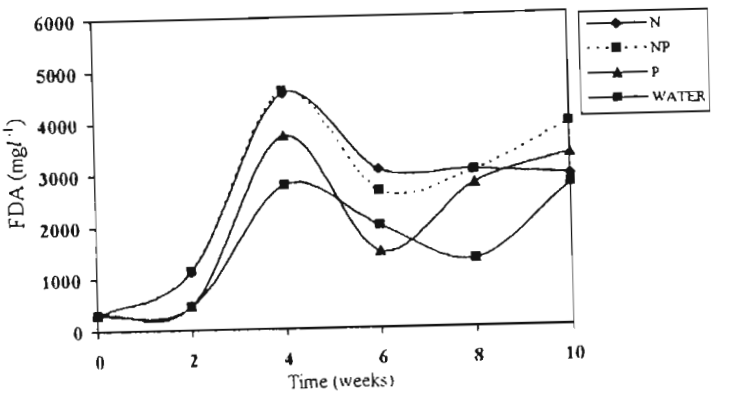


(F)

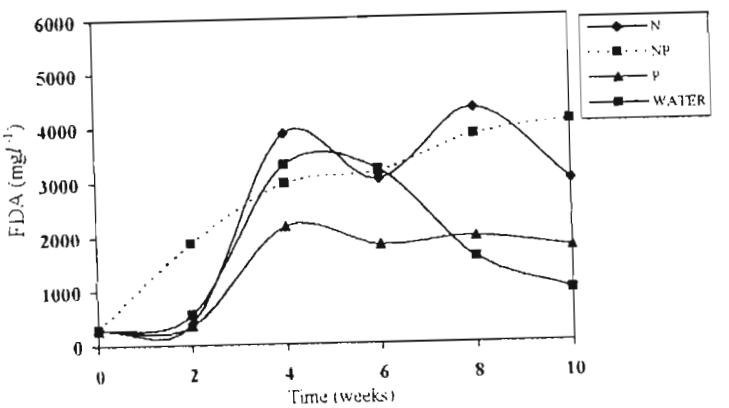
Figure 8.4 (D) - (F) : Changes in FDA activity (an estimate of total microbial biomass) of (D) heavily contaminated, (E) moderately contaminated and (F) uncontaminated soil extracts over 10 weeks, after supplementation with 20% (v/v) composted pine-bark and different nutrient treatments (N=Nitrogen, NP=Nitrogen + Phosphorus, P=Phosphorus, W=Water only) (n=3).

The overall activity in the uncontaminated soil was lower than that observed in **Figures 8.4** C and **8.4** F, probably also due to the competition for nutrients.

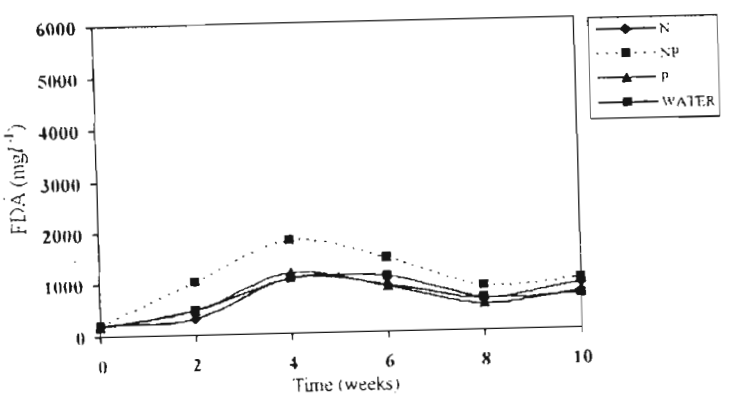
The soils of all three contamination levels, with composted pine-bark and soybean plants (**Figures 8.4 J - L**), were observed to have activity trends that were analogous with the soils which were mixed with composted pine-bark only (**Figures 8.4 D - F**). Surprisingly, the presence of the plants did not increase the microbial activity in the contaminated soils by any order of magnitude, even though the soil samples were taken from the rhizosphere.



(G)

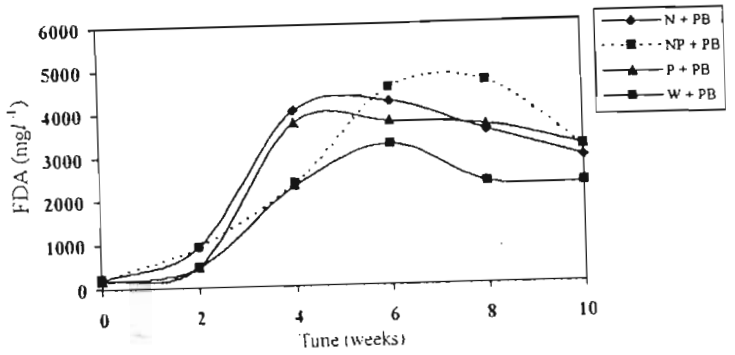


(H)

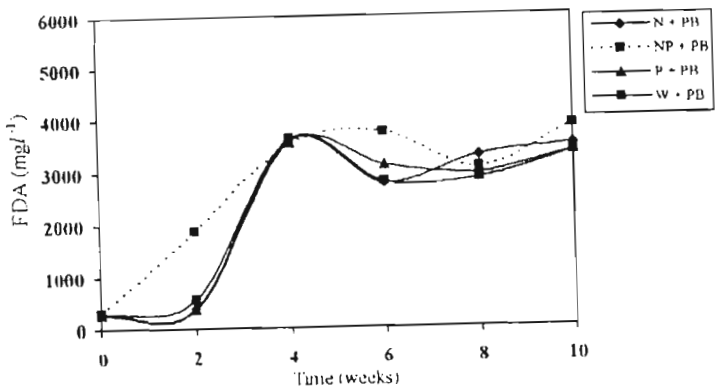


(I)

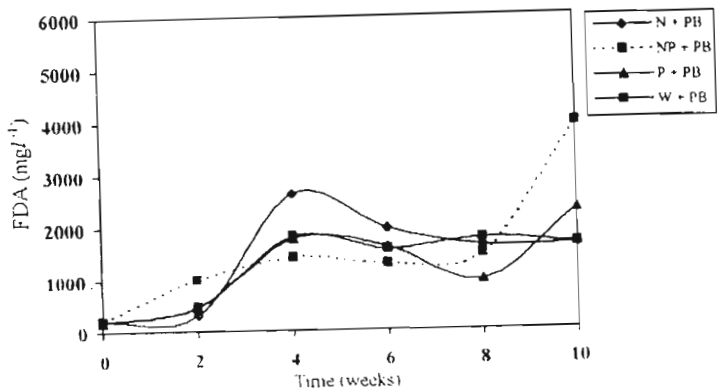
Figure 8.4 (G) - (I) : Changes in FDA activity (an estimate of total microbial biomass) of (G) heavily contaminated, (H) moderately contaminated and (I) uncontaminated soil extracts over 10 weeks, after supplementation with different nutrient treatments (N=Nitrogen, NP=Nitrogen + Phosphorus, P=Phosphorus, W=Water only) and in the presence of a soybean plant (n=3).



(J)



(K)



(L)

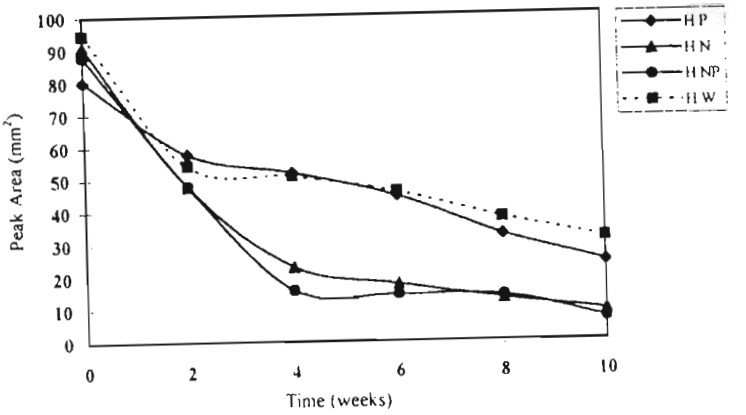
Figure 8.4: (J) - (L) : Changes in FDA activity (an estimate of total microbial biomass) of (J) heavily contaminated, (K) moderately contaminated and (L) uncontaminated soil extracts over 10 weeks, after supplementation with 20% (v/v) composted pine-bark, different nutrient treatments (N=Nitrogen, NP=Nitrogen + Phosphorus, P=Phosphorus, W=Water only) and in the presence of a soybean plant (n=3).

(vi) Total Petroleum Hydrocarbon (TPH) Disappearance in Soils

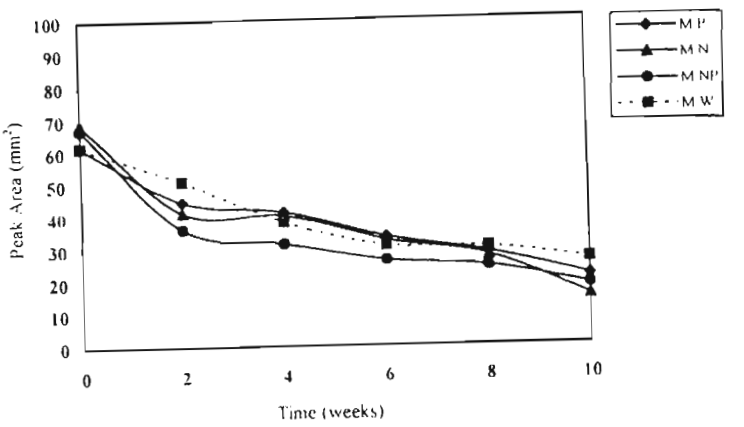
As expected, microbial activity was directly correlated with hydrocarbon degradation (**Figures 8.5 A-I**). The parallel hydrocarbon residue and microbial activity measurements gave strong support to the argument that biodegradation was the dominant component of the remediation process. Microbial activity increased dramatically at 4 weeks in most cases and between 50% and 80% of the total hydrocarbon degradation also took place during the same period.

In soil which was mixed with composted pine-bark (**Figures 8.5 D** and E), the hydrocarbon disappearance rates were not only faster, but the residues which remained at 10 weeks were also lower than in those treatments which did not receive composted pine-bark (**Figures 8.5 A** and B). The corresponding concentration decreases of hydrocarbons in the soils which received pine-bark and P or water compared to the soils without pine-bark correlated strongly with the higher levels of FDA activity which were observed in **Figures 8.4 D** and E, and **Figures 8.4 A** and **B.**

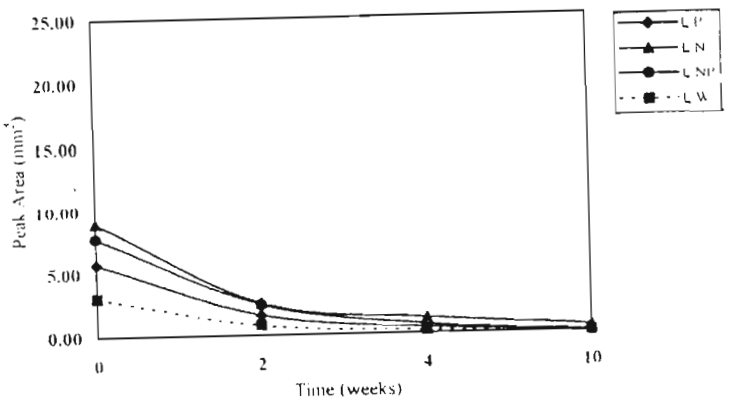
The presence of the soybeans in the contaminated soil appeared to enhance the degradation of hydrocarbons as dramatically as the addition of composted pine-bark. The residual concentrations after 10 weeks for the heavily contaminated soil with soybean plants (**Figure 8.5** F) were very similar to the hydrocarbon concentrations after 10 weeks in the heavily contaminated soil mixed with pine-bark (**Figure 8.5** D), and the disappearance rates were very close. The disappearance rates recorded for the moderately contaminated soil with soybean plants were not as rapid as those calculated in the soil which contained pine-bark although it was observed that the plants did not respond well to this soil (**Figures 8.5** E and G).



(A)

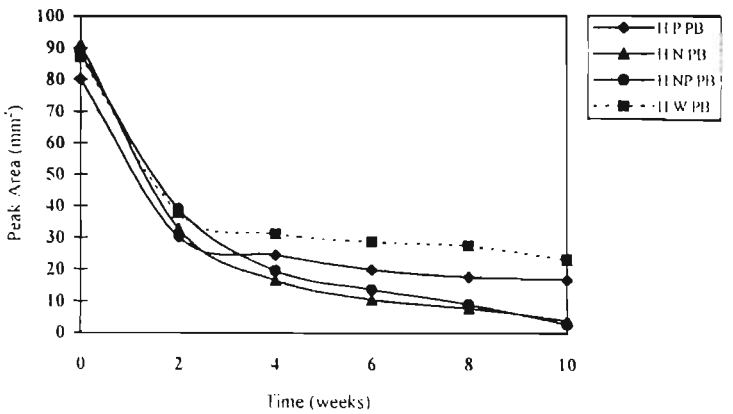


(B)

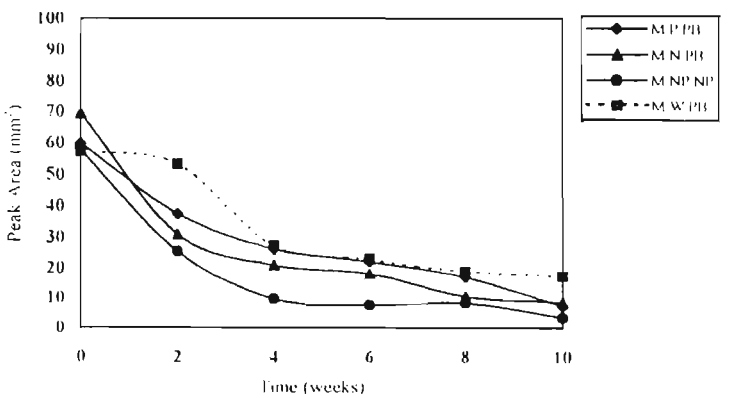


(C)

Figure 8.5 (A) - (C) : Changes in GC peaks areas (an estimate of total petroleum hydrocarbons, TPH) of (A) heavily contaminated, (B) moderately contaminated and (C) uncontaminated soil extracts over 10 weeks, after supplementation with different nutrient treatments (N=Nitrogen, NP=Nitrogen + Phosphorus, P=Phosphorus, W=Water only) (n=3).

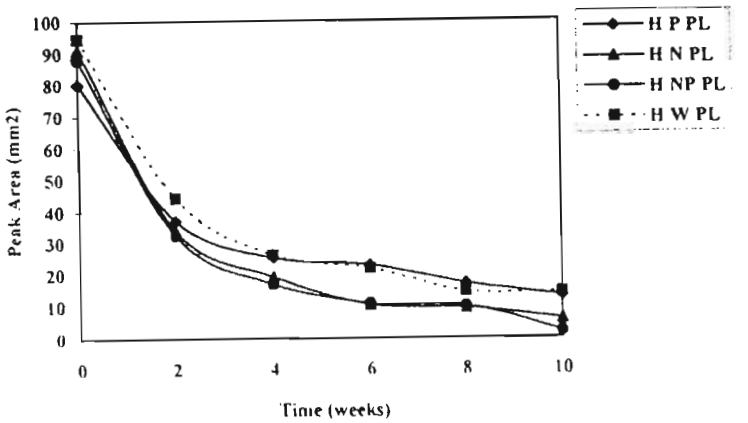


(D)

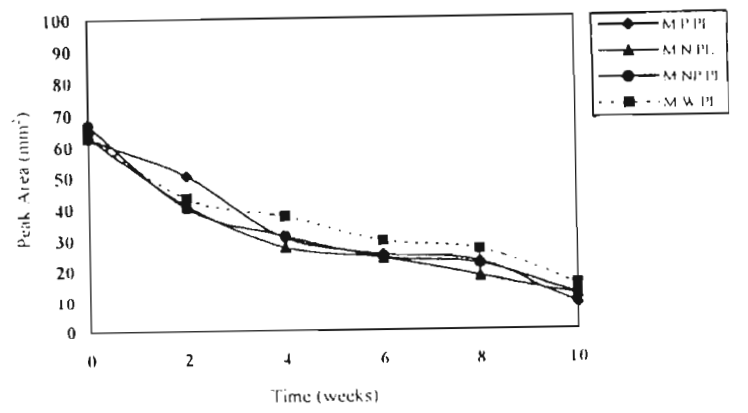


(E)

Figure 8.5 (D) - (E) : Changes in GC peaks areas (an estimate of total petroleum hydrocarbons, TPH) of (D) heavily contaminated and (E) moderately contaminated soil extracts over 10 weeks, after supplementation with 20 % (v/v) composted pine-bark and different nutrient treatments (N=Nitrogen, NP=Nitrogen + Phosphorus, P=Phosphorus, W=Water only) (n=3).

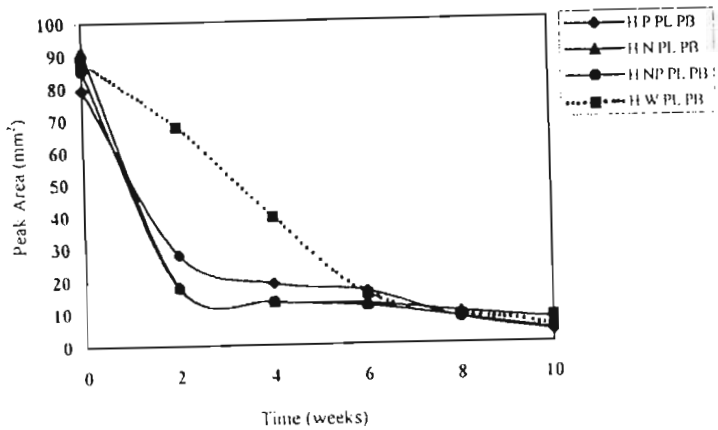


(F)

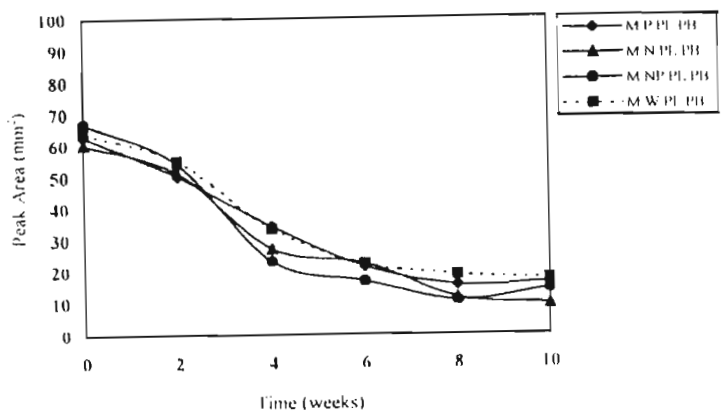


(G)

Figure 8.5 (F) - (G) : Changes in GC peak areas (an estimate of total petroleum hydrocarbons, TPH) of (F) heavily contaminated and (G) moderately contaminated soil extracts over 10 weeks, after supplementation with different nutrient treatments (N=Nitrogen, NP=Nitrogen + Phosphorus, P=Phosphorus, W=Water only) and in the presence of a soybean plant (n=3).



(H)



(I)

Figure 8.5 (H) - (I) : Changes in GC peak areas (an estimate of total petroleum hydrocarbons, TPH) of (H) heavily contaminated and (I) moderately contaminated with 20% (v/v) composted pine-bark and a soybean plant, which was supplemented with different nutrient treatments (N=Nitrogen, NP=Nitrogen + Phosphorus, P=Phosphorus, W=Water, PB=Pine-bark only) (n=3).

Finally, the simultaneous treatment of the soil with pine-bark *and* soybean plants did not appear to enhance the degradation rate of the total hydrocarbons in the soil to any greater extent compared with each individual treatment (**Figures 8.5 H** and **I**). The rates were slightly elevated in the moderately contaminated soil which could be attributed to the improved aeration and buffering capacity of the soil through pine-bark addition.

Both similarities and differences between treatments were, therefore, evident. In each case, tilling and watering alone increased TPH disappearance but not to the same degree as that effected by complete bioremediation treatments of supplementation with N or N plus P, composted pine-bark and/or plants. The responses of the plants to the contaminated soil were not always as anticipated although the response of the *soil* to the *plants* was generally favourable.

The degradation of oil in these experiments followed first-order (exponential) kinetics. Because of diffusion limitations and increases in the catabolic microbial populations, even the depletion of a homogeneous substrate is intermediate between first-order and zero-order (linear) kinetics. Kinetics are usually further complicated in the case of individual hydrocarbons as each is utilised at a different rate.

In this study, the tendency for utilisation to be slower after 4 weeks was caused not only by substrate depletion but also by the fact that the remaining hydrocarbons were probably more recalcitrant than the labile molecules (Bartha and Bossert, 1984a, 1984b; Bartha, 1986). To some extent, this was compensated for by the increasing numbers (enrichment) of the hydrocarbon-degrading microorganisms in the soil with time.

The C_6 to C_9 components of oil under these conditions were accounted for more by evaporation than by biodegradation, while biodegradation primarily removed the C_{10} to C_{40} components. This became clear when the

gas chromatograms of the untreated and treated soil samples were compared (**Figures 8.6 A - E**). The treatments (N + P) which promoted biodegradation were characterized visibly by the loss of components with longer retention times.

Biodegradation and evaporation contribute to the removal of petroleum hydrocarbons. Subtracting the loss of hydrocarbons in the controls from the loss recorded in the experimental soil samples strongly underestimates the true contribution of biodegradation and this should be taken into account when interpreting the results (Song *et al.*, 1990).

It is believed that the biodegradation of the higher aromatics and of the asphaltic compounds, few of which can serve as growth substrates by themselves, is dependent on a continued presence of saturated hydrocarbons to support the co-metabolic biodegradation of the former molecular types (Dibble and Bartha, 1979). Additional studies would be required to verify or reject this hypothesis. However, it would seem probable that the presence of the composted pine-bark and/or the plants would promote the co-metabolic biodegradation of the hydrocarbons due to the simultaneous degradation of root exudates or lignin.

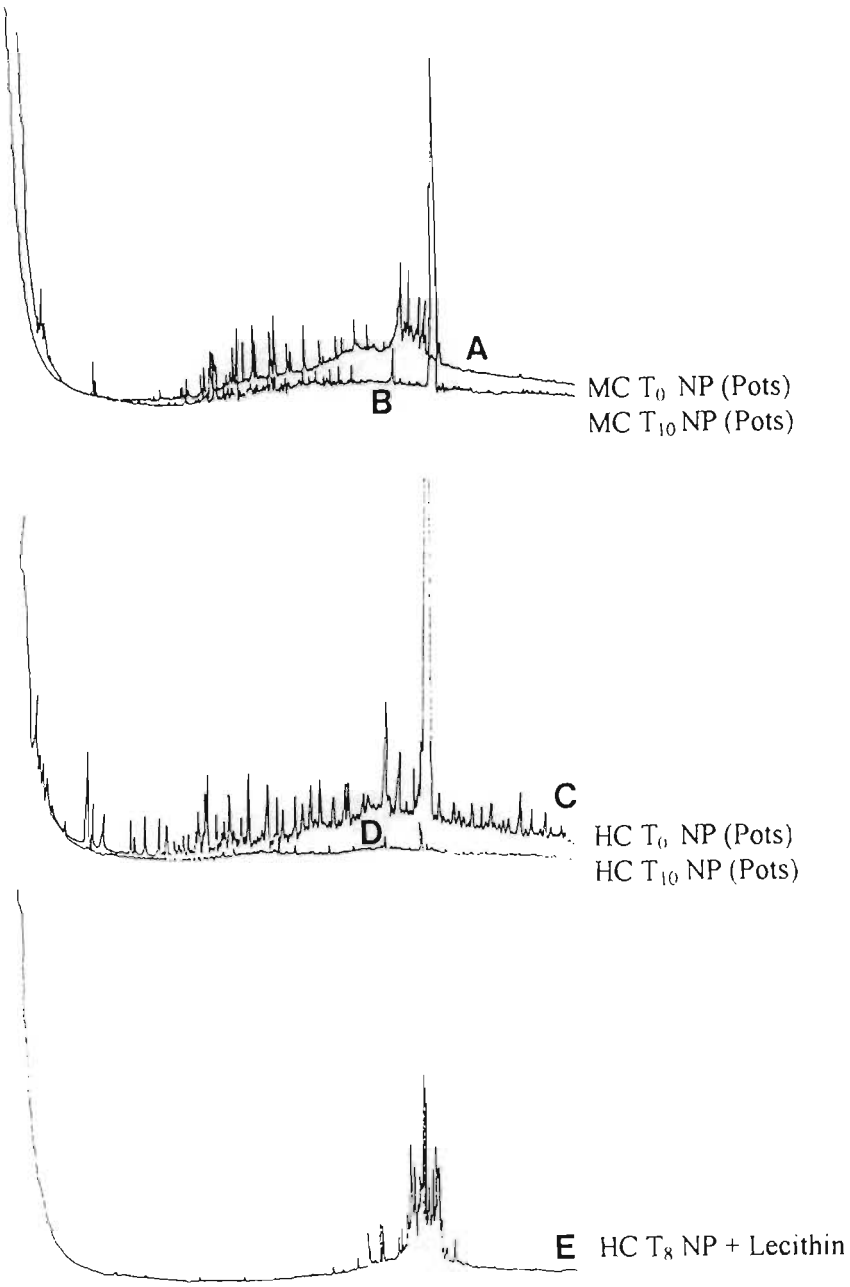


Figure 8.6 : GC traces of extracts of moderately contaminated soil :

(A) before treatment, at the start of Pot Trial 3; (B) after supplementation with N + P at 10 weeks.

GC traces of extracts of heavily contaminated soil :

(C) before treatment; (D) 10 weeks after supplementation with N and P; and

(E) after supplementation with N, P and lecithin after 8 weeks (n=3).

(vii) *Soil pH Changes*

The pH changes which occurred in the heavily and uncontaminated soil during the treatment period were not very dramatic (data not shown). The soils supplemented with LAN were within the acceptable pH range for microbial growth (pH 4.5 - 5.5), and remained relatively unchanged for 8 weeks. The pH values of the moderately contaminated soil, in contrast, were consistently lower (3.9-4.5) than the pH values of the other soils. This may explain the unfavourable response of plant growth in this soil. Lime was added at 6 weeks but appeared to have no effect. Organic acids formed by the degradation of hydrocarbons could have counteracted the effect of the liming, particularly since this soil had very low buffering capacity.

Pine-bark had neither a beneficial nor a detrimental effect on the gross pH. The presence of the soybean plants, however, appeared to significantly ($P<0.01\%$) increase the pH of the heavily contaminated soil. The cause of this effect was unclear as the moderately contaminated soil showed no change.

(viii) The Benefits of Adding Organic Matter to Contaminated Soils

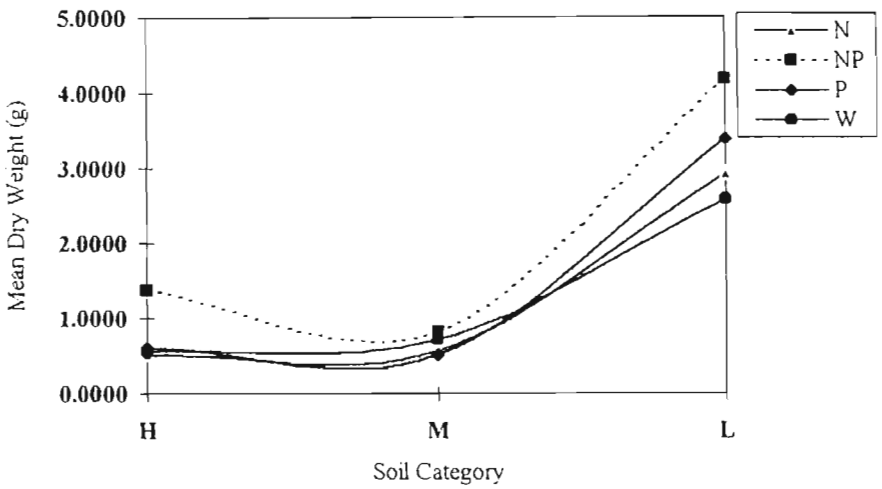
Organic matter in soil contributes to its humus content. Soils rich in humus are, generally, more productive than those poor in humus, as it has positive effects on the chemical, physical and biological properties of the soil. In addition to serving as a source of N, P and S, organic matter influences nutrient supply in other ways. Organic matter is required as a source of energy for molecular nitrogen fixation by microorganisms. The availability of phosphate in soils is often limited by fixation reactions, which convert the monophosphate ion ($H_2PO_4^-$) to various insoluble forms (Morgan and Watkinson, 1990). In contrast, the addition of organic residues to the soil often enhances the availability of the native soil phosphorus to higher plants (Handrick and Black, 1992).

Aluminium toxicity is a major problem in many acidic soils such as the one used in this study. However, acid soils rich in native organic matter, or

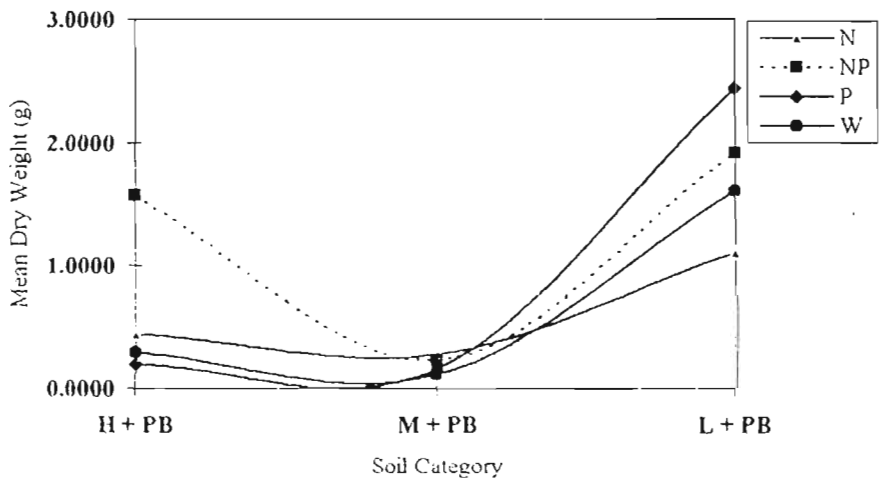
supplemented with large quantities of organic residues, have low Al concentrations and permit good growth of crops and microorganisms under conditions where toxicities would otherwise occur. The role of organic matter in alleviating toxicity effects is well documented (Handrick and Black, 1992).

Organic substances in soil also have a direct physiological effect on plant growth. Soils with low humus content tend to become hard and cloddy. Aeration, water-holding capacity and permeability are all improved by the presence of organic matter (humus), which helps the soil to maintain a loose, open, granular condition through which water is better able to enter and percolate downward.

The rationale for the addition of pine-bark to the soil was, therefore, to improve the physical properties of the soil, which readily compacted and had very little 'natural' organic matter, and to investigate whether the ameliorant would enhance biodegradation. The pine-bark appeared to alleviate the potassium deficiency in the soybean plants and also increased the buffering capacity of the medium by increasing the cation exchange capacity (CEC). The plants of all the pine-bark treatments were more vigorous and healthier than those without pine-bark (**Plates 8.5, 8.7, 8.8** and **8.9**) which indicated that the improved soil quality was extremely beneficial to the plants and their associated microorganisms. The biomass of all plants grown in pine-bark-supplemented soil were also significantly different from the comparable controls (**Figures 8.7 A** and **B**).



(A)



(B)

Figure 8.7: The mean dry weight of the soybean plants grown for 12 weeks in (A) heavily, moderately and uncontaminated soil, and (B) heavily, moderately and uncontaminated soil, with composted pine-bark, which was supplemented with different nutrients (N=Nitrogen, NP=Nitrogen+Phosphorus, P=Phosphorus, W=Water only) (n=3).

PLATE 8.7 (A)-(H) :

The plants grown in heavily contaminated soil displayed a variety of toxicicity or nutritional symptoms some of which appeared to be alleviated by the addition of pine-bark. Overall, however, plants grown in soil to which N and P had been added (A and F) were significantly larger than those grown without phosphorus (B and E), illustrating the need for both nutrients to be present.

Plants grown in contaminated soil without P were stunted and yellow in colour (C and G) while those grown without nutrients (D and H) were remarkably similar to the latter, which suggests that the nitrogen-fixing microorganisms are very sensitive to oil components.

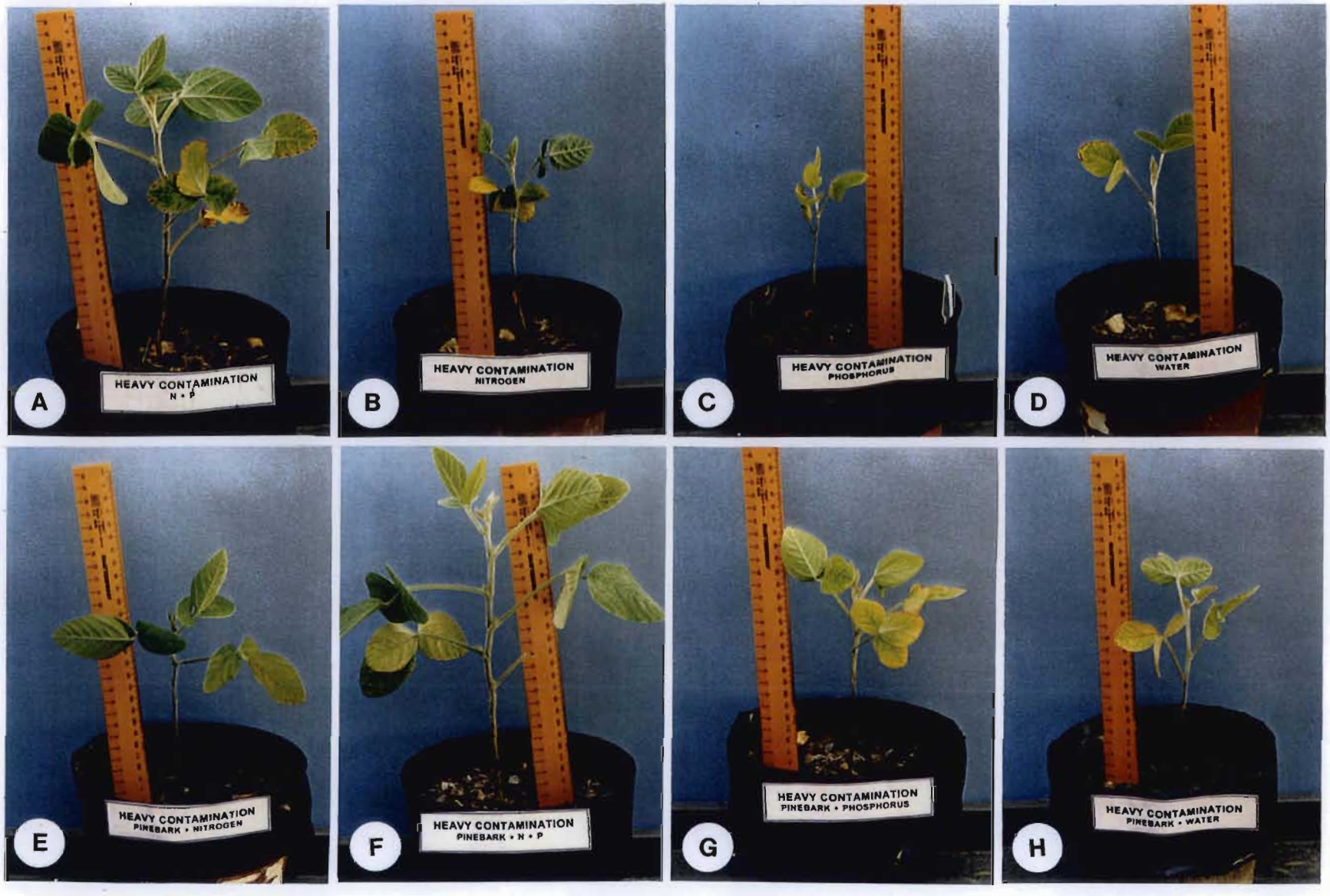

A
HEAVY CONTAMINATION
N + P
B
HEAVY CONTAMINATION
NITROGEN
C
HEAVY CONTAMINATION
PHOSPHORUS
D
HEAVY CONTAMINATION
WATER
E
HEAVY CONTAMINATION
PINEBARK + NITROGEN
F
HEAVY CONTAMINATION
PINEBARK + N + P
G
HEAVY CONTAMINATION
PINEBARK + PHOSPHORUS
H
HEAVY CONTAMINATION
PINEBARK + WATER

PLATE 8.8 (A)-(H) :

The trends observed in the development of the plants grown in heavily contaminated soil were reflected in those grown in moderately contaminated soil where the healthiest plants were those grown in soil to which nitrogen had been added (A, B, E and F). Those grown in soil to which P had been added (C and G) responded similarly to those grown in soil without added nutrients (D and H).

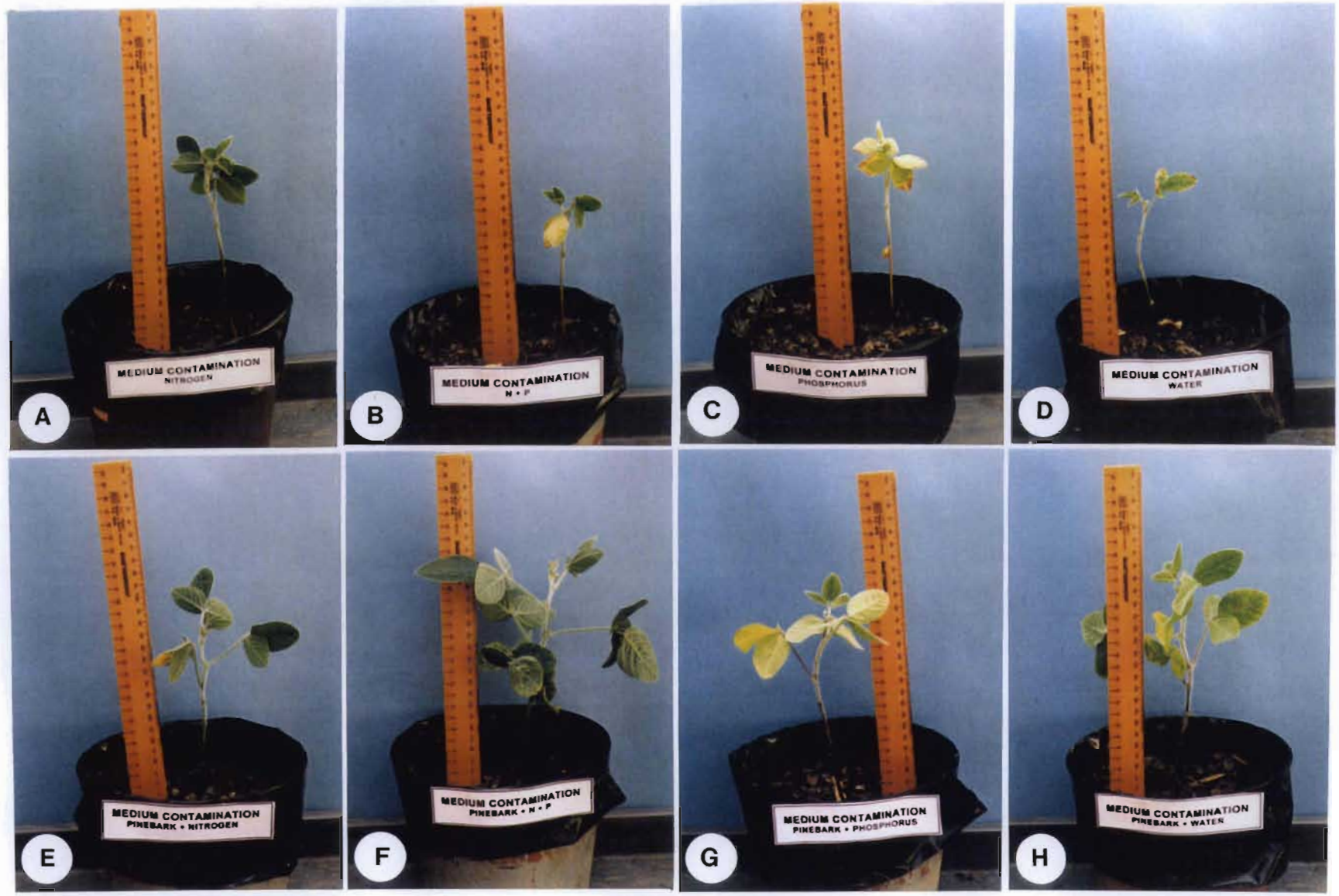

A
MEDIUM CONTAMINATION
NITROGEN
B
MEDIUM CONTAMINATION
N + P
C
MEDIUM CONTAMINATION
PHOSPHORUS
D
MEDIUM CONTAMINATION
WATER
E
MEDIUM CONTAMINATION
PINEBARK + NITROGEN
F
MEDIUM CONTAMINATION
PINEBARK + N + P
G
MEDIUM CONTAMINATION
PINEBARK + PHOSPHORUS
H
MEDIUM CONTAMINATION
PINEBARK + WATER

PLATE 8.9 (A)-(H) :

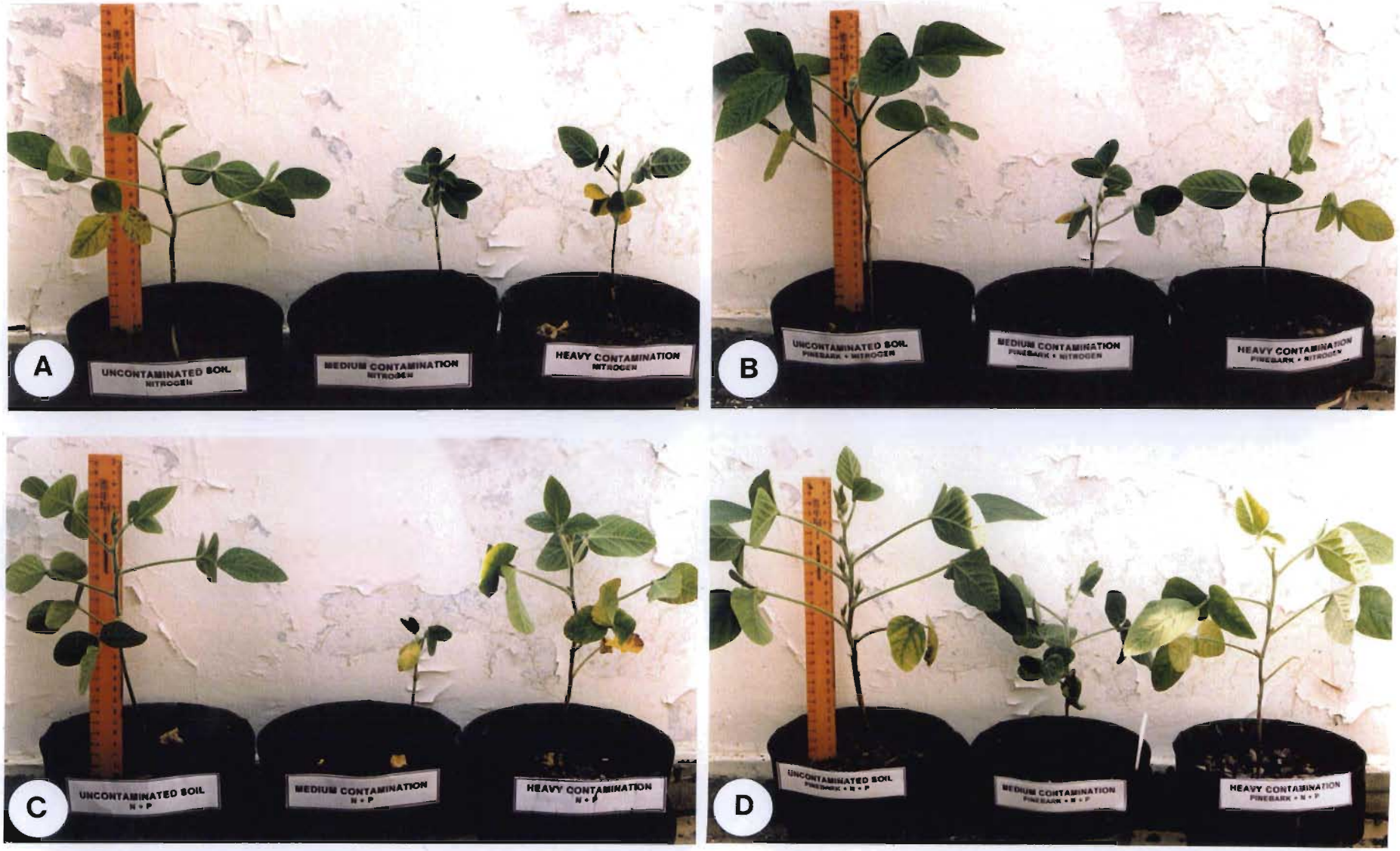
Soybean plants grown in uncontaminated soil (control) and soil + pine-bark were significantly larger in size and mass than those grown in heavily or moderately contaminated soil. There was little difference between treatments A-D and E-H, although a few symptoms displayed in the leaves observed in A, B, E and F were similar to those plants grown in contaminated soil.

Overall, the plants were tall, vigorous and had large leaves and thick stems. The soil which received P only or water (C, D, G and H) were as healthy as those plants to which N or N and P had been added (A, B, E and F). Nitrogen-fixing microorganisms were, therefore, able to provide necessary nitrogen to the plants under uncontaminated conditions.


A
UNCONTAMINATED SOIL
NITROGEN
B
UNCONTAMINATED SOIL
N • P
C
UNCONTAMINATED SOIL
PHOSPHORUS
D
UNCONTAMINATED SOIL
WATER
E
UNCONTAMINATED SOIL
PINEBARK • NITROGEN
F
UNCONTAMINATED SOIL
PINEBARK • N • P
G
UNCONTAMINATED SOIL
PINEBARK • PHOSPHORUS
H
UNCONTAMINATED SOIL
PINEBARK • WATER

PLATE 8.10 (A)-(D) :

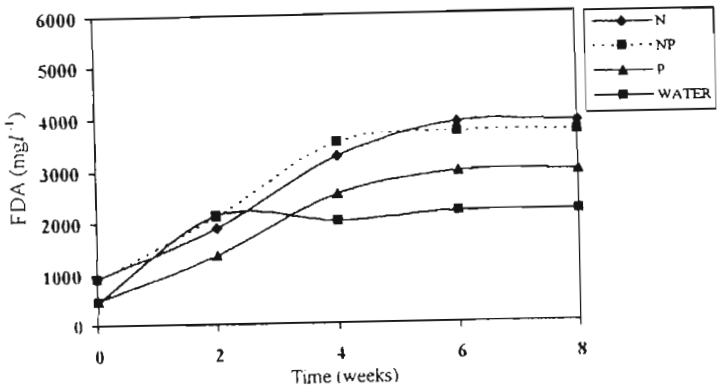
Examples of the plants grown in soil and soil + pine-bark of all contamination categories which received N (A and B) and N + P (C and D). Those grown in soil + pine-bark were generally taller with larger leaves, indicating that an increase in porosity is favourable for plant growth, despite the presence of oil components in the soil.


A
UNCONTAMINATED SOIL
NITROGEN
MEDIUM CONTAMINATION
NITROGEN
HEAVY CONTAMINATION
NITROGEN
B
UNCONTAMINATED SOIL
PINEBARK + NITROGEN
MEDIUM CONTAMINATION
PINEBARK + NITROGEN
HEAVY CONTAMINATION
PINEBARK + NITROGEN
C
UNCONTAMINATED SOIL
N + P
MEDIUM CONTAMINATION
N + P
HEAVY CONTAMINATION
N + P
D
UNCONTAMINATED SOIL
MEDIUM CONTAMINATION
HEAVY CONTAMINATION
PINEBARK + N + P

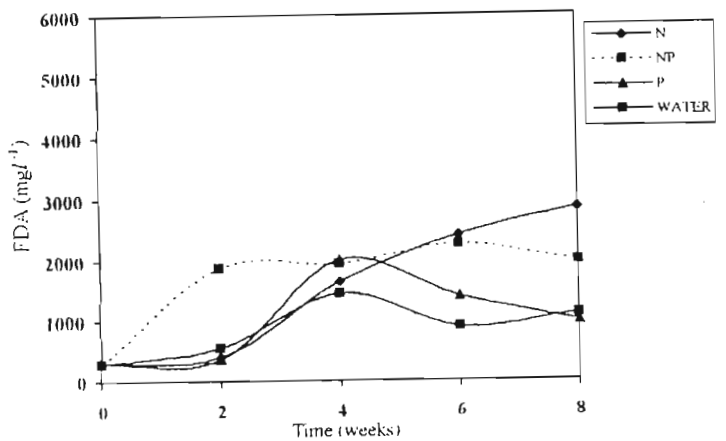
(ix) Supplementation of Contaminated Soil with Lecithin

Lecithin is a natural product which is extracted from soybean seeds and is used extensively in the food industry because of its emulsifying properties (Snyder and Kwon, 1987). The functionality of lecithin is a result of its structure. The lipophilic portion of the molecule arises from the hydrocarbon chains of the fatty acid molecules, and the hydrophobic portion arises from a phosphate group and whichever groups are combined with the phosphate. This bipolar nature of lecithin makes it an excellent surface-active agent which, in turn, allows it to function as an emulsifier, wetting agent and release agent, to modify viscosity, or to control crystallization (Snyder and Kwon, 1987). As a phospholipid with such valuable properties, it was considered to be ideal for the emulsification of oil without the possibility of toxicity, which many chemically synthesized surfactants/emulsifiers have to the microbial population. Due to time constraints, the trial was designed with heavily and moderately contaminated soil only. Uncontaminated soil was expected to behave in the same manner as previous treatments and was, therefore, excluded.

The addition of lecithin to the contaminated soil did not increase the microbial activity (measured by FDA activity) significantly compared with the other treatments (**Figures 8.7 A** and B). However, the concentrations of residual hydrocarbons which remained after 6 weeks were as low as those which remained in the other treatments after 8 weeks (**Figures 8.8 A** and B). It appeared that the lecithin facilitated hydrocarbon biodegradation through improved solubilisation and availability. In addition, the lecithin decreased the soil pH slightly (data not shown) which may have been the reason why activity was not enhanced to the extent that it was expected.



(A)



(B)

Figure 8.8: Changes in FDA hydrolysis activity over 8 weeks in (A) heavily contaminated and (B) moderately contaminated soil which was supplemented with (A) 5g kg^{-1} lecithin and (B) 2.9g kg^{-1} lecithin, and nutrients (N=Nitrogen, NP=Nitrogen+Phosphorus, P=Phosphorus, W=Water only) (n=3).

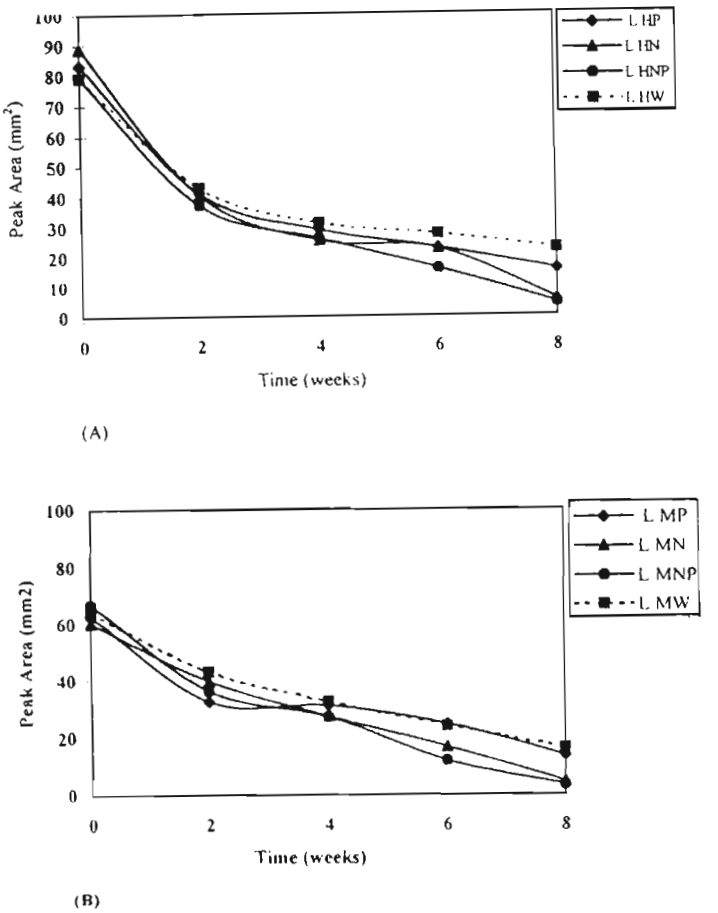


Figure 8.9: Changes in GC peak areas (an estimate of the total petroleum hydrocarbons, TPH) over 8 weeks in (A) heavily contaminated and (B) moderately contaminated soil which was supplemented with (A) 5g kg^{-1} lecithin and (B) 2.9g kg^{-1} lecithin, and nutrients (N=Nitrogen, NP=Nitrogen+Phosphorus, P=Phosphorus, W=Water only) (n=3).

The principal advantage of lecithin is that it seems to have a 'localised' solubilization effect, rather than a 'mass' effect at low concentrations, which aids availability but does not mobilise the oil in the same way as other surfactants which have the effect of "soil washing". This is probably because the lecithin is oleophilic and rather viscous and adsorbs to the oil and soil particles, rather than increasing the mass flow.

This positive result suggested that lecithin, combined with a nutrient source, could be a potentially valuable remediation supplement for soils contaminated with hydrocarbons, particularly when an *in situ* strategy is envisaged and the site manager is reluctant to add more chemicals.

(x) The Return of Nitrogen to Soil by Nitrogen Fixation or Green Manuring

One of the oldest agricultural practices is the growth of legumes for soil improvement. The yields of non-leguminous crops are usually greater when they are grown after legumes, due to an increased nitrogen capital (Foth, 1990). In these cases, the legume crop is harvested and the benefit to the following crops is a by-product.

In green manuring, a crop is grown for subsequent ploughing to add organic matter to the soil. This is particularly beneficial for sandy soils which are naturally very low in organic matter. In these soils, nitrogen results from soil organic matter mineralization, as added nitrogen fertilizer is often leached before the microorganisms or crop can use it. The value of decomposing legume material is, thus, to maintain long-term soil organic nitrogen status rather than to supply large amounts of mineral nitrogen for short-term uptake (Redman *et al.*, 1989).

The major effect of the green-manure crop is to increase the supply of nitrogen (and other nutrients), and provide an increase in the organic matter content of the soil. Other benefits are the protection of soil from erosion and reduced loss of nutrients by leaching. However, it has been difficult to

attribute the economic benefits of green manuring to any effect other than that of increased nitrogen supply (Redman *et al.*, 1989).

Although this investigation did not examine the potential of introducing the soybean foliage into the contaminated soil to improve the biodegradation rates of hydrocarbons, it is hypothesised to be another inexpensive but effective procedure which could complement other remedial approaches.

(xi) Statistical analysis

The statistical analyses revealed several interesting, but not entirely surprising, conclusions :

- For the dependent variable pH, comparisons which were found to be significant at the 95% confidence level were between all combinations of moderately, heavily and uncontaminated soils; between N/NP and W, and N/NP and P nutrient treatments; between pine-bark and no pine-bark treatments; and between lecithin and no lecithin supplementation;
- For the dependent variable PK, measuring TPH, comparisons which were found to be significant at the 95% confidence level were between the heavily and uncontaminated soil, and the moderately and uncontaminated soil, but not between the moderately and heavily contaminated soil. Also, significant differences were found to exist between nutrient treatments N/NP and W, and N/NP and P, but not between N and NP or P and W. Finally, the differences between pine-bark and no pine-bark were all significant, as were the differences between the plant or no plant treatments and lecithin versus no lecithin supplementation;
- For the dependent variable mgFDA, measuring microbial activity, comparisons which were found to be significant at the 95% confidence level existed between the heavily and uncontaminated soil, and the moderately and uncontaminated soil, but not between the heavily and moderately contaminated soil. The same trends for nutrient, pine-bark and lecithin treatments were as observed for the PK dependent variable

(above). However, no significant differences in microbial activities were calculated for the plant treatments, at all levels.

Thus, significant, high-order interactions comprised (overall) of : lecithin-contaminant; time-contaminant-nutrient; time-contaminant-pine-bark; time-contaminant-pine-bark-plant; and time-contaminant-lecithin effects **(Appendix 2)**.

8.8 Conclusions

Petroleum pollution of soil, generally, has strong negative effects on the plant community. The mode in which it acts on plants is complex and involves both contact toxicity and indirect deleterious effects, mediated by interactions of the chemicals with the abiotic and microbial components of the soil. Direct effects were observed very clearly in the SEM micrographs of the soybean roots where the solvent effects of low-boiling point hydrocarbons on the lipid membrane structures of the cells were made apparent. These compounds are readily removed from the soil by leaching and their effects are generally of short duration. The indirect effects, which include oxygen deprivation, are more lasting and, therefore, more severe. These occur through the exhaustion of oxygen by hydrocarbon-degrading microorganisms. Anaerobic conditions develop which facilitate the microbial generation of phytotoxic compounds such as hydrogen sulphide. Together with oxygen, the oil-degrading microorganisms compete with the plants for mineral nutrients. Other problems which result include changes in the physical structure of the soil, thus decreasing its capacity to store moisture and air (De Jong, 1980). All these negative effects manifest themselves either immediately or during the biodegradation of the polluting oil.

The lower germination rate of plant seeds in oily soil could be attributed to one or more of the following factors : increased water repellence by the contaminated soil making the soil more hydrophobic, thereby lowering the

available moisture for the seeds and the seedlings (Brown *et al.*, 1983); potential hazards of phytotoxic components still present in the soil; and intermediary products of microbial hydrocarbon degradation including fatty acids and terpenoid materials, with phytotoxic properties (Stevenson, 1966). The presence of volatile components in the soil used in the trials was not considered to be significant due to the mixing which occurred during the filling of the pots, and the high ambient atmospheric temperatures (>26°C). Therefore, it is believed that hydrophobic action was the main cause of reduced germination which was exacerbated by the presence of intermediate degradation products. The results of this study differed from those of Bossert and Bartha (1985) who reported that in soil treated with dissolved air flotation oil sludge, soybeans failed to germinate.

In summary, oil might inhibit plant growth due to increased water repellency, resulting in the lowering of available moisture for the plants. Plants, however, do differ in their sensitivity to phytotoxic components of oil and it appears from this study and those of other workers (Stevenson, 1966; Brown *et al.*, 1983; Bossert and Bartha, 1985; Huddlestone *et al.*, 1985) that soil with <1% (w/w) oil may be suitable for growth of certain plants. Uptake of elements is also characteristic for each plant type.

Soil pH is a major factor with respect to the reactions of heavy metals and the solubility and uptake of other nutrient elements. Under the acidic conditions of Natal Group Sandstone-derived soils, Al, Mn, Mg, Fe and Zn are all mobile and available to plants. The combined effects of the microbial degradation of oil components and the lack of buffering in the sandy soil caused the soil pH to drop, in some cases to below 4.0, which immediately compromised the effectiveness of the nitrogen-fixing bacteria, increased the solubility of metals, rendered phosphorus unavailable and reduced the growth rates of the plants whose ideal pH for growth is approximately 6.5. Although the relative role of bacteria versus fungi in the biodegradation of hydrocarbons in soil has not been determined, liming in this study favoured the biodegradation of oil, in agreement with previous findings (Lehtomäke

and Niemelä, 1975; Verstraete, Vanlooke, deBorger and Verlinde, 1975; Dibble and Bartha, 1979).

It must be realised that there may be a lag period after the addition of nutrients before degradation occurs. This may not necessarily be due to the type of N source, nor the direct toxicity of the added N, but simply that the added nutrients could cause a shift in the metabolism of the population away from the mineralization of the hydrocarbons. Alternatively, the population may have been adapted to the indigenous nutrient concentration and the sudden increase in nutrient supply may have been temporarily inhibitory. Such oligotrophic behaviour is common (Morgan and Dow, 1986). Whatever the explanation, the results thus far suggested that the added inorganic nutrients may stimulate, have no effect or may even inhibit the biodegradation of environmental contaminants in soil. All applications at individual biotreatment sites, therefore, require careful evaluation. The success of *in situ* biotreatment for the cleanup of contaminated sites depends upon the provision of suitable environmental conditions for biodegradation. Normally, either or both inorganic nutrients and oxygen are provided to the soil but, as has been shown in this study, there may be difficulties with many of the potential approaches, despite their apparent simplicity.

CHAPTER NINE

FIELD PLOT STUDIES

9.1 Introduction

Field plot experiments are an essential step in bioremediation studies to facilitate the expected full-scale site operations. Although the direct application of batch test data to field site design is not often a realistic practice, particularly from the point of view of the attenuation of pollutants by soils, field studies may be used as a diagnostic tool to :

- verify laboratory and controlled-environment plot results;
- identify field-oriented problems not detectable in the laboratory;
- integrate site conditions into a single system;
- evaluate erosion factors;
- evaluate odour and volatilisation problems; and
- evaluate more closely the soil-waste interactions.

Furthermore, the most useful method for evaluating whether microorganisms are actively degrading the contaminant involve not only sampling the site but also conducting experiments in the field. These field experiments require the addition of various chemicals to the soil/subsurface in a controlled manner to determine if their fate is consistent with what should occur during bioremediation (Rittman *et al.*, 1994). The addition of stimulants such as electron acceptors, electron donors and nutrients should speed biodegradation but not abiotic contaminant removal processes. Thus, when stimulants are added to one subsite but not to another, the relative rate of contaminant loss should increase in the stimulant-supplemented subsites. The contrast in contaminant loss between enhanced and non-enhanced subsites can be attributed to microbial activity. To apply this

approach requires an experimental site sufficiently uniform to have comparable subsites.

The pilot study described here was a 14-week trial conducted in three phases. The first phase consisted of tilling the plots and leaving them for two weeks without further treatment (PI T_0-T_2). This was intended to allow investigation of the loss of low molecular weight hydrocarbons due to volatilisation. The second phase consisted of : (i) placing heavily contaminated soil onto a plastic liner and mixing composted pine-bark into the soil (20% v/v) with the simultaneous addition of nutrients (landfarming); and (ii) tilling and adding inorganic supplements to moderately contaminated plots (*in situ* bioremediation) (PII T_2 - T_8). In the third phase, (iii) plants (grass) were introduced and permitted to establish, facilitated by nutrient augmentation (PIII T_8 - T_{14}). "Natural" bioremediation was carried out by leaving some plots undisturbed after the first tilling, or by adding water only.

9.2 Field Plots

The experimental site consisted of 11 vegetation-free plots (1m x 1m) with a border of 0.5-1.0 m (4 x Heavily contaminated HC; 4 x Moderately contaminated MC and 3 x Low contamination LC). Four different subplots (10 x 10 cm) within each plot were sampled at each sampling event to monitor changes in the oil concentration. A small hand-operated auger was used to sample the plots to a depth of between 15 and 50 cm. The soil samples were packed into clean polyvinyl flip-top containers, leaving no headspace so that volatilsation was minimised, sealed and taken to the laboratory. The soil samples were used immediately for the FDA hydrolysis bioassay **(Section 8.6.2.12)** and the measurement of pH. The soil was then frozen at -17°C until required for hydrocarbon quantification.

9.2.1 Phase I

All 11 plots were demarcated with wooden stakes and string and labelled. Due to the variability of the oil contamination and the topography of the site (**Plate 4.2 A-H** and **Figure 4.2**), four plots were selected in each of 'heavy contamination' (HC) zones and 'moderately contaminated' (MC) zones; and three plots in 'low contamination' (LC) zones. Plots in the MC zones had a layer of ash, approximately 10 cm thick, just below the soil surface (2-5 cm depth). This layer was removed with a garden spade before tilling. All plots were moistened to ~16-20% moisture (w/w), tilled with garden forks and spades to a depth of between 0.5 and 1.0 m (after sampling) and sampled once at 0 weeks (T_0) and again 2 weeks later (T_2).

9.2.1 ***Phase II***

9.2.1.1 "Land Treatment"

The soil in the first three HC plots (HC1, HC2 and HC3) was excavated to a depth of 0.8-1.0 m and placed onto a large piece of high strength polypropylene sheeting to prevent leaching and to facilitate mixing. Composted pine-bark (~20% v/v) was mixed into the soil of Plots 1 and 3 (HC1 and HC3), while Plots 2 and 4 (HC2 and HC4) were only tilled by hand. Plots 1, 2 and 3 were supplemented with a commercial slow-release N:P:K (3:2:1) fertiliser (Grovida) at a rate equivalent to 20 kg N ha^{-1} . A C:N ratio of 10:1 was calculated from the percentage carbon in the soil and assuming 50% carbon degradation. Plot 4 (HC4) was the control plot which received water only. The plots were covered with 80% shadecloth to prevent wind erosion and algal or plant growth.

9.2.1.2 *In Situ* Treatment

The soil in the MC plots was not excavated but was tilled and moistened to between 20 and 30% water holding capacity. Plots 1 and 3 (MC1 and MC3) were supplemented with the slow-release 3:2:1 fertiliser at a rate of 12 kgN ha^{-1} (C:N 10:1). Plots 2 and 4 (MC2 and MC4) received no supplementation. Additional fertiliser was added after 6 weeks.

9.2.1.3 "Natural" Remediation

Soil of low contamination was not excavated. The soil in Plot 1 (LC1) was supplemented once with 3:2:1 (Grovida) at a rate of 5kg ha^{-1} while Plot 2 (LC2) was tilled and Plot 3 (LC3) was merely moistened.

Soil in the above plots were sampled every fortnight for 6 weeks.

*9.2.2 **Phase III***

9.2.2.1 "Land Treatment"

Soil from Plots HC 1, 2 and 3 was sampled and returned to the excavated areas without the plastic liners or the shadecloth. Fertiliser was added at the same rate as before and the soil was mixed thoroughly. Grass from the site was planted into the plots by hand and watered. The grass was allowed to establish for a further 6 weeks.

9.2.2.2 *In Situ* Treatment

Plots MC 1 and 3 were fertilised at the same rate as before and grass from the site was planted by hand into each plot and allowed to establish for 6 weeks.

9.2.2.3 "Natural" Remediation

Plots LC 1, 2 and 3 were tilled and sampled every fortnight for 6 weeks. Grass was not planted into any of the plots.

As before, the plots were moistened to ~16-20% moisture (w/w), tilled and sampled every fortnight for 6 weeks. All observations were recorded photographically.

*9.2.4 **Analyses***

9.2.4.1 FDA hydrolysis bioassay

As described in **Section 8.6.2.12 (i)** and **(ii).**

9.2.4.2 Infrared Spectrophotometry (IRS) for Total Hydrocarbon (TPH) Determination

As described in **Section 8.6.2.12 (iii).**

9.2.4.3 Gas chromatography (GC)

As described in **Section 8.6.2.12 (iv).**

9.2.4.4 Soil pH

As described in **Section 6.2.1.7.**

9.2.4.5 Plant Survey

Plants from the experimental site, specifically grass species, were collected and taken to the Herbarium (Department of Botany, University of Natal, Pietermaritzburg) for identification. The most common grass types were then chosen for transplantation into the plots.

9.3 Results and Discussion

There are two types of sample-based techniques for demonstrating field biodegradation : measurements of field samples, and experiments run in the field. In most bioremediation protocols abroad, a third technique, modeling experiments, provides an improved understanding of the fate of contaminants (Rittman *et al.*, 1994). Detailed experimental protocols for carrying out the tests are usually developed and vary from site to site depending on the types of contaminants present, the geological characteristics of the site and the level of detail desired in the evaluation.

Techniques for evaluating *in situ* bioremediation generally involve removing samples of soil and/or water from the site and bringing them to the laboratory for chemical or microbiological analyses. Many of these techniques require comparing conditions at the site once bioremediation is under way with site conditions under baseline circumstances (controls) when bioremediation is not occurring. Baseline conditions are established in two ways. The first method is to analyse samples from a location that is hydrogeologically similar to the area being treated but is either uncontaminated or is outside the zone of influence of the bioremediation system. The second method is to gather samples before starting the bioremediation process and to compare them with samples gathered at several time points after the system is operating (NAS, 1994). To ensure maximum information collection, a combination of both methods was used in this experiment.

9.3.1 TPH Disappearance in Field Plots

9.3.1.1 Phase I

In Phase I, the soil was tilled to a depth of 0.5-1.0 m (**Plate 9.1 A** and **B**). Samples were taken, extracted and analysed for hydrocarbons only. At this stage, the measurement of biological activity was not taken as it was intended to estimate the loss of hydrocarbons by physical or chemical processes. Stimulation of microorganisms was judged to be unlikely, since the site had been contaminated for over two decades and it was probable that the other nutrients in the soil had either been depleted or displaced, thus precluding further biological degradation.

The total losses of hydrocarbons from the heavily contaminated soil during Phase I (2 weeks) were 29.35% (HC1); 28.15% (HC2) and 20.69% (HC3). Plot 4 showed a decrease of only 4.35%, which was primarily due to its site location. The soil was frequently waterlogged which should have effected solubilisation of the lower molecular weight hydrocarbons. Losses of between 20 and 40% in the first two weeks from untreated controls have been reported, depending on the composition of the contaminant oil (Dibble and Bartha, 1979).

The moderately contaminated soil showed hydrocarbon decreases of 10.87% (MC1), 11.07% (MC2), 17.80% (MC3) and 8.10% (MC4). The lower rates of disappearance compared to the other plots was explained by the fact that the initial oil concentrations particularly the concentrations of volatile low molecular weight hydrocarbons, were lower than in the heavily contaminated soil. The MC plots were also located in a drier area than the HC plots. A higher porosity, therefore, facilitated greater volatilisation with

time than was possible in the wetter soil, where the pores were filled with water and dissolved/adsorbed hydrocarbons. A slow, but steady decrease in TPH was observed in Plot 1 of the low contaminated (LC) soil. The rates of disappearance of hydrocarbons in the others were not significant ($P<0.05$).

9.3.1.2 Phase II

Phase II consisted of a two-pronged approach. The first was to excavate the heavily contaminated soil, which had been tilled in Phase I, and to lay it onto a thick plastic liner and mix it with composted pine-bark (McDonalds Seeds) (20% (v/v) and 20 kgN ha^{-1} of commercial slow-release fertilizer (N:P:K=3:2:1, Grovida). This was done because the heavily contaminated soil was more prone to anaerobiosis and leaching of soluble hydrocarbons due to its very high oil concentration. A 'land treatment' strategy (**Chapter 1**) was considered to be appropriate because a greater degree of control was possible in this type of approach. The soil was bulked-up by the addition of pine-bark and was covered with 80% shade-cloth to prevent wind and rain impacting the soil piles (**Plate 9.1 C-D**).

The second approach was to attempt *in situ* remediation for the moderately contaminated (MC) soil. This was considered possible because the concentration of oil in this area was not as high and, therefore, was a lower threat with respect to leaching and/or mobility than the HC soil. The soil was tilled and 12 kgN ha^{-1} added as described above, to two of the plots (MC1 and MC3). The other two plots were tilled and allowed to rehabilitate "naturally". It was accepted that after preliminary treatment of the heavily contaminated soil by landfarming, the soil would be returned to the site of excavation and treated *in situ*. The rationale was, therefore, to attempt to

reduce the contamination to at least that of the moderately contaminated soil, and thus be susceptible for *in situ* remediation.

Phase II progressed for 6 weeks during which time the hydrocarbon concentration was monitored. The HC soil which had been treated with nutrients and composted pine-bark showed further decreases in hydrocarbons, compared with the figures for Phase I, of 57% (HC1); 54% (HC3); 46% (HC2); and 18.6% (HC4). The rates of degradation (1.36% TPH day^{-1} and 1.29% TPH day^{-1}) were, thus, highest for those soils that were supplemented with pine-bark and fertiliser and were significantly faster ($P<0.01$) than those without pine-bark or nutrients (1.09% TPH day^{-1} and 0.44% TPH day^{-1}, respectively). This suggested that the addition of N, P and K to the soil effected rapid degradation which was enhanced by the presence of composted pine-bark and which was greater than the rate of degradation recorded with tilling alone. At the rate of disappearance measured in HC4, it should take at least three times longer than the treated plots to reach the same TPH concentration. With no intervention at all, remediation would take considerably longer.

The moderately contaminated plots showed similar trends. The total hydrocarbons decreased in the supplemented soils by 50% (MC1) and 48% (MC3) (~2.36% TPH day^{-1}). The disappearance rates (~0.77% TPH day^{-1}) were slowest in the unsupplemented plots (MC2 and MC4) where the oil concentrations decreased by 36.8% and 28%, respectively. The higher rates of TPH disappearance in the unsupplemented MC plots compared with the unsupplemented HC plot were possibly due to the higher percentage of low-molecular weight hydrocarbon residues and their toxicity to the hydrocarbonoclastic microorganisms. The measured rates of disappearance of oil from the soil were comparable to those recorded in the earlier work (**Section 6.2.3.2**), suggesting that similar mechanisms were in operation.

PLATE 9.1 (A)-(D) :

(A) and (B) show field plots undergoing Phase I of remediation, where soil was thoroughly tilled, within a square metre, and left for two weeks to determine the loss of oil by volatilisation and chemical oxidation.

(C) shows a heavily contaminated plot during Phase II, where soil was transferred to a plastic sheet, tilled and supplemented with composted pine-bark and nutrients, and covered with shadecloth. (The insert shows the heavily contaminated soil as it appeared on transferal to the plastic sheeting).


A
B
C
D

9.3.1.3 Phase III

Although many trends and significant effects were revealed in the pot trials which related specifically to nutrient interactions with the soil microorganisms, the major emphasis of that work (**Chapter 8**) was on phytoremediation which, in many respects, was successful. The mere fact that the plants survived in the compromised soil, in addition to the results which supported the hypothesis that the presence of plants was beneficial, was encouraging. However, the symptoms which were displayed by the plants indicated that the chosen plant type was, perhaps, inappropriate.

It was concluded that successful revegetation of the contaminated soil may ultimately rest on the type of flora indigenous to, or which would naturally revegetate the affected area. A decrease in species diversity and a shift in dominant plant types occurs during revegetation of impacted soils and a survey of the plants surviving on the experimental site was made as the next logical step in the field investigation (**Table 9.1**). Indigenous grasses which were found growing at the experimental site were then transplanted onto the plots.

Phase III, therefore, consisted of further fertilisation and the introduction of vegetation to the contaminated soil. The growth and establishment of the transplanted grasses were dependent upon natural rainfall and field temperatures over the 6 week experimental period. It was important to examine the success of these grasses as remedial agents in comparison with soybean, which was examined earlier in the pot trial.

Table 9.1 : Predominant grass species found after a survey of the experimental site.

Taxonomic Name	Common Name
Paspalum urvillei	Giant Paspalum
Digitaria eriantha	Finger grass
Chloris gayana	Rhodes grass
Panicum maximum	Guinea grass
Eragrostis curvula	Weeping lovegrass
Eleusine indica/coracana	Goose grass
Cynodon dactylon	Couch grass (Kweek)
Melinis repens	Natal redtop

Grasses, which have a genetic advantage that enables their establishment in an unfavourable soil environment, can penetrate and influence treatment to a depth of 3 m (Weaver, 1954). However, the growth and incorporation of leguminous crops, such as soybean are, potentially, a more useful means of exploiting biological nitrogen fixation as an additional or alternative nitrogen input to the soil.

There are two categories of root systems, namely tap roots and fibrous roots. Tap roots, like those possessed by soybean plants, are characterised by an enlarged central root that penetrates deeply into the soil, with lateral roots branching off the central axis. Fibrous root systems, being finer and more profuse, may offer a superior means of increasing the total rhizoplane surface area per m^3 of soil, when compared to a tap root system. The most intensely characterized fibrous root systems belong to the grass family. Sod-forming grasses, which produce horizontal stems, are known for their excellence in stabilising soil surfaces against erosive forces and are an ideal

vegetative cover for *in situ* treatment of contaminated soil (Shimp *et al.*, 1993).

It was found that, with the exception of the grass in HC4, the high daily temperatures (averaging >36°C) and sporadic spring rains spurred the growth of the grass which grew both rapidly and vigorously with few signs of toxicity or nutrient deficiency (**Plates 9.2** and **9.3**). It appeared that the grass was better suited to the conditions than the soybeans as it was, apparently, unaffected by the very low soil pH which predominated in this area.

Figures 9.1 A-C show the trends of hydrocarbon disappearance from the variously treated soils during the 14 week trial. Certain similarities and differences were evident. In each case, tilling alone increased the disappearance, compared to the untreated soil, but not to the same degree as the complete bio-treatments which consisted of fertilisation, amelioration, tilling and phytoremediation. Persistence of the hydrocarbons increased in relation to their molecular weight. During 14 weeks of bioremediation, the "complete" treatment lowered the hydrocarbon concentrations by an average of 86.8%. At the same time, the untreated contaminated soil experienced 42% removal, of which Phase I (tillage) accounted for a large portion.

Figures 9.2 A-E show the gradual changes which occurred in the hydrocarbon concentrations of HC1. The components changed significantly in response to each phase of treatment. Initially, the C_{10}-C_{20} components disappeared; later the fractions from the entire profile disappeared. The effects of volatilisation, nutrient addition, pine-bark supplementation and, finally, the establishment of grass in the plot clearly played individual contributory roles in the cleanup of the soil. Traces of similar extracts taken from HC2 (data not shown) revealed that without pine-bark, degradation was slightly retarded.

PLATE 9.2 (A)-(H) :

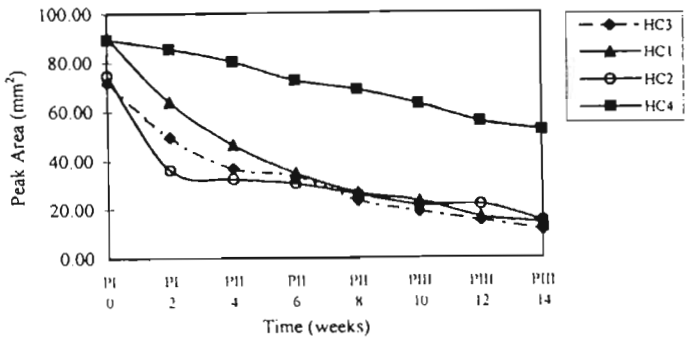
Phase III of the field trial involved the introduction of grass to the plots which had been partially remediated through Phases I-II. Slow-release nutrients were added to the soil during the growing period. (A) Plot HC1 at T_6 of Phase III, and (B) shows the same plot 6 weeks later, at T_{14}. (C) Plot HC2 at T_6 ; and at T_{14} (D). (E) Plot HC3 at T_6, and at T_{14} (F). (G) Plot HC4 at T_6, and at T_{14} (H).


A
B
C
D
E
F
G
H

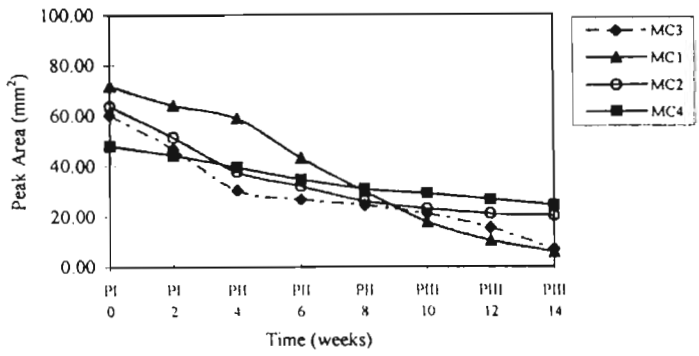
PLATE 9.3 (A)-(D) :

(A) Plot MC1 at T_8 of the trial, Phase III. (B) Shows the same plot 6 weeks later. Similarly, (C) shows plots MC 2 (foreground) and MC 3 (background) at T_8, and (D) shows the same plots 6 weeks later.

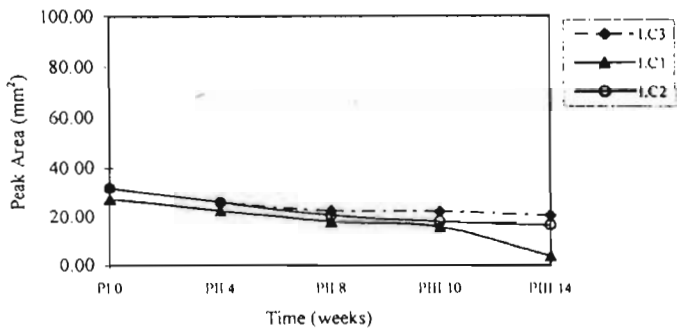

A
C
B
Đ



(A)



(B)



(C)

Figure 9.1: The change in peak area, indicating TPH disappearance, over 14 weeks (Phases I-III) of (A) heavily contaminated; (B) moderately contaminated and (C) low contamination field plots (n=4).

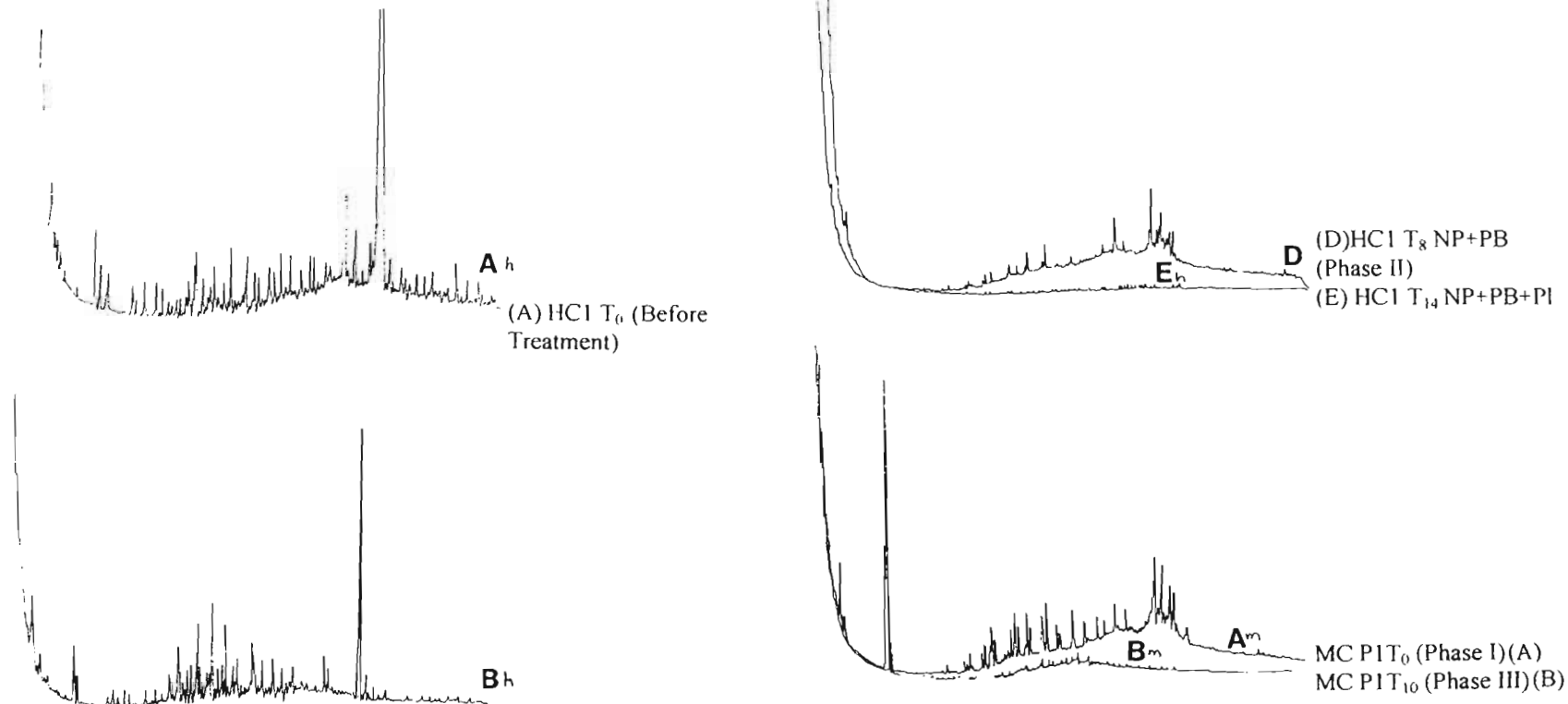


Figure 9.2: GC traces of extracts of moderately$_m$ and heavily$_h$ contaminated soil :

(A_m and A_h) before treatment; (B) after 2 weeks of tilling (Phase I); (C) 2 weeks after supplementation with nutrients N, P and composted pine-bark (T_4, Phase II); and (D) after 4 more weeks (T_8).(E) shows the oil components remaining in the extract after nutrient supplementation, pine-bark addition and grass establishment (end of Phase III, T_{14}) (n=3).

*9.3.2 **Microbial Activity (FDA).***

Microbial activity was not monitored during Phase I and Phase II of the pilot study due to logistical difficulties. However, in Phase III, it was possible to monitor this activity and, thus, formulate a theory to account for the processes which were effecting the changes in the soil with time. This was based on the premise that if the trends in the field reflected those observed in the laboratory, then specific conditions could be confidently and effectively applied in a full-scale bioremediation exercise.

As stated previously, it was of particular importance to measure the activity of the soil microbial community in response to the contamination concentrations and the treatments which the soil received, to determine whether the microorganisms were responding positively, and to identify any causal relationships between hydrocarbon disappearance, microorganism activity and soil pH. When microorganisms metabolise contaminants, they usually propagate. In general, the larger the number of active microorganisms, the more quickly the contaminants will be degraded (Rittman *et al.*, 1994). Thus, results correlating contaminant loss with an increase in the number of contaminant-degrading microorganisms provide one indicator that active bioremediation may be operating in the field.

Figures 9.3 A-C show strong positive changes in FDA hydrolysis activity in response, firstly, to hydrocarbon concentration and, secondly, to the degree of supplementation. The strongest activity responses were evident in the plots subjected to complete bioremediation treatment which were heavily contaminated and, therefore, had considerable labile carbon pools. The lowest activity was observed in contaminated but untreated soil.

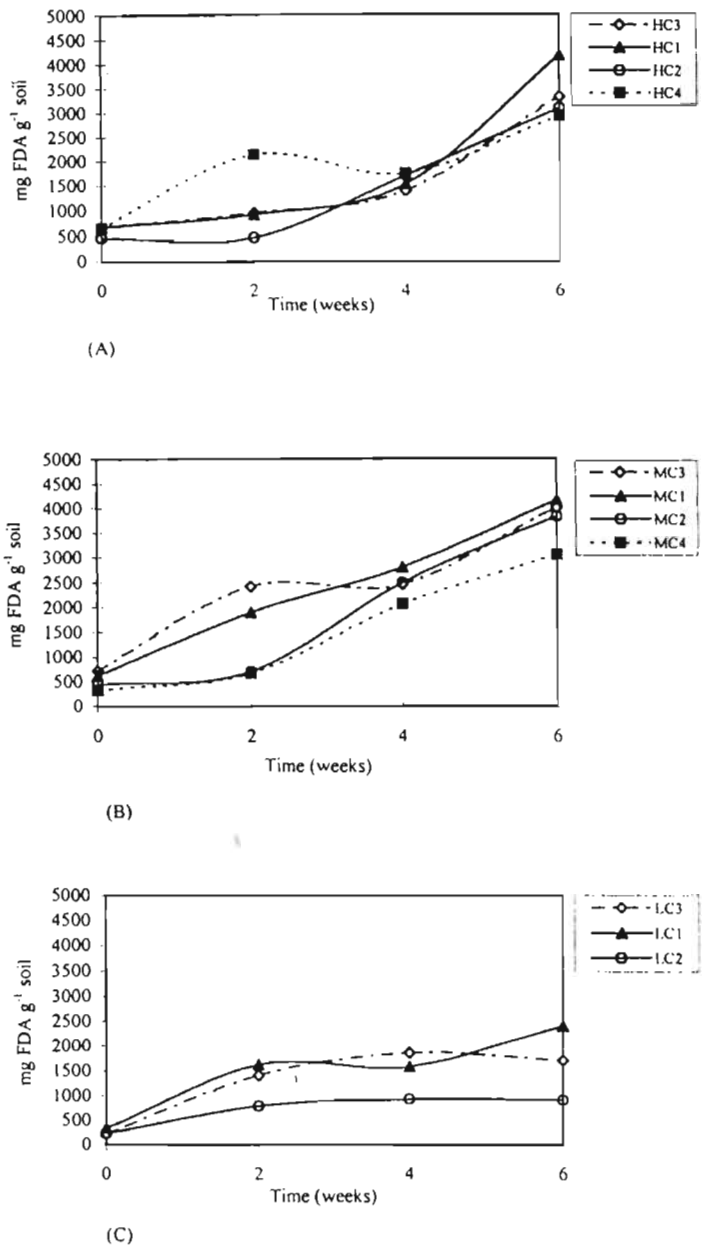


Figure 9.3 (A)-(C): The change in FDA hydrolysis activity that was measured over 6 weeks after the introduction of plants (Phase III) in (A) heavily contaminated; (B) moderately contaminated; and (C) low-contamination field plots (n=4).

Therefore, as expected, microbial activity was inversely correlated with hydrocarbon persistence. This was consistent with the trends recorded in the pot experiments (**Chapter 8**).

In all cases, where water and plants were present, microbial activity increased to rates which were close to those measured in the plots which received nutrient supplementation. The data gathered from the control plots (**Figure 9.3** C) verified the hypothesis that water addition could enhance microbial activity (LC3), while the introduction of oxygen alone, by tillage, was insufficient to encourage growth (LC2).

The trends observed in the plots were so closely correlated with those recorded in the pot trial that it could be postulated that the overall interactions were similar. Clearly, bioremediation would not take place without the introduction of nitrogen or oxygen (pinebark or tilling), which were the critical rate-limiting elements. Thus, aside from these treatments, no significant environmental factors were operative in the field which would preclude the *in situ* bioremediation of the impacted soil.

9.3.3 Soil pH

Soil pH values showed considerable fluctuations during the trial period (**Figures 9.4 A-C**). In the heavily contaminated soil, all the plots exhibited different responses with time. The only similarities were between HC1 and HC3, which were treated identically, where initial soil pH values of approximately 5.5 were measured. Both pH values later decreased (after about 4 weeks) to approximately 4.5. With the introduction of plants, the pH values increased again. HC4 gave the opposite trend where the initial and final soil pH values were both 3.5. A possible explanation could be that this particular plot was frequently flooded due to its location, and had to be drained regularly. This may have caused a build-up of hydrogen sulphide and other anaerobic products which are capable of acidifying the soil. In

contrast, HC2 showed only minor pH fluctuations (between 5.0 and 5.5) over 14 weeks even though it was also supplemented with nutrients. The pine-bark could have played a buffering role in Plots 1 and 3, assuming its higher cation exchange capacity (CEC).

The trends which were observed in the MC plots were different to those of the HC plots. The MC plots which were supplemented with nutrients experienced increases in soil pH with time, while the pH values of the plots which received water only were consistently low. Since the degradation of hydrocarbons results in the formation of organic acids, and the growth of plants is known to acidify soil (Handreck and Black, 1993), then a possible explanation for this could be the action of nitrifying bacteria which responded to the nutrient supplements. The predictable pH decline in response to degradation and fertilisation was demonstrated in LC1 (**Figure 9.4C**), which experienced a shift in pH that differed from the insignificant fluctuations measured in the control plots LC2 and LC3 (**Figure 9.4C**).

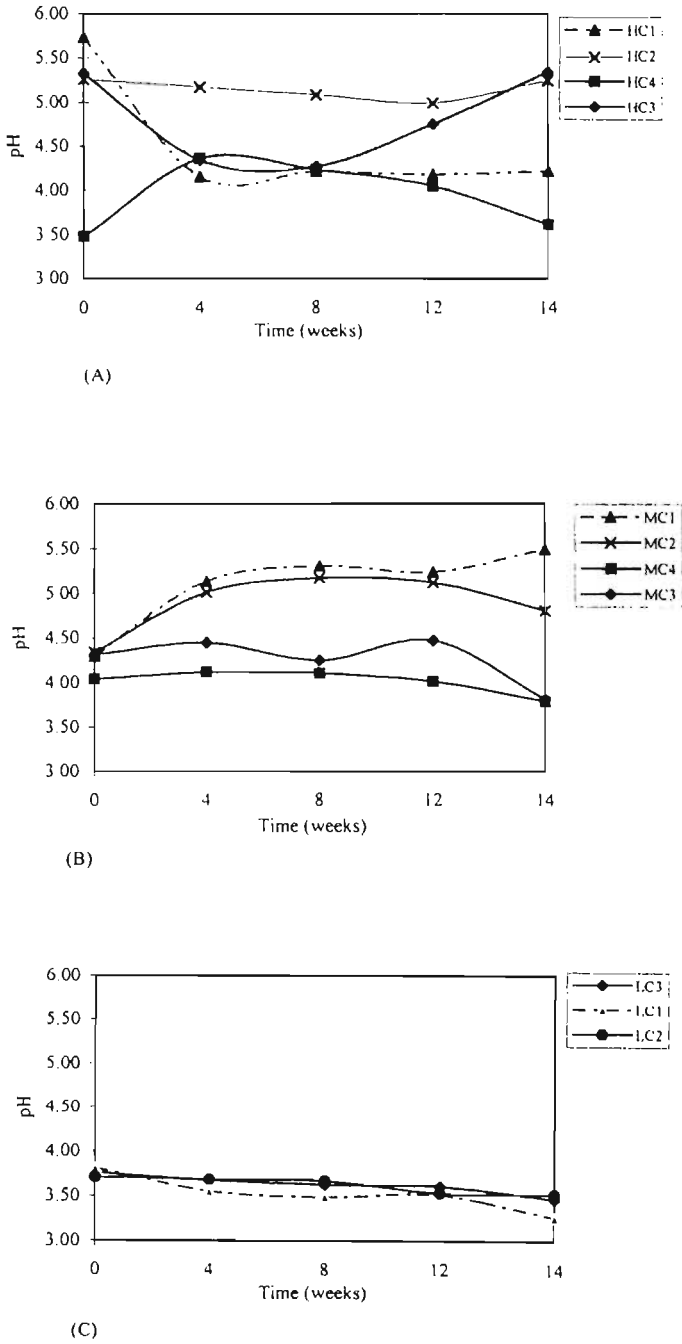


Figure 9.4 (A)-(C): The soil pH changes which occurred over 14 weeks (Phases I-III) in (A) heavily contaminated; (B) moderately contaminated and (C) low contamination field plots (n=4).

9.4 Conclusions

Interesting results were serendipitously discovered during a study of the improved growth of rice and soil N content in soil to which oil residues had been applied (Rasolomanana *et al.*, 1987). Hypothesising that the increased growth was due to the initial 'removal' of the oil residues from the rhizosphere by specific microorganisms, the researchers isolated and studied the dominant diazotrophic bacteria from the rhizosphere of an actively nitrogen-fixing rice plant growing on the polluted soil. Although the rhizosphere did not contain more than 10^5 N-fixing bacteria g^{-1} soil, a strain of *Bacillus polymyxa R3* was found to be dominant. This strain grew and reduced oil residues only in the presence of glucose or exudates from an axenic plant. It was found that the presence of *R3* diminished the inhibition of rice growth, due to the oil compounds, and its nitrogenase activity increased in the presence of these residues. This co-metabolism of oil components in the presence of exudates and their stimulating effect on nitrogen fixation may provide an explanation for the observed positive effects of the disposal of oil residues on arable lands. It could also be hypothesised that rhizosphere co-metabolism greatly enhanced soil organic matter turnover and humification rates.

In this study, grass species, from in and around the most contaminated area of the site, were planted in the field plots and allowed to establish for a period of 6-8 weeks. They were considered to be good candidates to test the hypothesis that the presence of plant root systems may enhance removal of hydrocarbons by stimulating chemical and biological processes because :

a) the grasses used were already established on the site and were clearly adapted to the climatic conditions of the area, and thus required very little attention with regard to irrigation etc.;

b) the fibrous roots of grasses provide the maximum surface area of any vegetation m^{-3} of soil for enhancement of microbial activity;

c) the unmanipulated genetic diversity of these grasses may give these species the necessary genetic advantage enabling them to establishment in an unfavourable soil environment; and
d) the potentially deep roots of these grasses can penetrate and influence treatment to a depth of 3 m below the surface (Weaver, 1954).

Since it is well established that the rates and extents of humification in grassland soils is greater than non-vegetated soils, or even forested soils (Stevenson, 1982), it is possible that oil incorporation into soil humus in a grassland system would continue as the soil organic content increased and the supply of potential humic building blocks increased, during grass root proliferation in an oil-impacted environment. Hansen and Schnitzer (1969) recovered, with chemically aggressive extraction procedures, many large (4-6 ring) PAH compounds from humic acid. Their contention was that significant amounts of compounds occur in the "nuclei" of soil humus material. Root growth not only provides exudates that are readily available for humus synthesis but also root senescence supplies substrates for microbial metabolism which indirectly contribute to humification (Bollag and Loll, 1983).

The results of the field study provided evidence of the accelerated disappearance of hazardous organic compounds in the rhizosphere although the actual cause of the disappearance was not confirmed. The parallel measurements of hydrocarbon residues, microbial activity and pH changes in the contaminated soil facilitated a description of the fates and mechanisms at work in hydrocarbon-compromised soil. Thus, after consideration of the success of phytoremediation and the complex but significant effects of the interactions identified in **Chapter** 8, it can be concluded that it would be extremely desirable if the degradative capacities of plants were increased. The possibilities for exploitation are many and well within the capability of most laboratories. Interest of the author, in particular, focuses on the exploitation of the plant as a "self-sustaining

CHAPTER TEN

CONCLUSIONS AND REMEDIAL RECOMMENDATIONS

10.1 Bioremediation Process Selection

In any case of contaminated soil or groundwater, a logical format can be used to identify the bioremediation technology most appropriate for site-specific conditions. The procedure considers the type of contaminant, subsurface conditions, groundwater conditions, types of development on and adjacent to the site, permit requirements and relative costs. Furthermore, the procedure helps to indicate : (1) additional information that may be necessary for a final decision to be made; and (2) the types of detailed investigations and evaluations that will give other essential information. Very often, the final selection of, and design for, an appropriate bioremediation process will depend on the results of detailed studies.

Figure 10.1 indicates the major steps for determining the most appropriate bioremediation technology for a site. The steps involve an assessment of : the chemical composition of the soil or sludge, whether the constituents are considered hazardous or nonhazardous and physical handling characteristics. Site information will identify the choice of *in situ* or on site treatment strategies and the engineering considerations for such a technology.

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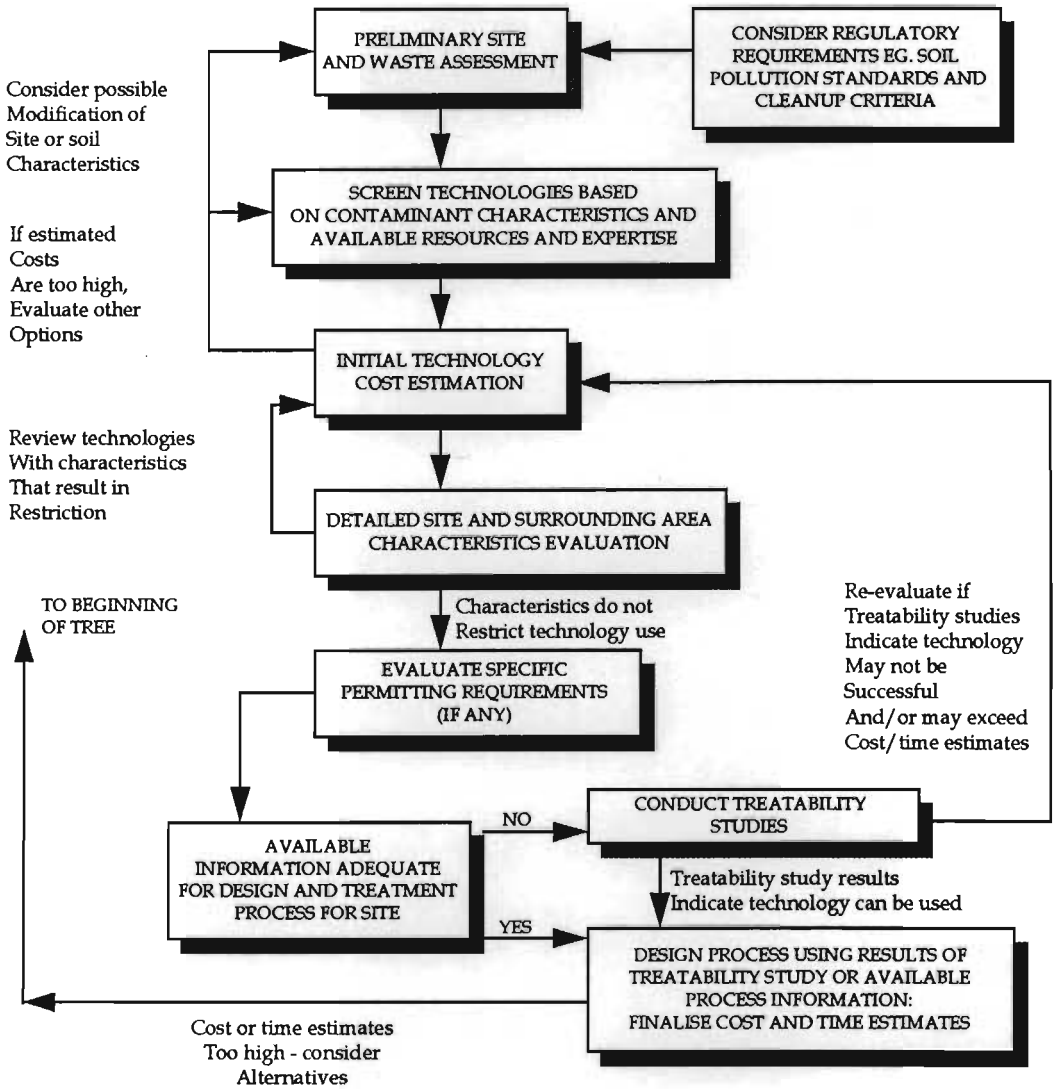

Figure 10.1: The decision-making stages involved in evaluating the best practical clean-up option for a contaminated site.

10.1.1 A Screening Protocol for the Bioremediation of Contaminated Soil

A screening protocol for the assessment and execution of bioremediation involves several steps (Rogers, Tedaldi and Kavanaugh, 1993) :

- Information about the pollutant(s) and the contaminated media must be collected during the site characterization and feasibility study stages (**Chapters 4, 5, 6 and 7**);
- A treatability study must be performed to develop information about the potential effectiveness of bioremediation for specific pollutants and media and to optimize process parameters (**Chapter 8**); and
- Finally, contaminant degradation rates and scale-up parameters should be considered before the full-scale design phase (**Chapter 9**).

The first stage assesses the appropriateness of bioremediation for a particular situation. This involves the evaluation of the contaminant's chemical properties; the chemical properties of the soil at the site; the physical properties of the site; and, the microbiological properties of the soil. Decisions at the end of this phase which should be taken are the choice of bioremediation from current methods, and the use of electron acceptors which will determine the metabolic pathways taken by the microorganisms to degrade the contaminants.

The second stage is the treatability study which identifies the design criteria to be used. Information about the bioremediation kinetics is required (i.e. rates of disappearance of the pollutants) and from this the feasibility of attaining the desired end-point is evaluated. Once this is clear, the operating parameters are determined and the costs for the full-scale implementation are estimated.

The final design phase facilitates the definition of a treatment approach for all the contaminant phases, allows plans and specifications to be proposed and considers all role-players and contractors. At this stage, the results of

the feasibility and treatability studies are used to design structures which will ensure proper and timely delivery of nutrients and electron acceptors at specific loading rates to the contaminated medium.

10.1.2 Choosing a Bioremediation Method

The method of bioremediation to be employed is chosen based on the information gathered during the characterization phase of a project (stage 1). *In situ* treatment offers the benefit of not requiring movement of contaminated soils to establish the appropriate conditions for contaminant degradation. Typically, nutrients, water and oxygen have to be supplied by injection wells to stimulate the indigenous organisms to degrade the waste. *In situ* bioventing may offer a less intrusive, yet effective, approach under certain circumstances. Implementation of *ex situ* bioremediation avoids the difficulties imposed by hydrogeological constraints (Brown, Norris and Brubaker, 1985).

In situ systems are generally applicable where the hydrogeology of the site permits the transport of water, nutrients and/or oxygen through the subsurface and permits the hydraulic containment of the contaminant (Raymond *et al.*, 1989). In general, sites with conductivities $\geq 10^{-4}$ cm s^{-1} and fairly homogeneous stratigraphy are good candidates for *in situ* bioremediation (Thomas and Ward, 1989; USEPA, 1990). If the contamination is shallow and can be excavated easily, or site physical and/or chemical characteristics prohibit *in situ* bioremediation, an *ex situ* system may be preferred (**Table 10.1**).

Table 10.1 : Favourable and unfavourable factors affecting bioremediation (from Bouwer, 1992)

Favourable Chemical and Biological Factors	Unfavourable Chemical and Biological Factors
Small number of organic contaminants	*A complex mixture of compounds, organic and/or inorganic
Non-toxic concentrations	Toxic concentrations
*Diverse microbiological populations	Sparse microbiological activity
*Suitable electron acceptor condition	Absence of appropriate electron acceptors
pH 6-8	*pH extremes
Favourable Hydrogeologic Factors	**Unfavourable Hydrogeologic Factors**
Granular porous media	Fractured rock
*High permeability (>10^{-4} m s^{-1})	Low permeability
Uniform mineralogy	Complex mineralogy or high clay/organic carbon content
Homogeneous media	Heterogeneous media
*Saturated conditions	Unsaturated strata, or intermittently saturated conditions

* Site conditions

10.2 Recommendations for Full-Scale Bioremediation on the Experimental Site

Before applying bioremediation, the source of contamination must be identified and mitigated, major accumulations of free product must be removed, removal of structures impeding remediation must be commissioned and mechanisms for plume containment must be installed.

10.2.1 Initial Remedial Actions

10.2.1.1 Removal of Pollutant Sources and Free Product

The first step in a full-scale remediation exercise is the removal of the contaminant source. In the present case, the greatest source of pollution was the oil-recycling plant itself, which was disassembled and the tanks and yards were hydrocleaned. Some parts were removed and sold.

The next highest contaminant source consisted of the effluent dam and the effluent reservoir, situated in the northwest section of the site. The crumbling (and visibly leaking) brick reservoir may have a large "oil reservoir" underneath it due to its long-term use for effluents and cooling

water for the oil recycling operation. It was not certain how long the reservoir had been leaking but a large amount of oil sludge could have accumulated at the bottom and may be a continuous source of pollution if not removed. The HC4 plot was in the region just below the reservoir, and was very heavily contaminated. On several occasions, tar-like material which appeared to originate from the reservoir base was observed to be oozing out of the soil in that area. The reservoir should, therefore, be drained and dismantled to remove (by excavation) any sludge which may be present and to expose the soil beneath it which could be remediated. Alternatively, the reservoir should be drained and assessed with respect to its pollution potential. If it is not found to be a contamination source, the structure should be repaired and re-lined to prevent any future environmental impact.

Any free product contaminating the watertable (both regional and perched) should be removed by direct pumping using the existing monitoring wells, or by installing injection and extraction wells to effect directional migration of the floating product.

10.2.1.2 Can Any Oily-Phase Residue Support a Plume ?

Monitoring wells can provide misleading information about the course of bioremediation. Pumping, or seasonal changes in regional water tables, can lower groundwater elevations below the depth interval occupied by the oily-phase contaminants. Water produced by monitoring wells may be clean, but contamination will return when recharge raises the regional water table elevation. Changes in the state of rivers nearby could also alter the slope of the water table (hydraulic gradient), which in turn would change the trajectory of the plume of contamination. Although bioremediation can remove the compounds of concern (eg. BTEX) from the subsurface, it may leave behind significant amounts of oily-phase hydrocarbons. Even after

extensive *in situ* bioremediation, pockets of fine-textured material may still contain high concentrations of contaminants.

If the conditions on the site should change by, for example, a pH shift as a result of the tenant's activities, some compounds and metals could become mobilized and be carried into the groundwater particularly during spring/summer. At the present state of science, only long-term monitoring can determine if natural biodegradation will prevent the regeneration of a plume of contaminated groundwater (Wilson, 1993). It is, therefore, recommended that the groundwater is monitored quarterly for a period of 2 years.

10.2.1.3 Plume Containment

In situ bioremediation, typically, requires periods in the order of months to years to reduce contaminants to acceptable concentrations. During that time, the contaminants must not be allowed to spread outside the contaminated zone and thereby escape treatment.

A plume can be contained by physical or hydrodynamic controls or a combination of both. Physical controls include low-permeability vertical walls installed to block the transport of the plume or to inhibit the flow of clean groundwater into the contaminated area. These walls are usually comprised of a mixture of bentonite and soil or bentonite and cement. Synthetic sheet curtains are also used, on a limited basis, for containment. Physical barriers are effective with shallow aquifers underlaid by a solid confining layer of bedrock or clay (LaGrega *et al.*, 1992), conditions applicable to the experimental site.

Hydrodynamic controls are especially useful with *in situ* bioremediation since stimulating supplements may be added with the control water. Hydrodynamic controls consist of combinations of injection and extraction

wells and/or infiltration galleries which manipulate groundwater to prevent plume movement. The wells are sited so that their radii of influence (area of water drawdown or mounding) overlap, thus allowing water control within the entire treatment zone as well as effective manipulation of the level of the water table. The radii of influence are estimated by repeated application of steady pumping rates with drawdown equations appropriate to the specific aquifer conditions, calculated by a qualified hydrogeologist. Plume direction, shape and migration speed can each be manipulated and can effectively regulate the retention time and nutrient delivery within the biostimulation zone (Knox *et al.*, 1986; Barcelona *et al.*, 1990).

In the event of very heavy rains, this option should be investigated. A long-term alternative may be the planting of poplar or eucalyptus trees on the northern boundary of the site to act as a buffer zone and to serve as a dewatering system. Aprill and Sims (1990) found, in column studies, that the cumulative volumes of leachate collected from unvegetated reactors were significantly higher than those collected from vegetated reactors. Loss of water in the form of leachate from the vegetated systems ceased after the plants had established a sufficient root and shoot mass. Transpiration losses via the leaves then became the dominant determinant in affecting the water status of the system, as represented by the decreasing water content of the soil in the presence of vegetation as both the time and biomass increased. Transpiration losses in the field should, therefore, retard the downward migration of water and, thus, the leaching of soluble parent compounds and intermediates.

10.2.2 Viable Bioremediation Options

There is the potential to 'over study' a problem and, similarly, to 'over-engineer' a site. This has occurred in many cases abroad, particularly the United States, where clients are beginning to question the applicability of the current cleanup standards, methods and costs. Billions of dollars have

been spent cleaning sites to the standards and time frames laid down by the federal regulators. As a result, bioremediation has become 'big business' and some of the engineered solutions which can achieve cleanup quickly and uniformly are, in the opinion of the author, often unnecessarily complicated. If time is not a major constraint, the following relatively unsophisticated steps should achieve satisfactory cleanup of the site within a period of approximately 18 months.

Three bioremediation options were considered to be appropriate for the experimental site on the basis of the criteria outlined in **Table 10.1** and the results of the bench and pilot-scale studies. These approaches not only account for the general lack of South African regulatory requirements for cleanup or appropriate experience in bioremediation, but also aim to clean up the site without requiring major engineering accomplishments. The technology is still in its infancy and legislation does not yet compel industry to practice sound cleanup procedures. Thus, the technologies selected are relatively uncomplicated, and as inexpensive as possible. Some options have been investigated while others are based on the experienced gained from field work.

10.2.2.1 Option 1 : *In Situ* Biostimulation

The transfer of laboratory research results to the field is often a frustrating and unsatisfying activity. Part of the problem has to do with the comparable levels of inquiry in the laboratory and in the field. Laboratory studies deal with biochemical and/or physiological processes. Appropriate controls ensure that only one variable is responsible for the results obtained. During field-scale implementation of bioremediation technology, several processes operate concurrently i.e. biodegradation, partitioning, dilution and volatilization of the contaminant. Experimental controls during the full-scale execution of *in situ* bioremediation are often omitted because the technology is applied uniformly to the affected area. As a result, performance

monitoring that targets the contaminant or nutrient chemistry cannot confirm that the biological protocol developed in the laboratory was responsible for pollutant removal at full scale. Scale-up in this case was successful, at least to pilot scale, and can be practiced with confidence.

Whenever bioremediation is applied to a specific site, the question arises as to whether the remediation specialist will employ species of microorganisms which are inoculated into the soil and/or water or whether indigenous organisms will be the species of choice. These two approaches play key roles in the success or failure of a project. Both have merits but the indigenous microorganisms generally are advantageous for *in situ* soil and groundwater treatment. Introduced species, however, may outperform the indigenous microorganisms in a bioreactor and in some land applications. Considerable time can be saved in the project by making a comprehensive site evaluation including a thorough bioassessment or treatability investigation. In this study, a diverse population of indigenous microorganisms were shown to be capable of degrading the organic components of oil under enhanced 'natural' conditions, thus obviating the need for an exogenous culture(s) (**Sections 5, 6, 7, 8** and **9**).

Although many feel that it is time that genetic engineering and selection of specific catabolic species 'come of age', effective use *in situ* has still to be proved. As the field experience correlated with carefully controlled laboratory work, it was apparent that the indigenous populations were already acclimated to the existing environment and have adapted to utilize the pollutants as C and energy sources. Thus, it is probable that they would have the competitive edge over introduced species. Inoculated species have been reported to rapidly decrease in number and are often undetected within a few days. A number of factors for this have been implicated :

(a) the contaminant concentrations may be different from the concentrations to which the inocula have been adapted to in the laboratory;

(b) the added microorganisms are possibly removed by predation; and

(c) naturally-occurring compounds may be present which inhibit the growth of the inocula (Goldstein *et al.*, 1985).

For 'engineered' bioremediation, once the microorganisms have been identified, the critical success determinant is how well the subsurface materials can transmit fluids. As mentioned, for systems which circulate groundwater, the hydraulic conductivity in the contaminant area should be of the order of $\geq 10^{-4}$ cm s^{-1}. For systems which circulate air, the intrinsic permeability should be $>10^{-6}$ cm^2. (For both types of systems, the contaminated area will be much more difficult to treat if it has crevices, fractures or other irregularities which facilitate fluid channeling around the contaminated material).

Studies of the geological cross section of the site revealed a complex patchwork of layers, lenses and fingers of different materials. Indeed, two overriding characteristics of the subsurface are that it is intricately heterogeneous and difficult to govern the flow of water; and chemical transport is so complex that it is impossible to predict these properties quantitatively or even to interpolate them with accuracy from sparse observations without complicated computer modelling software, which was not available. Nevertheless, conditions were found to be close to ideal on the experimental site; the permeability of the soil (**Table 4.1**) in the contaminated areas was well within the accepted limits for *in situ* suitability and it was, therefore, not considered to be a limiting factor.

Nutrients would be easily delivered by controlling groundwater flow by injection wells or infiltration galleries coupled with downstream production wells (**Figure 1.4**). In the most common configuration, groundwater is withdrawn from extraction wells downgradient from the biostimulation zone and supplemented with the nutrients required for optimum microbial activity, treated if necessary, to remove free product and reintroduced to the soil using infiltration galleries or injection wells. Water from an external

source is used if the flow of withdrawn water is insufficient to control the subsurface flow or if it is impractical to reinject the groundwater. The rate of nutrient delivery to the bioremediation zone would, therefore, be more frequently limited by the solubility of the nutrients in water and the reinjection flow rate than the heterogeneity of the geology.

Often, when the limiting nutrients are added to the soil in liquid form, excessive microbial growth may occur around the injection zone, causing plugging of the permeable medium and restricting aqueous flow. Without the occasional addition of high concentrations of hydrogen peroxide, which clears the blockages rather successfully, plugging may cause bioremediation rates to slow. An alternative answer to this problem would be to use frequent, low additions of the proposed slow-release nutrients [**Section 8.6.2.7**] in the infiltration gallery. Only water would be added and circulated through the gallery without on-line addition of soluble nutrients. Nitrogen and phosphorus would be released as needed. This would minimize bacterial "blooms", decrease the amount of nitrate released into the water and, thus, economize on fertilizer by better utilization and distribution of nutrients. The results of the research conducted in this study indicated that this approach should be very effective, since over-fertilization can easily occur, particularly if it is assumed that 100% of the carbon in the contaminant will be catabolized.

Because the soil and its subsurface are complex and incompletely accessible, knowledge of the fates of contaminants will always be limited. The situation is compounded in *in situ* remediation technologies since the amount, location and type of contamination frequently are unknown. Without knowing the starting point for remediation, defining the finishing point is difficult. Errors in measurements, artifacts imposed by extrapolating laboratory results to the field and an inherent shortage of data further complicate the evaluation and create uncertainty about the success of a remediation process. However, based on the results obtained in this study,

with summer soil temperatures and with complete bioremediation treatment (tilling/amelioration with pine-bark or a similar bulking agent, nutrients and legume cultivation on the surface, and injection wells for remediating the subsurface), reducing the oil in soil from 80g C kg^{-1} soil to <10 g C kg^{-1} soil and subsequent revegetation of the site could be attempted in approximately 6 months. Further reductions to ~1g C kg^{-1} (1000 ppm) soil could probably be achieved in a year to 18 months.

10.2.2.2 Option 2 : Solid Phase Land Treatment, Including Fungal Remediation

Both surface soil and on-site land treatment processes are among the more widely used bioremediation technologies. Surface soil land treatment involves maintaining the contaminated surface soils intact with, if needed, the addition of nutrients to promote biodegradation and adjustment of the pH toward neutrality; tilling the soil periodically to increase the availability of oxygen and nutrients to the soil microorganisms and, possibly, irrigating to assure adequate moisture for microbial degradation.

Surface soil land treatments do not have a liner, unlike on-site land treatment units. The latter is a constructed unit which contains the contaminated soil being remediated and has walls, a drainage system and a leachate collection system (**Figure 1.3**). This type of treatment facility is usually constructed adjacent to the site requiring bioremediation to minimize transportation costs and to provide better technical and managerial control of the process. Both surface soil and on-site land treatment systems have been used successfully to remediate spill sites, industrial wastes and residues, and soils at surface impoundments (B. R. McGugan, personal communication).

Composting (soil piles) as a bioremediation option for contaminated soils and other wastes/residues is similar to the conventional process used for

composting of leaves and refuse (**Section 1.3.2**). The main differences are that high temperatures are rarely achieved in contaminated soil bioremediation composting and the purpose is degradation and loss of target organic compounds rather than organic matter stabilization to produce a soil conditioner.

Typical composting systems are the Windrow or the 'Beltsville' system. The Windrow system is an open system which incorporates periodic turning of the compost pile in the absence of forced air. The 'Beltsville' system is an open pile equipped with an air distribution system under the pile. Air is sucked through the pile from the atmosphere and exhausted through a blower. Bulking agents are commonly added to increase the porosity and assist the flow of air to maintain aerobiosis. Because of the mixing and aeration in these systems, volatilization is a significant factor in the removal of organics. Thus, monitoring and management of the gaseous emissions would be necessary at locations where air quality concerns exist.

An investigation of the feasibility of composting processes for the remediation of the contaminated soil investigated in this case study is in progress (B. R. McGugan, University of Natal, Pietermarizburg). An exhaustive review is, therefore, not appropriate here. However, this approach was identified by the author as another possible solution for the remediation of the contaminated site. In this study, other waste products which could be utilized as ameliorants or alternate nutrient sources to encourage co-metabolism are also being assessed for their effectiveness in the remediation process.

Land adjacent to the experimental site is available for composting. It is predicted that the same degree of success would be achieved under those conditions investigated in the pot trial, using pine-bark as an ameliorant and a Windrow system design.

10.2.2.3 Option 3 : Encouraging Wetland Development or a Buffer Zone for the Intrinsic Bioremediation of Contaminated Soil

The following is not a method which has a predicatble outcome, but is rather presented as an idea which, conceptually, has great value. It relies on intrinsic bioremediation, which implies that although it is the most inexpensive and uncomplicated option, clean-up of the site would not necessarily occur. The suggestion is to encourage the development of a natural wetland, or to develop a buffer zone of trees along the northern border of the site.

A wetland is an "ecotone" (Hammer and Bastian, 1990) - an "edge" habitat, a transition zone between dry land and water, an environment that is neither clearly terrestrial nor aquatic. There is no single definition for wetlands but they are defined by the U.S. Fish and Wildlife Service (1979) as a "transition between terrestrial and aquatic systems, where water is the dominant factor determining development of soils and associated biological communities and where, at least periodically, the water table is at or near the surface, or the land is covered by shallow water." Specifically, it requires that wetlands meet one of the following conditions :

- areas supporting predominantly hydrophytes (at least periodically);
- areas with predominantly undrained hydric soil (wet enough for long enough to produce anaerobic conditions that limit the types of plants that can grow there).

Site selection for a wetland is based on geological, geotechnical, hydrogeological, and other environmental information. Ideally, the sites should be flat to gently sloping. Wetland complexes naturally occur at topographic lows which receive runoff waters from various sources and have adapted to the substances carried by runoff, using them to help support some of the highest recorded productivity rates. The northern portion of the experimental site was frequently wet, due to the water table

being very close to the surface. In one area adjacent to the effluent dam, a small wetland is already developing naturally. Since the effluent dam forms a hydraulic continuum with the perched water table, it is envisaged that if the containment berms of the dam were excavated to become gently sloping, a wetland would probably develop along the entire northern boundary.

Wetlands can provide effective, natural attenuation for many types of pollution. They can effectively remove or convert large quantities of point source and non-point source pollutants including organic matter, suspended solids, metals and excess nutrients (Nichols, 1983). Natural filtration, sedimentation and other processes help clear the water and sediment of many pollutants, whilst some of the contaminants are immobilized chemically and will remain unless disturbed (Hammer *et al.*, 1990). Chemical reactions and biological decomposition processes break down complex compounds into simpler substances which are removed for biomass production by wetland plants through adsorption and assimilation (**Section 8.2.3**).

Another advantage of this system is the ability of wetland plants to provide oxygen to waterlogged roots (Karwase, 1981). It has been shown that bog plants are able to transport oxygen to anaerobic soil (Armstrong, 1964). Flood-tolerant and wetland plants, therefore, have adaptations which enable them to maintain an aerobic environment in the rhizosphere and simultaneously detoxify the soil. Also, their normal metabolism allows them to control or tolerate anaerobic metabolism. Even when grown in unsaturated soil, some wetland plants operate under anaerobic conditions and have higher concentrations of anaerobic by-products than non-wetland plants (Shimp *et al.*, 1993). This abundant by-product of the plant growth process which increases the dissolved oxygen content of the water and also of the soil in the immediate vicinity of plant roots (**Section 8.2.9**) would increase the capacity of the system for aerobic bacterial decomposition of the pollutants as well as its capacity to support a wide range of oxygen-utilizing

aquatic organisms, some of which, directly or indirectly, may utilize additional pollutants. In areas with a high or fluctuating water table (as in this study), such processes will be important for successful bioremediation.

Wetland plants have two important functions with respect to polluted environments. Firstly, within the water column, stems and/or leaves significantly increase the surface area for the attachment of microbial populations; and secondly, wetland plants have the ability to transport atmospheric gases (including oxygen) down into the roots to enable their roots to survive in an anaerobic environment. Some incidental leakage occurs, producing a thin, aerobic region (the rhizosphere) surrounding each root hair. Some chemical oxidation undoubtedly occurs in this microscopic region but, more specifically, the rhizosphere supports large microbiological populations which effect beneficial transformations of nutrients, metallic ions and other compounds (**Sections 8.2.3 - 8.2.8**). The juxtaposition (on a microscopic scale) of an aerobic region, surrounded by an anaerobic region, multiplied by the almost infinite area of rhizosphere boundary is crucial to nitrification-denitrification and numerous other significant pollutant transformations (Brix and Schierup, 1990). In addition, wetlands support microbiological populations that catabolize pollutants and have short generation times, high division rates and considerable genetic plasticity, all of which permit these organisms to rapidly adapt to and exploit new nutrient and/or energy sources (Brix and Schierup, 1990) and which would contribute towards a successful cleanup programme.

Useful emergent wetland species include many members of the cattail, reed, rush, sedge and grass families which are common to South Africa. *Phalaris* spp. (e.g canary grass), *Spartina* spp., *Carex* spp. (sedges) and *Juncus* spp. (rushes) all have potentially high uptake and production rates. They are widespread, able to tolerate a range of environmental conditions and can alter their environment in ways suitable for waste treatment. Rhizosphere processes are largely unknown (Guntenspergen *et al.*, 1990) but presumably

have positive impacts and warrant further investigation. Species of *Typha* (cattails), *Azolla* and *Glyceria* (manna grasses) support nitrogen-fixing microorganisms in their rhizospheres (Bristow, 1975) and some wetland species are also associated with mycorrhizal species (Sondergaard and Laegaard, 1977). Other, more aesthetic, plants such as the calla lily (*Zantadeschia aethiopica*), canna lily (*Canna flacida*), elephant ears (*Colocasia esculenta*), yellow iris (*Iris pseudacorus*) and ginger lily (*Hedychium coronarium*) are also flood-tolerant and could render treatment areas more attractive.

Useful emergent wetland species for this purpose include many members of the cattail, reed, rush, sedge and grass families which are common to South Africa. *Phalaris* spp. (e.g canary grass), *Spartina* spp., *Carex* spp. (sedges) and *Juncus* spp. (rushes) all have potentially high uptake and production rates. They are widespread, able to tolerate a range of environmental conditions and can alter their environment in ways suitable for waste treatment. Rhizosphere processes are largely unknown (Guntenspergen *et al.*, 1990) but presumably have positive impacts and warrant further investigation. Species of *Typha* (cattails), *Azolla* and *Glyceria* (manna grasses) support nitrogen-fixing microorganisms in their rhizospheres (Bristow, 1975) and some wetland species are also associated with mycorrhizal species (Sondergaard and Laegaard, 1977). Other, more aesthetic, plants such as the calla lily (*Zantadeschia aethiopica*), canna lily (*Canna flacida*), elephant ears (*Colocasia esculenta*), yellow iris (*Iris pseudacorus*) and ginger lily (*Hedychium coronarium*) are indigens, flood-tolerant and could render treatment areas more attractive.

Trees can also increase degradation by two other mechanisms of soil oxygenation. If sufficient numbers of trees are planted as a buffer zone, the groundwater level may be lowered, which increases the volume of unsaturated soil and the oxygen supply by the soil pathway. As a result, trees can increase microbial activity. Many trees in a closed canopy stand

will transpire more than a precipitation equivalent of 1 m of water per year, if it is available. Their effect on the water table may, thus, be considerable in sub-humid climates where there is a considerable deficit of precipitation over transpiration (Shimp *et al.*, 1993). The capacity of plants to transpire large quantities of water is significant and has been exploited in the dewatering of sludge (Cunningham and Berti, 1993), prevention of downward water flux through landfill caps and containment of contaminated water down-stream of a problem site. Some sites, although polluted, do not pose an obvious environmental risk unless there is off-site migration into a waterway. Pollutants slowly leaching from these soils into a shallow aquifer and then into a small stream have been targeted by this root-intercept strategy (Cunningham and Berti, 1993b). Thus, as an alternative to encouraging a natural wetland, it may be worthwhile to plant a zone of trees along the nothern boundary to both lower the water table and enhance the attenuation of pollutants in the plume.

The current use of adapted trees for degrading contaminants identifies their potential to remediate contaminated soil in a more passive manner than the usual 'pump-and-treat' technology or on-site schemes (Tripathi and Shukla, 1991; Green and Upton, 1992). While most studies to date have examined herbaceous species such as reeds (*Phragmites* spp.), rushes (*Scirpus* spp.) and cattails (*Typha latifolia* L.) (Reddy and Smith, 1987; Hammer, 1989; Cooper *et al.*, 1990), there are some reports of forested swamps being used for nutrient removal of municipal wastewater discharges (Reddy and DeBusk, 1987; Richardson and Davis, 1987)

The greatest problem in South Africa is the lack of detailed information from long-term experience of these systems. Although research has shown that wetlands, in particular, can provide effective treatment (C. Breen, personal communication), this option generally remains unknown outside the scientific community. The contaminant degradation mechanisms in wetlands are similar, if not identical to, the microbial transformations which

occur in treatment plants, lagoons or other conventional biological treatment systems. The latter often require large inputs of energy, detailed operating procedures and subsequent high costs to maintain optimal environmental conditions for suitable microbial populations within a relatively small area. The low capital and operating costs, effectiveness, and self-maintaining attributes of wetland treatment or buffer zone development result from the complex of plants, water and microbial populations in an area large enough to be self-sustaining without significant energy or other maintenance inputs.

Regardless of application, wetlands have the potential to improve any landscape. Unless heavily polluted, water is one of the most compelling of all design elements. For both practical and aesthetic reasons, wildlife (especially birds) and people are enticed to water. Therefore, whether properly encouraged in the right environment, or designed, installed and managed, wetlands can introduce an important functional and aesthetic element to the landscape.

10.2.2.4 How 'Clean' is Clean ?

Societal and political demands have demanded a clean environment in harmony with economic prosperity, and remediation specialists have risen to the challenge abroad by accelerating technology transfer and development with a significant number of successes at field scale. Unfortunately failures have been recorded. Responding to the environmental challenge of contaminated soil first requires an answer to the question "how clean is clean?". Thus, a central issue when planning any remedial action is to select the target criteria. General concentration limits for a given site to protect human health and the environment have not been set in South Africa, with the exception of water (Department of Water Affairs and Forestry, 1995). To date, these have been determined on a case-by-case basis. Unfortunately, there is very little toxicological information to

underpin these decisions and to assess the hazards. There is, in fact, very little local information available on soil pollution or cleanup.

While there is no debate about the need for improving the environment, the discussion is, however, whether South Africa should adopt the standards and criteria of, for example, the USA or the Netherlands. The major component of the cleanup costs in these countries is related to the requirement that sites must be cleaned to concentrations such that any future use is not precluded; this is frequently taken to be "background" or "pristine" conditions (Linz, 1994). Recently, changes in endpoints have been suggested that lessen this requirement but still safeguard human health and the environment. These new requirements may appear reasonable but the proposed approach still does not recognize scientific reality, namely that many chemicals in soil are immobile or may not pose adverse environmental or health impacts even though they are present in concentrations that exceed the specified (ideal) targets.

Scientific reality is particularly important when it concerns the selection of a remediation technology for a site cleanup. For example, there is growing evidence that bioremediation not only reduces the total concentrations of organic components in soil but simultaneously reduces the biotoxicity of the contaminated soil (Linz, 1994). It would, therefore, be appropriate for the existing data from treatability, field and other studies to be evaluated to determine the extent to which remediation not only removes chemicals, but also reduces the mobilities and toxicities of the residual chemicals. This may reveal whether remediation methods protect the environment and human health even when background concentrations are not exceeded. To answer the question of "how clean is clean?", the scientific approach must be based, not on the analytical detection limits of the pollutants, but on a combination of the results of toxicological and biological tests that can directly assess the concentrations of chemicals in soil or water which do not adversely affect human health and the environment.

The standards and guidelines that currently exist internationally may, in terms of the above arguement, be inappropriate. Site cleanliness should rather, in the opinion of the author, be determined on a site-specific basis by a 'relative-risk' evaluation (Bachmann, 1991). This would involve identification of the chemicals at the site, the potential pathways of concern, the points of exposure, the types and amounts of exposure, and the relative risk that does or may exist. To determine these site-specific risks, sound scientific methods are required.

South Africa, whilst in a relatively weak position internationally with regard to soil pollution standards/guidelines and cleanup legislation, is perhaps in a strong position to seize the challenge and act immediately. Scientists also have the benefit of hindsight without the hindrance of existing legislation.

10.3 The Relationship between Experimental Design/ Methodology and Full-scale Bioremediation

Bioremediation, even of recalcitrant compounds, has been identified as a legitimate option in this case, but there are numerous pitfalls in evaluating its efficacy for any other soil, sediment, groundwater or industrial wastestream. Use of control plots or control reactors in the greenhouse and laboratory can, as illustrated, convincingly demonstrate the removal of chemicals. Extrapolating the results from laboratory or pilot-scale experiments to give valid predictions of field-scale behaviour is essential, yet reliable methods to do so have remained elusive.

There are several principles which must be considered before attempting to predict the outcome of a full-scale remediation scheme.

10.3.1 The Multi-Disciplinary Approach

Bioremediation knowledge spans classical disciplines and requires inter-disciplinary cooperation. As a result, the fields of chemistry, microbiology, ecology and all of the engineering disciplines have evolved through the interactions so essential in bioremediation. The concepts used by these scientists have been so successfully applied in manufacturing that application of these same approaches to environmental remediation systems is entirely logical. However, the complexities of many remedial systems, together with the adaptive nature of biological systems, pose new challenges that extend beyond those faced before by traditional South African scientists.

The difficulty of linking biological behaviour with analytical parameters and full-scale predictability has always been a problem area. As a result, classical scientific philosophy has embraced the concept of the "controlled experiment" in ever-increasing levels of reductionism. This trend has led to great insights at the mechanistic level but at the price of uncoupling the experiment and its activity from its *overall environment* (Needham, 1935). Therefore, the goal of bioremediation experimental linkage requires that this mechanistic knowledge must be ordered into a logical network of relationships, which describe the interactions between the basic mechanisms. These relationships come from comparing the observed phenomena in various environmental experiments, while formulating and testing hypotheses to discover the nature of these interactions.

If the goal was to relate the biological responses observed in different experiments to discernible quantities, then there are two basic philosophies that should be considered :

- Create experiments that are geometrically, mechanically, thermally, chemically and biologically similar and where differences in the responses can be directly attributed to comparative effects; or

- Work with systems that are dissimilar in some way but where the dissimilarities can be described by predictive models.

The question then arises - can biological activity be compared between systems if sufficient knowledge of the physical, mechanical and thermal processes are known? The notion that only complex microcosms are satisfactory for biological similarity and linkage implies that linkage-based studies necessarily lead to very complicated, expensive and time-consuming efforts or, alternatively, the conclusion that only observation in the field is useful (i.e. no microcosm, no matter how complicated, can ever mimic or be used to predict the *in situ* condition). The implications of each of these suppositions have significant impacts on the economics and response times of using bioremediation, and the competition of this technology with alternatives (Litchfield, 1991; Woodyard, 1991; Rao *et al.*, 1992).

10.3.2 Physical Structures and their Experimental Influence

That contaminated environments are physically diverse is obvious. Compartmentalized models to anticipate the phases in which a contaminant may reside are important in the development of predictive models which incorporate mass-transfer and reaction mechanisms. Bulk as well as specific components of oil partition between air, water, sediment/soil and related biotic compartments as time progresses, following the introduction of oil to the environment. The physical properties of oil (**Section 2.4**) determine the equilibrium concentrations of the oil in these compartments.

Unfortunately, the limitations of chemical and biological analytical methods have generally prevented the calculation of mineralisation/transformation rates in multi-compartment, mixed culture microcosms. Thus, experiments to simulate the environment are often controlled to reduce or eliminate variations in conditions which can have major effects upon biological kinetics (Blackburn, 1989; DiGrazia, 1991). Experimental strategies which include disturbances and perturbations may increase variability and make

statistical comparisons more difficult, but they offer a more realistic linkage to field-scale behaviour.

From the experience obtained in this case study, it was apparent that to make a realistic prediction of the effects of a treatment or a specific technological approach on a large scale remediation effort, one requires relatively complex experimentation due to the potential number of interacting factors. Therefore, unless the physical conditions of the site are consistent or homogeneous, it would be risky to over-simplify the experimental approach. However, this does not imply technological intricacy nor major expense.

10.3.3 Chemical Complexity

Petroleum oils consist, generally, of three primary distillate fractions which are obtained from crude oil. Each of these fractions (paraffins, naphthenes, aromatics and polars) consist, in turn, of a number of compound types. Frequently the contaminated site, as in this case study, has been exposed to a wide number of petroleum products over a number of years. This leads to complex residual concentrations in the soil. The oil, therefore, has an opportunity to become weathered and transformed by a variety of physical, chemical and biological processes over time.

When oil is spilled onto soil, evaporation and permeation tend to deplete the lower boiling point molecules, leaving viscous films that are subject to continuous physical stress and chemical attack. While photo-oxidation at the soil surface may convert the 2-4 ring aromatics, biodegradation in the subsurface may convert the saturates and aromatics, leaving higher molecular weight aromatics and polars relatively unchanged. Oil spilled at a site thus progressively increases in aromaticity and polarity approaching a more 'asphalt-like' composition as time proceeds. The depth of *analytical*

characterization of the oil present at a site is, therefore, critical to understanding its response to treatment.

10.3.4 Biological Factors and Predictability

10.3.4.1 Detection of Biodegradation

The overall objective of bioremediation is to remove the hydrocarbons from the contaminated medium and restore it to a prior state, thereby reducing the risk of adverse human health or ecological effects. High intrinsic variability in the oil may require a large (quantifiable) decrease before a reduction can be detected with statistical confidence. Such changes may be slow, so there could be value in employing indirect measures of success.

Since the basic rationale for bioremediation is that for microbial degradation to be stimulated the right environmental conditions or the right organisms must be provided, an early detectable measure of success is likely to be an increase in microbial metabolism or an increase in microbial numbers (Brown and Braddock, 1990; Brown *et al.*, 1991; Lindstrom *et al.*, 1991). Unfortunately, most methods necessitate being carried out on a bench and so the rates obtained in such experiments could be difficult to extrapolate to field-scale. Nevertheless, activity comparisons of bioremediation treatments and controls should provide indications of the success or failure of a specific strategy. Similarly, enumerations might be used to compare bioremediated and unsupplemented plots in relation to general environmental factors, such as the weather.

Studies at the bench, of samples with a well-characterized history collected from the field, offer another approach for the assessment of biodegradative responses. For such an approach to work, the source of the oil must be known, and some of the initial material must be available for analysis. Also, for the approach to be quantitative, relatively inert, conserved internal

markers must be identified; hopanes seem to serve this role in crude oils Butler *et al.*, 1991) and phenanthrenes fulfil this role in diesel fuels (Douglas, 1992). This approach cannot delineate into, for example, primary metabolism and cometabolism, the processes which contribute to the disappearance of hydrocarbons but it does provide a way of assessing the ultimate limits of biodegradation in a particular environment. Unfortunately, it can only be used if a contaminant is relatively uniform in chemical composition throughout the contaminated area as may occur in a spill from a single source. For many sites, and this case study is one, this may not be a realistic assumption.

10.3.4.2 Species Diversity and Activity

There are thousands of isolates recorded which are capable of growth on hydrocarbons (Atlas, 1981; Austin *et al.*, 1979; Bartha and Atlas; 1977; Focht *et al.*, 1990; Leahy and Colewell, 1990). Such oil-degraders have been isolated from many environments and thus may be ubiquitous (Aeckersberg *et al.*, 1991). Several generalisations can be made :

- For the relatively simple hydrocarbons, the insertion of oxygen into the molecule seems to be the rate-limiting step for biodegradation;
- the inserted oxygen is (preferentially) molecular oxygen, although there is no doubt that anaerobic biodegradation can and does occur (Aeckersberg *et al.*, 1991);
- there is evidence that some organisms which can degrade aliphatics do not seem able to degrade aromatics, and *vice versa* (Focht *et al.*, 1990); and
- biodegradation of alkanes, alkenes and simple aromatics (up to at least 4 rings) can be complete i.e. mineralisation results in water, microbial biomass and carbon dioxide (Walter *et al.*, 1991).

Analysis during an experiment may be able to show the above trends and so reveal the mechanisms at work (and hence the "stages") during a bioremediation exercise.

10.3.4.3 Biodegradability of More Resistant Compounds

An evaluation of the biodegradation of semi-recalcitrant compounds can be approached in a number of ways. One approach is to analyse the molecular composition of petroleum products and address the specific degradation of individual components, usually by monocultures. Another approach is to attempt to classify the molecules in the petroleum into broad groups, and to study the degradation of each by microbial associations. Such approaches should provide important insights into the specific metabolism of well-characterized microorganisms or they could shed light on the phenomenon of co-metabolism.

An alternative approach is to study the biodegradation of complex mixtures of hydrocarbons by correspondingly complex mixtures of organisms. This is undoubtedly the approach which would be recommended in cases where precise metabolic information is not an essential part of the solution, and is the approach which was adopted throughout this study. This work focussed on whether or not hydrocarbons were being biodegraded, and their rates of disappearance rather than what microorganisms were involved. Such an approach is relatively new for microbiologists, perhaps because of the belief that such work is not reproducible. The current recognition, however, of the importance of *in situ* processes has demanded a re-evaluation of such views and the movement towards the applied approach and away from the fundamentalist approach has been directly proportional to the nature of each project.

10.3.4.4 The Effect of Microbial Ecology

Microbial ecology addresses the development of methods and techniques to characterize the microbial populations and their interactions in active environmental samples.

One important question in making bench tests with the aim of later linkage to field treatments is whether the diversities of the microbial communities in samples used in replicate tests are representative of the overall microbial diversity of the impacted area. This is a very difficult issue to resolve as each site, sample, problem and condition is unique. The laboratory culturing of microorganisms may result in a very low percentage of the total microbial community, as in general, a very large proportion of the microorganisms in a sample are not yet culturable. However, experiments with only a few grams of soil or sediments should provide an adequate basis for determining the range of compounds that can be degraded at a given site by the indigenous populations (**Chapter 5**).

An obvious assumption can be made in the fact that significant differences occur within and between sites, especially between pristine and impacted sites. Adaptation of the microbial population is a critical factor in determining the ability of the microorganisms to degrade the contaminants. Often, weeks or even years are necessary before the indigenous populations are able to catabolise the molecules in a complex mixture. The range of compounds that can be immediately catabolised cannot, therefore, be accurately predicted *except* by using samples from the target site. One might, thus, expect a direct relationship between the growth rates of microorganisms and the rates of hydrocarbon degradation, which is, in fact, the case (**Chapter 6**).

10.3.4.5 The Impact of Analytical Methodology

Because of the chemical complexity of oil, selection of the type of analytical method used has a major impact upon the quantitative results of bioremediation experiments ("a result is only as good as the method used"). Apart from the costs of certain procedures and the consequential financial constraints which they could place on the project, results from the same type of detector (e.g. in a GC or HPLC) of soil extracts in different solvents or the

use of different fractionation methods will give different results and, thus, make comparison of experimental data difficult. Identification of appropriate methods can frequently take up a large portion of a project. For example, interferences with heavy metals or salts can render a seemingly universal method invalid.

Selection of suitable analytical techniques for monitoring hydrocarbon bioremediation is therefore vital, for several reasons. Non-specific analysis of the 'bulk' oil phase is of interest for identification of the location and extent of contamination (usually at the site investigation stage). Conversely, more specific compound information may be necessary because it can be used for risk analysis, kinetic modelling and for determining structure-function relationships which are important in developing experimental protocols. The choice of every analytical method is thus based on a balance between the desired information and the cost required to acquire it. At one extreme, visible indicators of contamination such as oil sheens on water or black oily soils have been used. At the other extreme, the elucidation of a biochemical pathway may require measurement of the concentration of a particular compound at the part per billion level in a multi-component mixture. Whilst the former requires, at most, an observational rating system, the latter may require weeks of sample preparation combined with sophisticated measurement and data interpretetion. Even more "middle ground" choices could involve selecting between simple gravimetric analysis and the more sophisticated Fourier Transform Infra-Red Spectrophotometry (FTIR). The most frequently recommended method for quantitative oil analysis in the literature is the combination of gas chromatography (GC) with mass spectroscopy (MS) i.e. GC/MS. However, it requires the use of very sensitive, expensive equipment and demands a high degree of operator expertise.

Generally, selection *is* a balance of time, cost, expertise and resources which are sometimes not easy to establish. But, time and energy spent during the planning stage of the project should ensure that the data and/or information

being gained through analysis are directly relevant to the hypotheses, as they are fundamental to the decision-making process.

10.3.4.6 Impact of Statistical Design and Analysis

Similarly, sound experimental design and the ability to statistically compare results are central to the issue of experimental linkage (Blackburn *et al.*, 1993). Statistical design consists of three interrelated components : treatment design, experimental design and sampling design.

Experimental design consists of methods to reduce the variability ("noise") in the data. A study may initially focus on screening a large number of treatments. The objective is to identify those treatments which are worthy of further investigation and those which are not. A second objective is to determine the optimal settings for certain process variables (e.g. the rates and frequency of application of an effective bioremediation agent).

In bench-scale experiments, unit-to-unit variability is avoided by mixing the substrate prior to dividing it amongst the reactors or pans. Variability within units could be due to edge effects and/or vertical zone differences. It may, therefore, be extremely difficult to obtain a representative sample from a reactor. Samples can be grab samples or composite samples. Grab samples are generally obtained and analyzed separately but this is usually expensive. A more practical and less expensive approach is to do composite sampling where several grab samples are taken (usually randomly) and are mixed thoroughly prior to analysis (this is a physical averaging process). The sampling design concerns the number of samples required and the frequency of sampling. It is directly related to the meaningfulness of the statistical tests that may be used on the data. Bioremediation studies are mostly "longitudinal" (Blackbird *et al.*, 1993). i.e. the effect of the treatment occurs over time. How the sampling is done depends on whether the 'difference' or 'rate' modeling is of principal interest.

10.4 Bioremediation Experimentation - is it Worth the Time and Effort ?

There are several issues that have arisen from the above discussion of whether bioremediation experiments can lead to accurate predictions, the experienced gained through the use of such processes, and associated commercialization of site remediation. More specifically, it has been dicovered that where petroleum oils are compositionally complex, quantitative analytical results and conclusions depend, not only on the general methods chosen, but also on the assumptions and slight variations ('error') accepted within methods. Because of the possible impacts of apparently minor variations in the methods or results, meaningful comparisons of results by different investigators may be difficult. Another important issue is the fact that contaminated sites may have great variability due to the diverse products spilled randomly over, perhaps, a century; weathering processes lead to compositional changes; and pollutant distribution on the impacted soils may be site-specific on scales as small as a few metres. The impacts of this chemical variability on the bioremediation process are relatively unknown.

Bench-scale methods may be used to identify biodegradation potential and rates of site samples but the linkage of these results back to the field is often limited. This is influenced by the problems of changing the environment and the population structure by sampling as well as in the biodegradation assay.

Statistical design is central to the success of both field and bench experiments as well as to the comparisons between them. A balance must *constantly* be found between the number of treatments studied and the precision of estimating treatment effects and rate models. Since bench-scale experiments are less variable than field-scale experiments, screening should be done at bench-level, whereas field studies should be confirmatory and

narrowly focussed. Treatment design must be complemented by sound experimental design to minimize error (data variability).

In summary, it may be stated that reliable predictive linkage of bioremediation experiments is, and has been, the most challenging part of a case study. It requires a large-scale effort and could involve the development of innovative new methods before the ultimate goal is achieved. However, the trends obtained in this study at the bench very closely resembled those which occurred in the field. This promising result served, not only as the assurance that the simplistic approach was both realistic and effective but that bioremediation was achievable *in situ* at the experimental site.

10.5 The Future of Bioremediation in South Africa

10.5.1 The Field of Bioremediation as a Whole

The major areas, in which current (global) research is moving rapidly, are the application of molecular genetics to develop new bacterial strains capable of either more complete destruction of compounds, or of metabolizing compounds otherwise refractory to microbial metabolism; and the development of new engineering techniques to enhance the rate and extent of biodegradation (Colwell, Levin and Gealt, 1993).

Whilst the field of molecular microbiology is not well-supported in South Africa (except in the fields of yeast biotechnology and medical microbiology) (FRD 1994) since it is regarded as a 'luxury' science, interest in the science and technology of bioremediation has recently emerged from a few companies and University Departments. It has been recognized that bioremediation will rapidly become as important an industry in Southern Africa as it has in Europe and the USA. A growth rate in the market for bioremediation of hazardous wastes in the USA of 65% per year has been

forecast (Glaser, 1992) and, although growth of this kind is not expected in South Africa in the short term, it is recognized that in the long term an equivalent situation is unavoidable.

Much progress is dependent upon the development of suitable regulatory policy with regards to soil and groundwater pollution standards, the enforcement of suitable waste disposal standards/alternatives, and the information and experience available to South African industry. The significance of the regulatory infrastructure will become more evident, particularly in real estate transfer issues (which is of particular relevance in terms of the land restitution programme). If landowners become accountable for the condition of their property, property titles will not be legally transferred unless the liability for contamination is legally included. Site investigations and subsequent cleanups will become a routine practice, along with the compulsory environmental impact assessment (EIA) prior to new developments as well as regular environmental audits on existing operations.

Since the antiquated technologies of burial or dumping at sea are no longer alternatives which are economical or in favour with the public, biotreatment will rise to the forefront as the method of choice for remediation of waste in the future. We are all moving, idealistically perhaps, toward the concept of sustainability. Therefore, while most compounds (given enough time) are degraded by microorganisms, the thrust for the next decade must be to promote the efficiencies of indigenous microorganisms and *in situ* practices so that the rates of degradation will outpace the rates of deposition.

10.6 Current Barriers to the Development and Application of Remedial Technology in South Africa

In addition to the experimental limitations already emphasised throughout this document, a more extensive analysis of barriers to development and

application of innovative technologies in connection with soil and water contamination in South Africa was made. The outcome demonstrates that in addition to barriers of a technical nature, including for instance geology, there are also several non-technical barriers of importance to the criteria for choice of technology in each case where remediation would be identified as an option.

These barriers may be divided into four groups :

- legislative barriers
- institutional barriers
- financial and market barriers
- technical barriers

All of the non-technical barriers are considered as important as the technical barriers. Morover, it is characteristic of the non-technical barriers that they can be eliminated to a certain extent by changing the law or regulatory structures. This is not true of the technical barriers, the present technical limitations of which can only be eliminated by technological development.

With the present technological level, there is for each problem a direct connection between the situation and the choice of technology.

10.6.1 Development of New Remedial Technology

Development of new or innovative remedial technology in South Africa is primarily restrained by the lack of willingness to invest. The assessment was made that there is presently great uncertainty as to how the market will develop and some hesitation towards large-scale investments in advanced treatment facilities prevails. Moreover, no platform from where a coordinated research and development effort for the treatment of contaminated soil can be made has been established and no forum where innovative technology can be tested and demonstrated is available either.

Thus, the development of remedial technologies is not considered possible without public support and control.

A preliminary identification of areas of effort within development and commercialization of soil treatment methods has also been made. It is clear that while methods on a bench scale have been developed for the major part of the present types of contamination, programmes supporting further development of techniques peculiar to South African conditions, for pilot and full scale implementation are estimated to be required to a larger extent. With respect to off-site treatment of contaminated soil, advanced facilities for thermal and physico-chemical methods have been established abroad. Implementation of such technologies is a matter of whether the South African market is considered to be sufficiently large. If off-site treatment of contaminated soil is aimed at, investments in programmes for further development of, particularly, second-generation microbiological methods primarily in closed systems such as reactors, are considered necessary.

10.6.2 Barriers to the Implementation of Innovative Technology

Barriers are defined as conditions determined by legislation, regulations, administrative practices, technologies etc., making the application of innovative technology unattractive in any specific situation. Thus, the individual barriers are not necessarily quantitatively or qualitatively comparable (Andersen, 1996). It should be noted that in the review of the literature, it was established that some of the barriers are more significant abroad than here, and vice versa.

Thus, it can be established that the choice of remedial technology should be made on the basis of an assessment of technical as well as non-technical factors. Being aware of the constraints should, however, serve to encourage rather than discourage innovations which can overcome these barriers.

Table 10.2 : Overview of barriers to the application of remedial technologies in South Africa

LEGISLATIVE BARRIERS
No legislation for remediation of soil
No soil pollution standards
No code of practice for contamination
on sensitive land-use areas before registration/rezoning
INSTITUTIONAL BARRIERS
Differing local authority objectives
Cleanup criteria
No guidelines for the documentation of cleanups
Guarantee in connection with application of technology
Neighbour relations and surroundings
No guidelines for landfill disposal (the usual method) of contaminated soil
Lack of awareness (of risk/liability)
Lack of appropriate technical skills/experience
TECHNICAL BARRIERS
Heterogeneous geological conditions
Time consumption of the cleanups
Composite contamination
Extent of contamination (area requiring remediation)
FINANCIAL AND MARKET BARRIERS
Cost-benefit with the alternative of cost-effectiveness (innovative technologies with inherent development costs more expensive than traditional solutions such as landfilling)
Lack of willingness to invest

10.6.3 Possibilities of Removing Barriers

10.6.3.1 Legislative Barriers

The removal of legislative barriers is, in most cases, possible if political consensus can be reached. These processes take much time and perseverance in any country, but the benefits of having a stronger regulatory infrastructure which protects the health of people and the environment are generally indisputable.

10.6.3.2 Administrative/Institutional Barriers

As is the case with legislative barriers, it is also possible to remove the institutional barriers deriving from such legislation. This may be done by changing the methods for administration of the law, an example of which could be the creation of soil criteria or guidelines for cleanups where present uncertainty constitutes a barrier to the application of remedial technology.

10.6.3.3 Market Mechanisms

Financial and market barriers can be removed, for instance, by means of a decision (policy) to the effect that the principle of the cheapest solution should not be taken into consideration in the choice of remedial technology, whereas requirements for cleaner technology should be decisive.

10.6.3.4 Technical Barriers

Most of the technical barriers, unlike the previous categories, cannot be removed. They can, however, be overcome by technological and educational development :

"Any technical problem can be solved; it is merely a matter of price" (Quote: P. Harremoes, The Technical University of Denmark)

The physical conditions of a soil contamination case are determined by geology (e.g. soil type and permeability), which are parameters which can be handled by means of significant technical progress only.

10.6.4 Barriers to the Development of Innovative Technology

Similar to the assessment of barriers to the *application* of remedial technology, an identification of the barriers to *development* of innovative technologies has been made.

Table 10.2 : Identified barriers to development of new or innovative remedial technology.

LEGISLATIVE BARRIERS
No regulatory incentive
INSTITUTIONAL BARRIERS
Cleanup criteria for soil
Lack of appropriate technical education/skills
TECHNICAL BARRIERS
Heterogeneous geological conditions
Composite contamination
FINANCIAL AND MARKET BARRIERS
Market unpredictability
Cost-benefit with the alternative of cost-effectiveness
Insufficient market volume without legislatory framework

The major constraint to the development of new technology is the fact that off-site treatment or disposal (usually landfilling) is lower in price than any other option in South Africa. Furthermore, at the current state of legislation, the market volume is very small. Consequently, it cannot be expected that all the foreign technologies will be transferred to South African conditions.
It was realized that the development of innovative remedial technologies is required. The development of technologies which will be suited to South African conditions is thus estimated to be a matter of will, especially since there is no regulatory requirement or incentive to do so.

The goal of this, the first study of its kind in South Africa, was to take up the challenge and find sound but inexpensive bioremediation options which could achieve the same endpoint as those requiring extensive engineering at a very high cost. The objective was to always be aware of the unique

constraints of the local (South African) situation viz. the technological limitations, cost and time constraints, the lack of soil cleanup criteria or standards, the sociological problems and the climate and geology peculiar to the region. Within these exceptional conditions, the objectives were achieved and this South African bioremediation experience was borne.

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APPENDIX 1: POT TRIAL III AND FIELD TRIAL RAW DATA

TREATMENT	mgFDA T0	mg FDA T2	mg FDA T4	mg FDA T6	mg FDA T8	mg FDA T10	Pk Area 0
HN	311.2	870.455	2762.5	4384.66	4583.55	3370.45	91.109
HN	316.94	955.682	3836.36	4361.93	3873.83	3475.57	89.885
HN	308.11	986.932	3458.52	4236.93	3052.27	3356.25	93.002
HNP	309.21	941.477	3725.57	4373.3	2717.05	3160.23	87.962
HNP	299.54	901.705	2097.73	4719.89	5052.27	3094.89	88.633
HNP	289.36	927.273	3736.93	2251.14	5157.39	3691.48	79.486
HP	292.94	455.682	2305.11	3009.66	3089.2	2310.8	79.674
HP	296.66	478.409	2288.07	1432.95	2634.66	1148.86	78.343
HP	321.11	503.977	2282.39	3339.2	3021.02	2861.93	80.233
HW	323.42	441.477	2677.27	3643.18	1094.89	753.977	84.663
HW	321.17	481.25	2484.09	969.886	1211.56	498.295	94.515
HW	330.07	467.045	2023.86	952.841	1086.36	597.727	85.071
MN	325.36	219.886	2458.52	4234.09	3342.05	2682.95	51.426
MN	289.87	322.159	2759.66	3523.86	3160.23	2685.8	60.125
MN	288.54	657.386	3790.91	3296.59	3711.36	3092.05	68.682
MNP	286.42	1600.57	3430.11	3790.91	4276.7	2342.05	68.665
MNP	288.01	1901.7	3211.36	2938.64	3807.95	3361.93	64.548
MNP	290.5	2126.14	2816.48	2560.8	4378.98	2489.77	67.177
MP	310.12	327.841	3151.7	2771.02	1944.32	1552.27	69.869
MP	305.4	424.432	3540.91	3106.25	2941.48	2626.14	56.304
MP	301.99	325.11	3293.75	1262.5	2961.36	1342.05	61.552
MW	299.3	620.455	3768.18	2336.36	3157.39	1506.82	61.692
MW	294.9	515.341	3651.7	1594.89	2881.67	4367.61	63.781
MW	268.32	546.591	3143.18	2539.77	2947.16	1361.93	66.131
LN	291.11	228.409	1799.43	1396.02	840.96	688.636	8.71
LN	278.96	486.932	1688.64	1231.35	1009.18	572.159	10.24
LN	271.8	239.773	1887.5	1248.3	2361.93	1430.11	11.084
LNP	282.7	1038.07	3026.7	1163.07	1873.3	657.386	11.108
LNP	285.5	1103.41	3043.75	1092.05	1708.52	327.841	6.008
LNP	283.22	932.955	1878.98	1452.84	1413.97	461.364	6.1154
LP	281.71	523.864	1288.07	1001.14	1285.23	779.545	6.1249
LP	290.3	481.25	1842.85	1236.93	1106.25	790.909	10.961
LP	285.44	430.114	1785.23	850.568	688.636	609.091	5.7
LW	220.1	200.45	1188.64	950	1441.48	1319.32	3.01
LW	280.62	896.023	1586.36	2160.23	1540.91	421.591	9.026
LW	230.35	353.409	1575.02	640.341	1484.09	1188.64	8.7
HN PB	210.98	870.455	4157.39	2543.75	4540.91	3719.89	77.66
HN PB	207.89	1503.98	3739.77	3589.2	4543.75	3822.16	89.885
HN PB	221.33	1555.11	3555.11	3680.11	4288.07	4026.7	89.85
HNP PB	260.59	1509.66	3859.09	4245.45	4390.34	3543.41	88.633
HNP PB	201.37	901.705	3052.27	3702.84	4458.52	3353.43	79.486
HNP PB	210.66	927.273	4086.36	4063.64	4103.41	3111.93	87.962
HP PB	186.64	455.682	3796.59	3114.77	3589.2	3870.45	80.233
HP PB	197.35	478.409	3171.59	2734.09	3535.23	2614.77	78.343
HP PB	185.22	503.977	3140.34	4055.11	4182.95	3271.02	78.343
HW PB	189.01	441.477	3200	3898.36	4253.98	3117.61	79.566
HW PB	193.51	481.25	2915.91	3424.43	4302.77	3168.75	94.515
HW PB	144.32	467.045	3100.57	3327.84	4333.52	3722.73	79.874
MN PB	201.6	219.886	3847.73	3986.93	4330.68	3913.07	51.426
MN PB	220	322.159	3836.36	3705.68	3918.75	3697.16	60.125
MN PB	311	657.386	4009.66	4046.59	4498.3	3688.64	60.125
MNP PB	340.53	1600.57	3961.36	3918.75	4316.48	4299.43	68.665

MNP PB	326.49	1901.07	3728.41	2762.5	4458.52	3421.59	64.548
MNP PB	312.87	2126.14	3873.3	4518.18	4475.57	4060.8	64.548
MP PB	299.54	327.841	2489.77	3515.34	4481.25	3208.52	61.552
MP PB	289.36	424.434	3077.84	4313.64	2131.82	3552.27	56.304
MP PB	301.75	325	3080.68	4151.7	3347.73	3003.98	61.692
MW PB	402.02	620.455	2759.6	3768.18	3947.16	3680.11	63.781
MW PB	321.11	515.341	2123.3	3003.98	1393.18	2833.52	63.781
MW PB	311.64	546.591	2055.11	3697.16	3060.8	2182.95	66.131
LN PB	317.89	228.409	2711.36	1753.98	2086.36	1424.43	11.31
LN PB	399.84	486.932	2742.61	1765.34	1941.48	1935.8	10.24
LN PB	289.87	239.773	2205.68	2197.16	2413.07	2529.55	8.02
LNP PB	294.11	1038.07	2276.7	2259.66	2427.273	1810.8	10.05
LNP PB	296.1	1103.41	2489.77	1296.59	2359.09	964.205	7.293
LNP PB	239.62	932.955	2771.02	2151.7	1702.84	1543.75	8.94
LP PB	220.3	523.864	2066.48	1876.14	1915.19	1356.25	9.12
LP PB	262.33	481.25	2057.95	725.568	2611.93	1753.98	10.961
LP PB	211.65	430.114	1316.48	1444.35	1762.51	813.636	10.33
LW PB	280.62	200.99	1157.39	1461.36	2285.23	915.909	8.69
LW PB	230.35	896.023	1279.55	1566.48	2100.57	1654.55	9.026
LW PB	211.94	353.409	1972.73	1160.23	2217.05	1083.52	8.17
HN PL	311	870.455	5685.8	3850.57	2575	2080.68	91.33
HN PL	316	1239.77	2745.45	3205.68	2907.39	2779.55	89.885
HN PL	308.11	1271.02	5194.32	3134.66	3870.45	3611.93	91.33
HNP PL	309.21	1577.84	4427.27	5200.06	2773.86	2663.07	85.36
HNP PL	299.54	901.705	4626.14	4458.52	3631.82	2986.93	88.633
HNP PL	289.36	927.273	4742.61	4279.55	3668.75	3305.11	89.885
HP PL	292.94	455.682	4447.16	1739.77	3646.02	1850.57	56.304
HP PL	296.66	478.409	3839.2	830.682	4759.66	2208.52	78.343
HP PL	321.11	503.977	2913.07	1878.98	2126.14	2472.73	79.42
HW PL	323.42	441.477	3839.2	2367.61	2634.66	1321.25	88.08
HW PL	321.17	481.25	4378.98	1484.09	2896.02	1680.11	94.515
HW PL	330.07	467.045	437.55	2103.41	1023.86	2708.52	85.071
MN PL	325.36	219.886	4319.32	2861.93	4367.61	4177.27	60.08
MN PL	289.87	322.159	3538.07	2856.25	4282.39	2515.34	60.125
MN PL	288.54	657.386	3802.27	3353.41	4316.48	2262.5	68.682
MNP PL	286.42	1600.57	2327.84	3396.02	3989.77	3262.5	68.665
MNP PL	288.01	1901.7	3407.39	2722.73	4262.5	3771.02	64.548
MNP PL	290.5	2126.14	3143.18	3373.3	4330.11	3935.8	67.177
MP PL	310.12	327.841	2648.86	1623.3	2131.82	1128.98	69.869
MP PL	305.4	424.432	844.886	1646.02	2075	2117.61	56.304
MP PL	301.99	325	3049.43	2171.59	1640.34	1964.2	61.552
MW PL	299.3	620.455	2557.95	2901.7	1549.43	1075	61.692
MW PL	294.9	515.341	2486.93	2518.18	1634.66	932.955	63.781
MW PL	268.32	546.591	2881.82	4185.8	1527.7	839.205	66.131
LN PL	291.11	228.409	717.045	1245.45	813.636	660.227	8.09
LN PL	278.96	486.932	1117.61	654.545	822.159	384.659	10.24
LN PL	271.8	239.773	1398.86	822.159	1038.07	796.591	11.812
LNP PL	282.7	1038.07	2555.11	643.182	552.273	464.205	11.108
LNP PL	285.5	1103.41	1523.86	1819.32	1134.66	600.568	7.293
LNP PL	283.22	932.955	1378.98	1839.2	1273.86	467.045	6.1154
LP PL	281.71	523.864	1427.27	751.136	1316.48	847.727	6.1249
LP PL	290.3	481.25	961.364	609.091	432.955	788.068	10.961
LP PL	285.44	430.114	1165.91	1282.39	842.045	603.409	6.039

LW PL	220.1	200	575	611.932	1026.7	413.068	6.467
LW PL	280.62	896.023	799.432	1029.55	401.705	910.227	9.026
LW PL	230.35	353.409	1827.84	1606.25	461.364	717.045	11.325
HN PB PL	210.98	870.455	4060.8	3467.05	4330.68	3347.73	91.109
HN PB PL	207.89	955.682	4188.64	4367.61	3583.52	2813.64	89.885
HN PB PL	221.33	986.932	3839.2	4819.32	2637.5	2455.68	93.002
HNP PB PL	260.59	941.477	3771.02	4538.07	4736.93	3290.91	87.962
HNP PB PL	201.37	901.705	3029.55	4555.11	4600.57	3307.95	88.633
HNP PB PL	210.66	927.273	3682.95	4495.45	4609.09	2768.18	79.486
HP PB PL	186.64	455.682	3719.89	3739.77	3685.8	3336.36	79.674
HP PB PL	197.35	478.409	3677.27	3711.36	3404.55	3319.32	78.343
HP PB PL	185.22	503.977	3873.3	3842.05	3833.52	2745.45	80.233
HW PB PL	189.01	441.477	2782.39	2410.23	3773.86	2455.68	84.663
HW PB PL	193.51	481.25	2640.34	4123.43	3001.14	2256.82	94.515
HW PB PL	144.32	467.045	1515.34	3168.75	2867.61	2117.61	85.071
MN PB PL	201.6	219.886	2907.39	4131.82	3467.05	3441.48	51.426
MN PB PL	220	322.159	2515.32	3444.32	3251.14	3307.95	60.125
MN PB PL	311	657.386	2867.61	3217.15	3120.45	3248.3	68.682
MNP PB PL	340.53	1600.57	2785.23	3907.39	2793.75	2756.82	68.665
MNP PB PL	326.49	1901.7	3120.45	3381.82	3793.75	3478.41	64.548
MNP PB PL	312.87	2126.14	3549.43	5035.23	3486.93	2870.45	67.177
MP PB PL	299.54	327.841	3597.73	3441.48	4123.3	2972.73	69.869
MP PB PL	289.36	424.432	3381.82	3114.77	2961.36	2947.16	56.304
MP PB PL	301.75	325	3677.27	2771.02	3376.14	2884.66	61.552
MW PB PL	402.02	620.455	3717.05	2475.57	3560.8	3455.68	61.692
MW PB PL	321.11	515.34	3361.93	2512.5	3969.89	2802.27	63.781
MW PB PL	311.64	546.591	3799.43	3376.14	4023.86	2259.66	66.131
LN PB PL	317.89	228.409	1827.84	1910.227	2143.88	1759.66	8.09
LN PB PL	399.84	486.932	2040.91	2370.45	1864.77	1251.14	10.24
LN PB PL	289.87	239.773	3032.39	2623.3	1248.3	1523.86	11.812
LNP PB PL	294.11	1038.07	2736.932	2722.727	1316.48	1665.91	11.108
LNP PB PL	296.1	1103.41	1967.05	2253.98	1546.59	1038.07	7.293
LNP PB PL	239.62	1932.955	2194.89	2930.11	1583.62	1165.91	6.1154
LP PB PL	220.3	523.864	2055.11	1631.82	2427.27	901.705	6.1249
LP PB PL	262.33	481.25	1109.09	1898.86	2452.84	1018.18	10.961
LP PB PL	211.65	430.114	2228.41	1307.95	2049.43	981.25	6.039
LW PB PL	280.62	200	2003.98	2001.04	2177.27	1663.07	6.467
LW PB PL	230.35	896.023	1768.18	1859.09	1188.64	1359.19	9.026
LW PB PL	211.94	353.409	1700	861.932	1921.59	950	11.325
LHN	311.2	870.45	1478.41	3978.41	3918.75		89.885
LHN	316.94	955.68	1677.27	2753.98	3853.41		87.962
LHN	308.11	986.93	254.91	3083.52	3887.5		88.633
LHNP	309.21	941.48	2430.11	3665.91	2986.93		79.486
LHNP	299.54	901.7	881.82	3611.93	4174.43		79.674
LHNP	289.36	927.27	3009.66	3285.23	3915.91		78.343
LHP	292.94	455.68	1447.16	3197.16	2944.32		80.233
LHP	296.66	478.41	1359.09	2171.59	2407.39		84.663
LHP	321.11	503.98	1319.32	2253.98	3529.55		85.071
LHW	323.42	441.48	2040.91	2870.45	1251.41		79.566
LHW	321.17	481.25	1856.25	1441.48	3032.39		79.874
LHW	330.07	467.05	2552.27	1700	2259.66		78.333
LMN	325.36	219.89	1870.45	2353.41	2722.73		51.426
LMN	289.87	322.16	1660.23	2381.82	2555.11		60.125

LMN	288.54	657.39	1415.91	2492.61	3282.39		68.682
LMNP	286.42	1600.57	1702.84	2538.07	1521.02		68.665
LMNP	288.01	1901.7	2094.89	2444.32	1864.77		64.548
LMNP	290.5	2126.14	2035.23	1802.27	2566.48		67.177
LMP	310.12	327.84	1654.55	1532.39	1146.02		69.869
LMP	305.4	424.43	2506.82	1330.68	807.95		56.304
LMP	301.99	325	1870.45	1342.05	1021.02		61.552
LMW	299.3	620.45	1555.11	1057.95	1103.41		61.692
LMW	294.9	515.34	1464.2	853.41	1327.84		63.781
LMW	268.32	546.59	1339.2	771.02	913.07		66.131

Pk Area 2	Pk Area 4	Pk Area 6	Pk Area 8	Pk Area 10	pH T0	pH T2	pH T4
49.228	21.31	19.872	11.063	9.4018	5.57	5.2	4.57
47.998	25.298	14.892	11.999	9.0487	5.38	5.03	4.88
46.422	22.386	17.728	15.638	9.4468	4.85	5.5	4.67
49.657	19.571	17.808	16.944	5.4994	5.21	5.23	5.28
47.962	12.115	11.288	10.589	5.0326	5.33	5.55	5.42
45.662	15.799	13.859	13.825	10.323	5.34	4.58	4.56
57.877	55.257	41.865	27.16	26.40	3.61	3.73	3.89
50.835	39.623	36.787	35.83	18.41	3.26	4	3.72
64.846	60.503	54.814	34.911	27.57	3.45	3.93	3.91
52.089	49.392	46.72	33.451	24.16	4.16	4.23	3.77
50.546	55.124	44.545	39.716	32.63	4.11	4.1	3.71
60.23	48.073	46.412	40.389	37.85	4.32	4.34	3.74
44.292	42.244	29.57	25.942	12.90	4.34	3.98	4.06
39.394	38.685	30.402	29.79	13.79	4.28	3.9	4.1
40.543	39.875	37.147	26.976	17.84	4.2	4.03	4.62
30.075	28.243	26.63	26.672	26.07	4.34	4.08	4.05
41.625	30.496	24.765	24.706	17.50	4.22	4.23	4.16
36.922	35.816	27.222	21.111	14.32	4.35	4.1	3.87
44.256	37.97	34.562	25.743	23.00	4.04	4.4	4.02
44.116	43.35	32.6	27.776	15.83	4.17	4.03	3.95
45.582	42.957	33.476	32.37	24.362	4.23	4.09	3.94
47.611	38.805	28.428	26.21	28.106	4.47	5.15	3.94
50.89	38.977	35.825	33.55	24.677	4.05	4.01	4.15
54.517	37.112	28.274	30.84	25.414	4.12	4.16	3.89
2.5116	2.1941	0.7464	0.7084		3.88	5.37	4.81
2.5684	0.8056	0.6753	0.6942		3.58	4.99	4.67
2.566	0.7084	0.6942	0.0592		3.75	5.25	4.69
2.4973	0.7037	0.6824	0.0782		3.76	5.04	4.71
2.431	1.24	0.49	0.0289		3.74	4.89	4.99
2.3955	0.97	0.24	0.0948		3.69	5.09	4.52
2.4215	0.46	0.0569	0.04		3.49	4.97	5.15
2.4191	0.22	0.0758	0.02		3.59	5.22	5.04
1.61	1.48	0.46	0.04		3.6	5.12	4.9
0.81	0.69	0.22	0.02		3.44	4.97	5.07
2.4286	1.65	0.6682	0.0616		3.57	4.87	5
1.024	0.71	0.51	0.034		3.81	5	5.09
29.96	7.61	6.68	4.83	4.01	5.57	5.34	5.24
22.839	20.033	14.479	11.65	3.98	5.38	4.64	5.11
45.71	22.48	10.68	6.88	4.22	4.85	4.86	5.09
67.2	24.691	12.795	3.98	2.39	5.21	4.53	4.48
26.156	16.287	13.809	12.181	3.01	5.33	4.48	4.31
23.91	18.21	14.6	10.95	2.89	5.34	4.37	4.39
30.47	26.77	18.19	16.33	15.98	3.61	4.28	4.3
29.521	18.623	16.685	16.01	15.23	3.26	4.08	4.14
30.482	28.31	25.22	20.91	19.27	3.45	4.17	3.95
34.311	32.38	31.45	28.49	27.99	3.66	4.28	4.13
38.811	31.063	25.4	25.31	14.276	3.56	4.26	4.04
39.84	30.217	29.01	28.5	27.3	3.71	4.07	4.12
28.525	14.97	12.77	8.3	6.88	4.34	4.79	4.71
38.294	24.059	20.564	8.8497	6.85	4.28	4.3	4.17
26.025	21.25	20.91	14.06	11.71	4.2	4.64	4.59
25.819	21.52	7.81	3.68	3.7	4.34	4.54	4.35

23.431	11.027	11.093	13.264	1.97	4.22	4.58	4.72
27.819	10.004	8.07	7.67	4.52	4.35	4.33	4.07
50.57	32.446	28.08	21.72	12.46	4.04	4.48	4.23
27.671	19.758	16.166	12.55	2.9072	4.17	4.71	4.41
34.425	26.8	22.35	16.37	6.02	4.23	4.35	4.3
57.43	32.375	21.62	17.21	16.91	4.47	4.46	4.37
45.11	18.628	17.799	14.908	12.612	4.05	4.33	4.16
57.32	31.162	29.99	24.45	22.19	4.12	4.23	4.29
3.41	1.99	0.27	0.16		3.88	4.45	4.09
5.973	0.8056	0.481	8.5914		3.58	4.44	4.15
6.881	3.79	3.31	3.01		3.75	4.46	4.18
8.55	2.25	0.71	2.09		3.76	4.48	4.18
6.008	5.81	0.746	0.5165		3.74	4.5	4.15
2.43	2.00	0.25	0.17		3.69	4.48	4.14
3.65	2.51	0.23	0.19		3.49	4.63	4.27
7.543	0.6824	0.5307	0.66		3.59	4.64	4.27
3.01	2.07	0.22	0.17		3.6	4.58	4.38
8.94	2.37	1.60	0.74		3.44	4.66	4.28
6.172	0.6682	0.497	0.399		3.57	4.53	4.31
7.33	1.65	0.89	0.35		3.81	4.55	4.31
29.96	16.05	14.42	4.08	4.42	5.57	7.2	6.14
18.14	13.252	12.245	11.79	8.20	5.38	6.21	5.9
53.51	28.14	4.74	12.48	5.33	4.85	6.73	5.49
50.20	24.04	8.08	7.81	2.36	5.21	6.19	4.78
19.164	12.198	11.596	10.875	0.97	5.33	5.97	5.59
27.291	14.339	12.89	11.529	2.98	5.34	5.91	6.18
31.653	27.876	23.74	15.99	11.86	3.61	4.31	4.42
22.55	18.211	18.07	17.159	15.515	3.26	4.27	4.09
57.19	29.22	26.39	17.79	11.97	3.45	4.34	4.38
67.68	39.40	31.00	19.99	19.49	3.66	4.54	5.45
32.854	18.012	14.536	9.9799	16.82	3.56	5.44	5.2
31.975	20.853	19.571	13.664	6.4282	3.71	4.62	3.78
51.34	27.24	22.87	18.21	11.56	4.34	4.02	4.44
33.728	22.26	20.945	15.524	4.60	4.28	3.98	4.13
37.868	32.515	27.156	19.988	19.06	4.2	4.03	4.58
42.938	29.456	28.504	19.664	16.35	4.34	3.97	4.34
41.739	28.319	23.602	24.011	13.57	4.22	4.18	4.55
36.162	35.565	20.126	21.815	6.85	4.35	3.96	5.75
32.813	25.149	23.379	22.37	3.70	4.04	3.98	4
72.224	37.858	27.897	25.267	8.39	4.17	4.01	3.92
46.277	28.304	23.895	20	13.91	4.23	4	4.56
41.192	38.846	24.575	27.845	21.38	4.47	4.01	4.58
42.659	32.706	28.311	23	18.78	4.05	4.09	4.13
45.933	40.993	23.355	23.094	6.09	4.12	3.95	4.21
6.453	3.573	0.7369	0.097		3.88	4.87	5.36
8.125	1.571	0.6966	0.064		3.58	4.99	4.9
11.084	1.6112	0.6919	0.088		3.75	4.9	4.85
1.666	1.613	0.7084	0.037		3.76	5.12	5
1.952	1.6515	0.6563	0.063		3.74	4.92	4.77
2.3196	1.696	0.7084	0.078		3.69	4.88	4.94
2.2177	1.492	0.7037	0.349		3.49	5.04	4.91
2.2557	1.115	0.7132	0.502		3.59	5.01	4.8
2.0377	1.822	0.7203	0.411		3.6	4.86	4.57

2.4215	1.697	0.7984	0.348		3.44	4.76	4.64
2.341	1.121	0.7084	0.161		3.57	4.91	4.96
2.3473	1.983	0.7165	0.109		3.81	5.46	4.79
18.152	14.226	8.0749	7.991	7.36	5.57	4.46	4.6
18.126	10.07	14.342	7.5963	7.391	5.38	4.96	5
18.154	15.72	15.027	13.558	6.7694	4.85	4.9	5.21
12.186	15.96	15.868	7.1414	5.338	5.21	4.71	4.65
17.197	11.508	7.5536	6.075	3.87	5.33	4.72	5.04
23.66	12.738	11.754	10.439	2.5163	5.34	4.55	4.67
28.06	20.073	14.283	6.857	2.3147	3.61	4.75	4.82
29.41	16.278	15.121	6.857	2.2486	3.26	4.82	4.45
26.51	20.699	19.77	9.6648	5.4828	3.45	4.46	4.38
28.298	17.896	15.17	9.608	9.089	3.66	4.22	4.11
29.428	26.224	15.37	7.9564	6.331	3.56	4.27	4.05
20.945	18.879	15.42	8.1555	8.00	3.71	4.19	4.12
28.369	25.813	15.119	6.4921	6.66	4.34	4.21	4.18
26.338	24.564	23.15	13.496	13.809	4.28	4.09	4.12
27.007	18.2242	16.349	14.839	7.8853	4.2	4.21	4.47
25.419	23.502	18.39	13.19	6.748	4.34	4.4	4.15
23.208	19.704	13.269	6.6627	24.08	4.22	4.24	4.6
20.659	19.41	18.798	12.333	6.9447	4.35	4.21	4.14
37.692	25.62	23.03	22.533	11.909	4.04	4.18	4.13
34.847	30.404	19.664	5.8477	3.68	4.17	4.2	3.98
30.414	25.056	21.445	18.306	15.99	4.23	4.33	4.07
36.96	18.363	17.233	18.875	18.07	4.47	4.25	4.39
32.581	26.981	20.929	18.05	13.57	4.05	4.28	4.1
30.75	21.776	18.166	17.749	15.75	4.12	4.3	4.14
8.09	3.573	2.56	0.507		3.88	4.63	4.4
5.973	2.44	1.571	0.5142		3.58	4.54	4.14
4.918	1.6112	1.61	0.5165		3.75	4.54	4.3
9.534	1.613	0.81	0.5497		3.76	4.83	4.17
7.293	1.6515	0.931	0.5663		3.74	4.47	4.19
5.155	1.696	0.94	0.5497		3.69	4.52	4.18
6.034	1.492	0.91	0.5473		3.49	4.77	5.23
7.543	1.115	0.866	0.5781		3.59	5.41	4.42
5.882	1.822	0.761	0.6137		3.6	4.61	4.23
5.697	1.697	0.883	0.5473		3.44	4.71	4.27
6.172	1.121	0.769	0.5568		3.57	4.85	4.33
6.681	1.983	0.994	0.5781		3.81	4.51	4.23
40.199	27.291	25	4.01		4.23		
42.311	23.91	22.16	6.891		4.8		
39.452	24.691	20.11	5.772		4.72		
36.44	26.156	15.77	2.98		3.98		
38.122	21.346	14.34	6.74		4.77		
36.93	30.482	17.01	3.01		3.84		
38.99	30.47	26.04	15.99		3.87		
45.33	24.805	20.18	16.5		3.99		
39.23	27.542	21.99	14.04		3.67		
36.555	34.311	26.549	18.07		4.77		
48.318	30.217	27.335	23.56		4.14		
44.162	28.743	28.116	25.662		4.02		
33.69	28.525	16.912	3.03		4		
46.818	26.025	14.563	6.99		4.81		

39.344	28.122	18.908	4.077		4.15		
39.802	25.819	12.054	3.68		4.07		
37.771	27.819	11.998	2.992		4.11		
31.585	27.999	12.003	3.923		4.89		
32.912	29.74	25.023	13.57		4.66		
31.89	31.653	23.447	15.862		3.57		
33.435	32.446	25.671	12.05		4.49		
40.199	34.425	24.665	15.75		5.3		
46.049	32.375	24.996	18.993		4.66		
43.002	31.162	22.368	15.024		5.53		

pH T6	pH T8	pH T10	DRY WEIGHT (g)	
4.76	4.81	4.76		
5	4.64	4.45		
5.59	4.58	4.85		
5.39	5.2	5.12		
5.38	5.09	5.1		
4.69	4.53	4.38		
3.84	3.79	3.81		
3.64	3.58	3.52		
3.76	3.91	3.94		
3.97	3.85	3.88		
3.98	3.58	3.59		
3.94	3.57	3.55		
4.15	4.09	4.05		
4	4.15	4.11		
4.43	4.01	3.99		
4.13	4	3.98		
4.57	3.93	3.91		
4.2	4.01	4		
3.9	3.98	3.95		
3.94	3.85	3.87		
3.91	3.85	3.85		
4.13	4.07	4.05		
3.96	3.76	3.8		
3.82	3.96	3.93		
5.15	4.77	4.18		
5.06	4.66	4.62		
4.69	4.54	4.35		
4.85	5.57	5.12		
4.78	4.89	4.87		
4.68	4.9	4.88		
5.01	5.34	5.22		
4.86	4.81	4.8		
4.85	4.92	4.9		
4.82	4.93	4.78		
4.85	4.88	4.83		
4.9	4.84	4.5		
4.63	4.22	4.1		
4.62	4.52	4.39		
4.6	4.49	4.45		
4.58	4.73	4.61		
4.59	4.74	4.89		
4.66	4.86	4.91		
3.94	4.61	4.69		
4.25	4.06	4.09		
4.02	4.1	4.34		
4.19	4.22	4.28		
4.18	4.37	4.45		
4.07	4.4	4.51		
4.75	4.4	4.44		
4.41	4.35	4.29		
5.01	4.46	4.12		
4.66	4.6	4.54		

4.52	4.56	4.51		
4.53	4.26	4.31		
4.26	4.35	4.28		
4.56	4.64	4.32		
4.28	4.32	4.28		
4.09	4.22	4.21		
4.3	4.34	4.29		
4.32	4.28	4.26		
4.06	3.94	3.89		
4.1	3.96	3.93		
4.12	3.99	3.82		
4.03	3.96	3.88		
4.05	3.98	3.89		
4.02	3.99	3.87		
4.27	4.24	4.51		
4.18	4.22	4.11		
4.38	4.41	4.33		
4.37	4.32	4.19		
4.29	4.24	4.39		
4.25	4.33	4.31		
6.38	5.72	5.5	0.506	
6.41	6.06	5.91	0.388	
6.69	5.93	5.89	0.442	
4.97	5.64	4.67	1.962	
6.85	5.52	5.37	0.831	
6.92	5.29	5.21	1.921	
4.08	4.26	4.21	0.209	
4.57	4.43	4.33	0.183	
4.66	4.09	4.01	0.21	
5.2	4.51	4.39	0.224	
4.32	5.16	5.01	0.322	
3.62	5.82	4.67	0.337	
4.11	4.88	4.53	0.348	
4.05	4.55	4.53	0.285	
4.23	3.95	4.05	0.185	
4.03	3.86	3.88	0.272	
4.21	4.01	4.34	0.217	
4.09	4.48	4.69	0.2	
3.79	4	3.99	0.202	
3.84	3.81	3.97	0.193	
3.91	3.83	3.81	0.085	
3.95	3.92	3.92	0.122	
3.9	4.02	4.03	0.107	
3.88	3.78	3.76	0.112	
4.77	4.57	4.56	1.057	
4.94	4.81	4.77	0.989	
4.57	4.64	4.63	1.277	
4.77	4.58	4.54	2.415	
4.51	5.2	5.21	1.576	
4.83	5.09	5.87	1.747	
4.84	4.53	4.52	2.071	
4.56	3.79	3.8	2.88	
4.53	3.58	3.6	2.361	

4.56	3.91	3.89	1.24	
4.76	3.85	3.86	1.171	
4.9	3.58	3.6	2.386	
4.56	3.57	3.61	0.559	
5.15	4.09	4.12	0.374	
4.53	4.15	4.11	0.593	
4.38	4.01	4	0.288	
4.47	4	3.98	1.534	
4.91	3.93	3.95	2.299	
4.39	4.01	3.99	0.717	
4.72	3.98	3.96	0.46	
4.44	3.85	3.82	0.64	
4.15	3.85	3.81	0.499	
4.35	4.07	4.04	0.711	
4.22	3.76	3.77	0.465	
4.25	3.96	3.99	0.515	
4.02	4.77	4.74	0.585	
4.6	4.66	4.7	0.585	
3.96	4.54	4.57	0.309	
4.1	5.57	5.39	0.485	
4.05	4.89	4.86	1.641	
4.11	4.9	4.89	0.416	
4	5.34	5.31	0.593	
4.03	4.81	4.83	0.511	
4.09	4.92	4.91	0.637	
4.1	4.93	4.9	0.553	
4.1	4.88	4.9	0.931	
4.16	4.84	4.88	2.632	
4.06	4.22	4.25	3.537	
4.15	4.52	4.55	2.592	
4.17	4.49	4.43	3.863	
4.19	4.73	4.77	4.261	
4.07	4.74	4.76	4.395	
4.26	4.86	4.89	2.316	
4.4	4.61	4.64	3.901	
4.07	4.06	4.07	3.912	
4.01	4.1	4.12	2.52	
4.19	4.22	4.25	2.977	
4.17	4.37	4.39	2.222	
	4.88			
	4.61			
	4.79			
	5.07			
	4.98			
	3.96			
	4.67			
	4.56			
	4.58			
	3.73			
	4.31			
	4.36			
	3.77			
	3.54			

	3.72			
	3.78			
	3.68			
	3.61			
	3.62			
	3.59			
	3.43			
	3.45			
	3.42			
	3.46			

SAMPLE	mgFDAT0	mgFDAT2	mgFDAT4	mgFDA T6	mgFDA T8	AREA T0	AREA PI T	AREA PII T	AREA PII T	AREA PII T	AREA PIII	AREA PIII	AREA PIII	pHPIT0	pHPIIT0	pHPIIIT1
HC1	870.4545	1589.205	4751.136	5052.273	4268.182	91.109	64.471	51.056	38.448	28.357	24.241	21.988	10.968	5.98	4.46	4.54
HC1	955.6818	1725.568	3941.477	4128.977	4538.068	89.885	58.725	40.986	33.416	21.554	18.081	14.539	14.513	5.63	4.05	3.82
HC1	986.9318	1282.386	3807.955	4785.227	4555.114	93.002	72.027	57.313	33.754	31.987	25.952	16.927	14.335	5.6	4.09	4.1
HC1	927.2727	1569.318	4225.568	4367.614	4495.455	87.962	60.479	35.631	33.517	24.834	25.035	13.442	17.434	5.73	4.02	4.39
HC2	455.6818	1978.409	3177.273	2861.932	3739.773	51.426	32.255	35.97	23.459	20.303	15.612	17.455	14.387	5.37	5.05	4.95
HC2	478.4091	1708.523	2969.886	3092.045	3711.364	88.633	39.294	31.096	32.508	24.879	20.99	25.059	13.103	6.07	5.65	5.36
HC2	503.9773	1444.318	3106.25	3361.932	3842.045	79.486	35.844	27.686	31.191	28.132	25.722	24.277	17.334	5.01	5.14	4.88
HC2	484.0909	1722.727	3160.227	3117.614	3717.045	79.674	37.574	34.863	34.522	30.38	23.488	20.249	15.076	4.6	4.86	5.14
HC3	986.9318	1441.477	3691.477	3969.886	4881.818	68.682	48.817	27.229	30.357	28.682	21.891	15.491	15.818	5.33	4.62	4.27
HC3	941.4773	1810.795	4827.841	3691.477	4435.795	60.125	49.52	45.127	43.514	24.234	16.981	12.847	10.551	5.34	4.38	4.02
HC3	972.7273	969.8864	2685.795	5330.682	5012.5	78.343	51.989	35.785	29.686	19.635	17.436	15.801	9.9799	5.32	4.1	4.36
HC3	992.6136	1407.386	2174.432	4543.75	4896.023	80.233	47.58	38.55	30.309	21.182	19.635	16.73	8.9208	5.34	4.26	4.43
HC4	910.2273	1961.364	2907.386	2626.136	2847.727	84.663	82.754	72.341	68.007	65.957	64.964	61.692	34.465	3.6	4.6	3.95
HC4	2782.386	2075	1765.341	2342.045	2668.75	94.515	92.113	89.641	67.824	62.889	52.589	43.936	34.475	3.26	4.43	4.12
HC4	2444.318	1211.364	3668.75	2685.795	2077.841	85.071	75.015	74.979	74.674	73.105	67.908	57.313	29.551	3.56	4.4	4.29
HC4	2421.591	1768.182	3438.636	2310.795	2512.5	93.063	91.876	83.673	79.501	72.693	65.745	58.252	28.99	3.51	4.01	4.57
MC1	219.8864	2941.477	4251.136	4540.909	3771.023	68.665	64.695	63.333	42.405	16.759	15.498	12.24	2.7864	4.22	5.56	4.87
MC1	322.1591	2833.523	4305.114	4398.864	4188.636	64.548	62.265	56.782	39.526	38.785	18.377	13.423	9.0321	4.35	5.46	5.58
MC1	458.5227	2637.5	3978.409	5455.682	4060.795	81.664	65.667	57.113	44.334	34.633	19.77	8.8426	8.6341	4.4	5.04	5.17
MC1	600.5682	2796.591	4094.886	4498.295	4003.977	72.849	63.798	58.451	46.883	29.511	17.474	7.1556	3.2129	4.23	4.48	5.6
MC2	657.3864	2288.068	4052.273	3052.273	3873.295	67.177	47.342	32.508	26.296	22.253	18.59	15.451	12.544	4.34	4.61	6.08
MC2	736.9318	2503.977	3844.886	2682.955	3114.773	69.869	50.472	33.695	29.824	25.206	20.86	17.612	16.872	4.28	5.07	4.62
MC2	626.1364	2719.886	2907.386	2850.568	2867.614	56.304	48.565	35.231	31.828	28.028	26.184	26.104	25.473	4.29	5.42	4.97
MC2	819.3182	2458.523	4492.614	2614.773	3376.136	61.552	59.313	49.689	40.071	28.736	26.177	24.907	6.2931	4.47	4.94	5.02
MC3	2535.227	2458.523	4055.114	3873.295	3288.068	61.692	56.366	29.895	27.167	25.213	23.123	18.23	6.7457	4.35	4.06	4.2
MC3	1901.705	2387.5	4290.909	3663.068	3711.364	63.781	50.98	27.433	26.94	25.265	17.635	14.28	9.8259	4.34	4.42	4.78
MC3	2776.705	2555.114	4265.341	2873.295	3739.773	66.131	41.986	31.584	27.061	23.815	21.27	18.287	7.1864	4.31	4.72	4.07
MC3	2512.5	2441.477	3421.591	3989.773	4123.295	49.228	38.448	33.036	25.724	23.419	22.241	10.866	4.9852	4.28	4.62	3.96
MC4	600.5682	1063.636	2330.682	2626.136	3467.045	47.998	42.509	38.228	35.008	30.127	29.954	26.003	23.32	4.04	3.94	3.96
MC4	682.9545	2893.182	3185.795	2310.795	3114.773	49.118	40.905	37.079	32.456	30.027	28.449	25.972	24.168	4.04	3.84	4.13
MC4	776.7045	1790.909	3177.273	2646.023	3376.136	49.657	46.028	40.424	33.361	31.129	28.091	24.673	22.649	4.03	4.16	4.39
MC4	620.4545	2484.091	3506.818	2055.114	2850.568	47.962	47.0548	42.396	38.336	32.368	31.386	29.045	27.612	4.05	4.55	3.93
LC1	1600.568	1771.023	3137.5	1768.182	1930.114	26.824	20.419	19.9	19.787	18.664	18.608	18.461	18.462	3.88	3.71	3.49
LC1	2126.136	1839.205	2651.705	1700	1631.818	27.22	20.32	16.249	16.223	16.221	16.217	16.181	16.122	3.58	3.52	3.49
LC1	1540.909	972.7273	2182.955	2040.909	1898.864	28.39	25.213	19.218	19.111	17.394	17.233	17.003	16.816	3.79	3.65	3.52
LC1	1242.614	1765.341	1668.75	1827.841	1859.091	26.425	24.604	16.631	16.366	16.102	15.558	15.185	11.338	4.01	3.72	3.47
LC2	1103.409	989.7727	526.7045	1606.25	910.2273	34.866	25.848	21.884	18.919	17.454	15.003	14.447	14.353	3.7	3.77	3.85
LC2	228.4091	918.75	708.5227	1109.091	1253.977	25.31	24.343	17.795	17.7	16.998	17.102	17.314	17.145	3.72	3.64	3.6
LC2	1029.545	867.6136	1208.523	1191.477	1307.955	35.626	27.686	21.876	18.564	16.831	16.459	16.115	15.746	3.74	3.68	3.62
LC2	827.8409	984.0909	1245.455	1103.409	930.1136	32.634	25.478	20.539	20.136	19.783	19.5	19.142	18.912	3.69	3.67	3.62
LC3	1038.068	1532.386	1310.795	2583.523	2370.455	38.932	26.434	24.548	21.869	21.651	21.832	21.451	20.983	3.75	3.7	3.68
LC3	2006.818	1532.386	2052.273	2043.75	2475.568	36.941	28.678	23.886	21.2	20.141	20	19.996	19.572	3.75	3.77	3.64
LC3	1583.523	2682.955	2040.909	2228.409	2194.318	28.463	26.589	22.919	22.571	21.804	21.692	21.6	21.503	3.77	3.64	3.6

LC3	1012.5	1697.159	1467.045	2001.136	2785.227	24.87	22.311	19.174	19.121	19.18	19.098	19.067	18.692	3.78	3.69	3.62

pHPIIIT2	pHPIIIT3
4.33	4.12
3.93	4.01
4.04	4.38
4.41	4.36
4.75	4.33
5.21	4.95
5.65	5.86
4.34	5.84
5.49	5.58
4.33	5.22
4.41	5.56
4.76	5.01
3.99	3.18
4.04	3.85
3.97	3.56
4.18	3.88
5.21	5.24
5.44	5.89
4.72	5.63
5.58	5.2
4.72	4.95
5.23	4.96
5.48	4.21
5.03	5.09
4.17	3.82
5.5	3.94
4.09	3.83
4.12	3.66
4.16	3.78
3.98	3.8
3.92	3.78
3.99	3.78
3.56	3.21
3.48	3.26
3.49	3.26
3.52	3.32
3.62	3.69
3.17	3.48
3.61	3.51
3.75	3.43
3.67	3.45
3.55	3.44
3.65	3.45