THE STUDY OF REHABILITAION OF HYDROCARBON CONTAMINATED SOIL USING BIOREMEDIAL MICROBES

By

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A dissertation submitted in fulfillment of the requirements for the degree of Master of Science in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand, South Africa.

August, 2006

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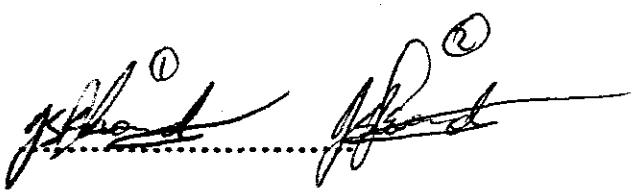
ABSTRACT

The study investigated the feasibility of bioremediation as a treatment option for chronically diesel-oil-polluted soil at petroleum and gas depot of Oilco (a company that is a division of Shell) situated at the east side of Empangeni which is in the Northern KwaZulu-Natal province, South Africa. To examine the efficiency of bioaugmentation, the contaminated site was treated with microbes, (previously isolated from the diesel-contaminated soil) to a depth of ±1, 2 meters, ±5 meters wide and 2 meters in length, plus the woodshavings as their nutrient source.

The effectiveness of bioremediation was observed over a period of 11 weeks and samples were taken at 15-day intervals. Over that period of 11 weeks, the changes in hydrocarbon concentrations were monitored in the soil and soil leachate and the accompanying changes in the soil microbial counts and activity. A significant reduction in the diesel-oil level could be achieved. The BTEX method was used in GCMS to check for changes in TPH. Prior to GCMS analysis the soil texture was analyzed using the Particle Size Determination method and the soil was observed to be sandy-loam (Day, 1995). For checking the soil microbial counts and activity the following groups of microbes were observed Aerobic Total Counts, *Nitrofyers, Nitrosofyers and Free-living nitrogen fixing bacteria* (Chan,*et. al*, 1993). The four groups of microbial counts were used as a biological parameter, and there was a correlation between each other as well as with the residual hydrocarbon concentration, indicating the importance of biodegradation. The effect of biostimulation of the indigenous soil microorganisms declined with time during the study.

DECLARATION

I declare that this dissertation hereby submitted to the University of Zululand for the degree of Master of Science has not been previously submitted by me for a degree at this or other University, that it is my own work in design and execution, and that all material contained therein has been duly acknowledged.



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22/02/2007 (16/04/2007)

Date

DEDICATION

I dedicate this work mostly to my father, uNdabezitha, uMthiya ngankomo abafokazana bethiya ngamahlahla, and my mother, uMavilakazi, Mphephethwa, if it wasn't for the encouragement from the two of you I don't think I would have come this far. You are undoubtedly the best parents one could ever ask for.

ACKNOWLEDGEMENTS

To the Almighty, thanks very much for the strength and courage and all the blessings, really in YOU there is no failure.

All the thanks to Dr. Basson, Prof. Kelbe, Prof. Lin and Mr. Mulder and Dr. Ngubane, without your assistance I wouldn't have done a thing. Prof. Kelbe and Dr. Basson without your constructive criticism I wouldn't have made the best out of it.

All my friends and family, thanks for every support throughout the course of the study. Thabile, you are the best for understanding everything and encouraging me. My gratitude goes to NRF for all the funds. Joan Otto and Dr. van Rossum thanks for all the assistance, may the good Lord bless you. Last but not least OILCOR without that spill in your premises I wouldn't have conducted such an interesting research thanks for allowing me to conduct my research within your premises and may your company grow even further.

May God bless you all!!!!!!!!

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Chapter 1

1. Introduction.

Biological processes which take place in natural environments can modify organic contaminants at spill location or during their transport in the subsurface. Such biological transformations, which involve enzymes as catalysts frequently bring about extensive modifications in the structure and toxicological properties of the contaminated soil. These biotic processes may result in the complete conversion of the organic molecule to innocuous inorganic end products, causing major changes that may result in new organic products, or occasionally lead to minor modifications. The available information suggests that the major agents causing the biological transformations in the soil, sediments surface water and ground water are the indigenous microorganisms that inhabit these environments (Westlake *et.al.*, 1974).

The amount of petroleum hydrocarbons that reaches the water table is often only a small fraction of the overall mass of the release from an underground storage tank. Yet this fraction is often the most troublesome and expensive to remove. One gallon of gasoline is enough to render one million gallons of groundwater unusable based on drinking water standards. It is through the groundwater that contaminants can seep beneath homes, be drawn into wells, and enter the homes of millions of people. Groundwater users are at risk when groundwater becomes contaminated (Noonan & Curtis., 1990).

Biodegradation can be defined as the microbially catalysed reduction in complexity of chemicals. In the case of organic compounds, biodegradation frequently, although not necessarily always leads to conversion of carbon, nitrogen, phosphorus, sulphur and other elements in the original compound to inorganic end products. Such a conversion of much

of an organic substrate to inorganic end product is known as mineralisation. Thus in the mineralisation of organic C (Carbon), N (Nitrogen), P (Phosphorus), S (Sulphur) and other elements, CO_2 or organic forms of N, P, S or other elements are released by the organisms and enter the surrounding environment (Benton, 1997). A cheap, effective and safe method for waste removal could possibly be done by microbial degradation (Venkanteswaran & Harayama, 1995).

Bioremediation is a treatment technology often used to clean up soils accidentally contaminated with petroleum hydrocarbons (HCs). It is an engineered process in which the natural biodegradation of petroleum HCs by indigenous soil bacteria, fungi, and protozoa is accelerated. Soil conditions for the optimal growth of bacteria are controlled through the addition of fertiliser to promote microbial activity and lime to control pH. Amendments in the form of organic matter (e.g., wood chips, sawdust, or peat moss) may be introduced to improve soil conditions (Ausma, *et. al*, 2002)

Bioremediation is the utilisation of microorganisms to remove pollutants from the environment. It is an acceleration of the natural fate of the biodegradable pollutants and hence a natural, or "green solution," to the problem of oil pollutants that causes minimal, if any, additional ecological effects. The most cost effective methods are generally *in situ* because these avoid costly movement of contaminated soils and waters. Bioremediation has become an important method for oil spill cleanup. Populations of hydrocarbon degraders generally are less than 1% of the total microorganisms in unpolluted environments but increase between 1% to 10% in environments exposed to petroleum pollutants. Mixed cultures of nongenetically engineered micro-organisms are commonly proposed as inocula for seeding to bioremediate oil contaminated soils and waters. A

genetically engineered hydrocarbon-degrading *pseudomonad* was the first patented organism in a landmark decision of the U.S. Supreme Court that greatly increased the economic potential of biotechnology. There is considerable controversy surrounding deliberate environmental release of genetically engineered microorganisms, and given the current worldwide regulatory framework for the deliberate release of genetically engineered microorganisms, it is unlikely that any such organism could currently gain the necessary regulatory approval in time to be of much use in treating an oil spill (Atlas 1995).

The loss mechanisms to which HCs are subjected in the soil during bioremediation include abiotic physical processes such as volatilization and leaching, as well as chemical and biological removal. These processes contribute to both a reduction in total hydrocarbon concentration (THC) in the soil and changes in the overall HC composition. The mobility and availability of HCs in soil depends on the physical and chemical properties of both the soil and the HCs present. (Ausma, *et. al.*, 2002)

The extent of hydrocarbon biodegradation in contaminated soils is critically dependent upon four factors, namely (1) The creation of optimal environmental conditions to stimulate biodegradative activity, (2) the predominant petroleum hydrocarbon types in the contaminated matrix and (3) the bioavailability of the contaminants to microorganisms. The petroleum hydrocarbon degradation is also affected by (4) the molecular composition of the hydrocarbons, characteristic which is directly related with the bioavailability of these compounds, and as a consequence, the biodegradation rate may be altered (Huesemann, 1995).

Bioremediation provides a means of enhancing the natural degradation process in which organic molecules are converted to biomass and harmless products such as carbon dioxide and water. Natural levels of nitrogen and phosphorus are often unable to support the microbial requirements following a sudden increase in hydrocarbon levels associated with an oil spill (Meyers *et. al.*, 1999). Natural communities of microorganisms present in the subsurface have an amazing physiological versatility. Microorganisms can carry out biodegradation in many different habitats and environments both under aerobic and anaerobic conditions. Communities of bacteria and fungi can degrade a multitude of synthetic compounds and probably a very natural product (Boonchan, *et.al.*, 2000).

Hazardous compounds persist in the surface because environmental conditions are not appropriate for the microbial activity that results in biochemical degradation. The optimisation of environmental conditions is achieved by understanding the biological principles under which these compounds are degraded and the effect of environmental conditions on both the responsible microorganisms and their metabolic reactions. Bioremediation potentially offers a number of advantages over traditional methods of treating toxic organic chemicals contaminating the environment. Such advantages include complete destruction of the contaminants, lower treatment cost, and greater safety and less environmental disturbances. Most studies on bioremediation involve either stimulating indigenous microbial populations that are known degraders to a contaminated site, a process also known as bioaugmentation (Boonchan, *et. al.* 2000).

Conversely, bioremediation is still not optimised to rehabilitate hydrocarbon contamination in soil environments. The findings of laboratory experiments cannot always be applied directly to the field. For instance growth and survival of microorganisms is affected by

environmental factors including temperature, soil type, nutrient and water availability. Many of these factors are not subject to stringent human control if at all in natural environment (Boonchan *et al.*, 2000).

Chapter 2

Literature review

2.1. Bioremediation

Indigenous, selectively adapted, or genetically altered microorganisms can potentially be used to degrade gasoline components dissolved in groundwater. The use of microbes to renovate contaminated aquifers is termed "Biorestoration." This technique, although not yet as well known or as widely used as either air stripping or carbon adsorption, is a very promising method for groundwater cleanup. Disadvantages of biorestoration include: (1) this technique cannot be used where a quick start-up is needed (biorestoration typically takes 4-6 weeks for acclimation; and (2) it is not successful in a start/stop mode; that is, it must be continued 24 hours per day, 7 days a week (Noonan & Curtis, 1990).

2.2. The use of bioremediation

Vidali (2001) provided a useful overview of bioremediation techniques and the advantages and disadvantages of the technology. Commercially available bioremediation products, including mixtures of oil degrading microbial inoculums and sources of nutrients, have been patented and marketed since the early 1970's (Linn, 1971; Zhu et al., 2004). Rapid commercialisation of bioremedial technologies occurred following the use of various bioremediation techniques after the Exxon Valdez spill in 1989, with venture capitalists taking advantage of growing markets (Macdonald, 1997). Low confidence in the efficiency of bioremediation, particularly when applied *in-situ*, may be due to the relatively few validated field trials that have been reported.

2.3. Health safety concerns

Given that commercially available inocula are freely available for bioremedial applications, there is still little guidance or regulation in the use of microbes for remediation, especially with regard to health and safety. This is surprising, given that physical works involved in the bioremediation of soils may be performed by a variety of workers and not necessarily by microbiologists. Therefore it is important that all those intending to perform bioremediation are aware of the risks associated with the use of microorganisms and are adequately trained in the use of protective equipment and hygiene practices. For example, microbial inoculums may be provided as dry powders which may be inhaled. In addition, there is an increasing interest in the use of nutrients and waste products as soil amendments to stimulate bioremediation (Vasudevan & Rajaram, 2001). However, the use of such amendments, particularly in batch *ex-situ* techniques, may provide a suitable environment for the considerable growth of potentially harmful moulds and fungi. Other risk factors include the metabolic products of microbial activity since partial or incomplete degradation of hydrocarbons that may result in compounds that are more harmful or toxic than the original compounds (Vogel & McCarty, 1985).

Concern has also been expressed that microorganisms isolated from soils that have considerable potential for metabolizing hydrocarbon substrates also pose human health risks in terms of opportunistic infections and allergenicity. Most notably, the potential use of organisms such as *Burkholderia* (previously *Pseudomonas*) *cepacia* has come under scrutiny due to the serious nature of the disease this organism can cause in cystic fibrosis and immunocompromised indivisuals (Holmes *et al.*, 1998; Berg *et al.*, 2005). Furthermore, Holmes *et al.*, (1998) suggests that potential exists for the evolution of multiple antibiotic resistant pathogenic organisms through horizontal gene transfer and that

further work is required toestablish the risks of widespread use of *B. cepacia* in agriculture and bioremediation.

2.4. Microbiology of bioremediation in groundwater

The degradation of contaminants in groundwater by indigenous microorganisms may be the only way to completely remove the pollutant. This is both cost-effective and feasible since the enhanced degradative potential of the microbial community may lead to biorestoration of a polluted site (Armstrong, 1991).

The long residence time of contaminants in groundwater provides microorganisms with ample opportunity to adapt to the carbon source (Ghiorse & Wilson, 1988). Adaptation refers to the increased rate of biotransformation of a compound due to previous exposure of the microorganisms to the compound (Armstrong, 1991). The number of bacteria in contaminated wells has been shown to be greater than the number in pristine wells (Armstrong, 1991). Shallow subsurface microbial communities have been shown to exhibit adaptation for growth and survival conditions which are poor in nutrients, survive in a wide range of nutrient concentrations, and may affect the chemistry of groundwater (Armstrong, 1991). Microbial communities that have previously been exposed to organic pollutants can biodegrade the compounds at much faster rates than microbes without previous exposure to pollutants (Armstrong, 1991).

The saturation zone consists of various types of anaerobic bacteria including heterotrophs, denitrifiers, sulphate reducers, methane formers, and hydrocarbon oxidisers (Ghiorse & Wilson, 1988). Bacteria that have been found to exist in groundwater environments include species of *Pseudomonas, Mycobacterium, Actinomyces, Bacterium,*

Pseudobacterium, Thiobacillus, and *Methanomonas* (Dunlap & McNabb, 1973). Species of *Pseudomonas, Mycobacterium,* and *Actinomyces* are also commonly found in the upper soil layers. The latter can grow in anaerobic environments by using nitrate or organic compounds as electron acceptors in place of oxygen. These three bacteria use a wide range of organic compounds as nutrients. There are various species of *Pseudomonas* that are capable of attacking and degrading a vast number of organic compounds; however, they may require aerobic conditions (Dunlap & McNabb, 1973).

There are several methods of removing gasoline constituents dissolved in groundwater, including air stripping, activated carbon adsorption, biorestoration, resin adsorption, reverse osmosis, ozonation, and oxidation with hydrogen peroxide, ultraviolet irradiation, and land treatment. All of these methods are capable of removing, destroying, or detoxifying all or some of the gasoline contaminants under the right circumstances. Air stripping and activated carbon adsorption, however, are the most cost-effective and widely applied in actual practice. Air stripping and/or activated carbon adsorption are applicable to most cases where gasoline has contaminated local groundwater. They offer the highest levels of effectiveness in reducing contaminants to low levels over a wide range of situations, as well as being fairly cost effective. Biorestoration is a technology that has only recently begun to receive attention; although promising, it has yet to be proven as a viable widespread method for controlling groundwater contaminants (Noonan & Curtis, 1990).

2.5. Microbiology of bioremediation in the soil

The heterotophic microorganisms found in the soil include naturally occurring populations that have the ability to degrade petroleum products (Englert *et al.*, 1993). Many genera of

hydrocarbon-degrading bacteria and fungi can be isolated from soil. In decreasing order, species of *Pseudomonas*, *Arthrobacter*, *Alaligenes*, *Corynebacterium*, *Flavobacterium*, *Achromabacter*, *Micrococcus*, *Nocardia*, and *Mycobacterium* appear to be the most consistently isolated hydrocarbon-degrading bacteria from soil (Englert *et al.*, 1993). All of *Pseudomonas*, *Arthrobacter*, and *Alcaligenes*, the top three most important hydrocarbon-degrading bacteria in soil, are either obligate or facultative aerobes. In decreasing order, *Trichoderma*, *Penicillium*, *Aspergillus*, and *Mortierella* are the hydrocarbon-degrading fungi most consistently isolated from soil (Englert *et al.*, 1993).

Members of the genus *Pseudomonas* are the most predominant group of soil microorganisms that degrade xenobiotic compounds in general (Glick & Pasternak, 1994). Biochemical assays have shown that various *Pseudomonas* strains can break down and detoxify more than 100 different organic compounds. In many cases, one strain can use several different related compounds as a sole carbon source (Glick & Pasternak, 1994). The biodegradation of complex organic molecules generally requires the concerted effort of several different enzymes. The genes that code for the enzymes of these biodegradative pathways are sometimes located in the chromosomal DNA, although they are more often found on plasmids. This is beneficial in soil bioremediation because plasmids can be transferred among different strains of bacteria, enabling many to biodegrade xenobiotic compounds that would not otherwise have the capability (Glick & Pasternak, 1994).

2.5.1. Soil as a microbial habitat.

The most extensive microbial growth takes place on the surfaces of soil particles, usually within the rhizosphere (the soil that surrounds plant roots). Even a small aggregate can have many differing microenvironments and thus several different types of microorganisms

may be present. One of the major factors affecting microbial activity in the soil is the availability of water. Water is a highly variable component of the soil, its presence depending on soil composition, rainfall, drainage, and plant cover. Water is held in soil in two ways, by adsorption onto surfaces or as free water existing in thin sheets or films between soil particles. The water present in soils has a variety of materials dissolved in it, the whole mixture being referred to as the soil solution. In well-drained soils, air penetrates readily and oxygen concentrations can be high. In waterlogged soils however, the only oxygen present is that dissolved in the water, and this is soon consumed by microorganisms. Such soils quickly become anoxic, showing profound changes in their biological properties. The nutrient status of a soil is the other major factor affecting microbial activity. In some soils carbon is not a limiting nutrient, but instead the availability of inorganic nutrients such as phosphorus and nitrogen limit microbial productivity (Madigan *et al.*, 2000).

2.6. Environmental conditions in the groundwater

There are several environmental conditions that influence the growth of microorganisms in the subsurface saturation zone. These factors are water availability, nutrients, oxygen, temperature, carbon source, and pH (Dunlap & McNabb, 1973).

Microorganisms require water for growth. The cells must be surrounded by a layer of water in order to permit nutrient and toxic product diffusion. As well, the cells must maintain an adequate amount of water in the cell in order to carry out metabolic and reproductive processes (Dunlap & McNabb, 1973). Water movement in the saturation zone is lateral towards the discharge point. The upper part of the zone of saturation exhibits the highest rate of movement, not exceeding a few years before discharge (Dunlap

& McNabb, 1973). The water in the saturation zone varies in its distribution, movement, and mineral content; however, it does not seem possible that limited water availability would pose a problem for microbes in the saturation zone (Dunlap & McNabb, 1973).

The groundwater must have the nutrients that are required for microbial synthesis of protoplasmic constituents and energy production used in metabolic activities (Dunlap & McNabb, 1973). Most pristine subsurface environments are oligotrophic which means there is a low supply of nutrients, and relatively low primary productivity of micro-organisms (Ghiorse *et al.*, 1988). This is because most readily metabolised compounds will be consumed by the microflora at the surface prior to reaching the unsaturated subsurface horizon above the groundwater (Ghiorse *et al.*, 1988). Sulphate is found in the upper regions of the groundwater; however, nitrogen and phosphorus are usually limiting nutrients. There may be regions which are deficient in ammonium, nitrate and phosphate (Ghiorse *et al.*, 1988). The absence of phosphorus and nitrogen may not imply limited growth or abnormal activity of the microbes unless there is an excessive amount of the carbon source (as with a polluted aquifer) (Ghiorse *et al.*, 1988).

Microbial activity in the groundwater is associated with activities that are likely to reduce molecular oxygen and other reducible substances (Dunlap & McNabb, 1973). Therefore, the concentration of oxygen and the oxidation-reduction potential of the groundwater have a large impact on microbial activity as well as the geochemistry of various substances in aquifers (Ghiorse *et al.*, 1988). Both organic and inorganic matter is oxidised in the saturation zone. This oxidation process consumes oxygen that may not be available in shallow anaerobic aquifers (Ghiorse *et al.*, 1988). The amount of dissolved oxygen in a

pristine aquifer has been shown to be several time that amount measured in contaminated aquifers (Armstrong *et al.*, 1991).

2.6.1. Deep subsurface microbiology.

Interest in the chemistry of groundwater and the potential leaching of pollutants and their transfer in groundwater aquifers had led to studies of the role microorganisms play in the deep subsurface terrestrial environment. The deep soil subsurface, which can extend for several hundred meters below the soil surface, is not a biological wasteland. A variety of microorganisms; primarily bacteria, are present in most deep underground soils. In samples collected aseptically from boreholes drilled down 300m, a diverse array of bacteria have been found including anaerobes such as sulphate-reducing bacteria, methanogens, and homoacetogens, and various aerobes and facultative aerobes. Microorganisms in the deep subsurface presumably have access to nutrients because groundwater flows through their habitats, but activity measurements suggest that metabolic rates of these bacteria are rather low in their natural habitats. Compared to microorganisms in the upper layers of the soil, the biogeochemical significance of deep subsurface microorganisms may thus be minimal. However, there is evidence that the metabolic activities of these buried microorganisms may over very long periods be responsible for some mineralization of organic compounds and release of products into the groundwater. The potential for *in situ* bioremediation of toxic substances leached from soil into ground water (for example, benzenes and agricultural chemicals) by deep subsurface microorganisms is of particular current interest (Madigan *et al*, 2000).

2.7. What are the main components of biochemical reaction rates in an *in-situ* project?

An *in situ* bioremediation project is made up of four major components: microorganisms, oxygen, nutrients, and environment. The microorganisms are the workhorse of the project. The bacteria use the organics that were released to the environment as a source of food. Chemical bonds in organic molecules act as the source of energy for the endemic bacteria and as building blocks for reproduction. Bacterial growth and reproduction occurs naturally, not because they comprehend the regulatory consequences of a contaminant plume (Nyer, 1998).

The large amount of time and money that has been spent on *in situ* projects has gone towards trying to determine whether the bacteria that are necessary for the degradation are present at the site or if specialised bacteria must be imported. The most expensive approach to try and answer this question is to try to identify the bacterial species that can degrade the specific compound found at the site. In reality, multiple organisms work in concert during the degradation of an organic compound. In field, a single bacterial species is never responsible for site bioremediation. If the compounds are degradable, then the natural bacteria at the site are usually able to degrade the compounds. The only times that bacteria need to be introduced to a site is when a toxic condition has existed at the site and has killed all of the natural bacteria. The cleanup of new spills may be enhanced by introducing mixed bacterial cultures. These circumstances assume that the organics are degradable. In general, petroleum hydrocarbons are degradable and chlorinated hydrocarbons are less degradable. The more chlorine substitutions on the organic compound, the less degradable the compound (Nyer, 1998).

While the bacteria are the key to bioremediation, at the present time it cannot be affected whether the appropriate bacteria are present at the site of the organic contamination. In most cases if the compound is degradable, the natural population has already adapted to the available organic compound and is using the compound as a food source. Simple microbial tests can be conducted on the soil to confirm the presence of viable bacterial populations and those that are capable of degrading the specific organic compound that was released (Nyer, 1998).

The real object of an *in situ* project is to enhance natural bacterial growth and reproduction. This is done by supplying the factor that is limiting the reaction rate of bacteria. The main limitations are oxygen, moisture, and nutrients, NH_3, and PO_4. It must also be ensured that the environment is suitable. Oxygen is the main rate- limiting factor in organic chemical degradation. The bacteria need large amount of oxygen to produce energy. Many *in situ* projects have required oxygen without nutrient addition. Moisture is the second most important factor. If the unsaturated zone contains too little moisture, then the bacteria will not have the microenvironments that they need to survive. The bacteria also require macronutrients and micronutrients to reproduce; the macronutrients are nitrogen in reduced form, NH_3, and phosphorus in the most oxidised form (PO_4). Micronutrients are almost always present in either the soil or aquifer and do not have to be considered in an *in situ* project. Nutrients are needed in situations where there is a need to grow a large bacterial population. This would be appropriate for large spills or when it is necessary to minimise the total project time. Both macro- and micronutrients can interact with the soil matrix (Nyer, 1998).

2.8. Microbial metabolism

During the process of *in situ* bioremediation, microorganisms use the organic contaminants for their growth. In addition, compounds providing the major nutrients such as nitrogen, phosphorus and minor nutrients such as sulphur and trace elements are also required for their growth. In most cases, an organic compound that represents a carbon and energy source is transformed by the metabolic pathways that are characteristics of heterotrophic microorganisms. It should be stressed however that an organic compound need not necessarily be a substrate for growth in order for it to be metabolised by microorganisms. Two categories of transformations exist. In the first, biodegradation provides carbon and energy to support growth and the process, therefore growth linked. In the second, biodegradation is not linked to multiplication, but to obtaining carbon for respiration in order for the cells to maintain their viability. This maintainance metabolism may only take place when the organic carbon concentrations are very low. Co-metabolic transformations also fall into the second category (Baker, *et al.* 1993).

It has been observed by a number of researchers that the number of microbial cells or the biomass of the species acting on the compound of interest increases as degradation proceeds. During typical growth linked mineralisation brought about by bacteria, the cells use some of the energy and carbon of the organic substrate to make new cells, and this increasingly larger populations cause increasingly rapid mineralisation (Baker, *et.al* 1993). Microorganisms need nitrogen, phosphorus and sulphur and a variety of trace nutrients other than carbon. These requirements should be satisfied as the species degrade the compound of interest. For the heterotrophic microorganism in the most natural systems usually sufficient amounts of N, S, P and other trace nutrients are present to satisfy the

microbial demand. Because carbon is limiting and because it is the element for which there is intense competition, a species with the unique ability to grow synthetic molecules has a selective advantage (Chan *et al.*, 1993).

Prior to degradation of many organic compounds a period is observed in which no degradation of the chemical is evident. This time interval is known as acclinitisation period or sometimes as adaptation or lag period. The length of the acclinitisation period varies and may be less than 1hr or many months (Chan, *et al.*, 1993).

Bioremediation of hydrocarbon-contaminated soils, which exploits the ability of microorganisms to degrade and/or detoxify organic contamination, has been established as an efficient, economic, versatile, and environmentally sound treatment. On-site-off-site and *in situ* systems may be used. Decontamination of polluted sites in cold climates has received increasing interest recently. Considerable oil bioremediation potential has been reported for a variety of terrestrial and marine cold ecosystems, including arctic, alpine, and antarctic soils; Alaskan groundwater; and antarctic seawater and sea ice. Environmental temperatures play a significant role in controlling the nature and extent of hydrocarbon metabolism. Temperature affects the rate of biodegradation, as well as the physical nature and chemical composition of hydrocarbons (Margesin & Schinner., 1999).

Monitored natural attenuation (intrinsic bioremediation) is becoming the accepted option for low-risk oil-contaminated sites and is a cost-effective remediation alternative as it has few costs other than monitoring costs and the time required for natural processes to proceed. Biodegradation is most often the primary mechanism for contaminant destruction; however, physical and chemical processes, such as dispersion, dilution,

sorption, volatilisation, and abiotic transformations, are also important. The most widely used bioremediation procedure is biostimulation of the indigenous microorganisms by the addition of nutrients, as input of large quantities of carbon sources (i.e. contamination) tends to result in rapid depletion the available pools of major inorganic nutrients, such as N and P. Several studies of the effects of biostimulation with mainly N-P-K or oleophilic fertilisers have reported positive effects on oil decontamination in cold ecosystems (Margesin & Schinner, 1999).

Microbial degradation of gasoline components can occur by aerobic respiration, anaerobic respiration, or fermentation. Aerobic microorganisms utilise oxygen in the process of decomposing hydrocarbons; anaerobes utilise inorganic compounds such as sulphate, nitrate, or carbon dioxide as terminal electron acceptors; and under fermenting conditions organic compounds serve as both electron donors and acceptors during microbe activity. Major gasoline components such as the aromatics and alkanes, as well as some minor constituents such as ethylene dibromide (EDB) and ethylene dichloride (EDC), have been shown to be more readily degradable under aerobic than under either anaerobic or fermenting conditions. By-products of anaerobic decomposition, such as methane and sulphide, and of fermentation reactions, such as organic acids and alcohols, may also pose greater system management problems than those associated with the aerobic decomposition products carbon dioxide and water (Noonan & Curtis, 1990).

2.9. Oil pollutants

Over a million tons of oil pollutants enter the marine environment each year as a result of accidental spillages and disposal of oily wastes. Most oil pollution problems originate from minor spillages which result from routine operations. Microbial degradation of

petroleum pollutants are a common natural process and are the reason oceans are not covered with oil. However, it is an immense challenge of microorganisms to degrade all components of petroleum mixture. Parts of this mixture are toxic to many microorganisms. As complexity of the mixture increases so does the resistance to biodegradation. Even at low concentrations, dissolved components of petroleum can disrupt the processes of some marine organisms. Microbial biodegradation of these compounds requires suitable growth temperatures and available supplies of fixed forms of nitrogen, phosphate and molecular oxygen. In addition, more rapid rates of degradation occur when there is a mixed microbial community than can be accomplished by a single species. Thus, in many cases environmental factors, rather than the genetic capability of a microorganism, limit the biodegradation of these pollutants (Atlas, 1981).

Petroleum is a complex mixture composed primarily of aliphatic, alicyclic, and aromatic hydrocarbons. There are hundreds of individual compounds in every crude oil, the composition of each crude oil varying with its origin. As a result, the fate of petroleum pollutants in the environment is complex. The challenge for microorganisms to degrade all of the components of a petroleum mixture is immense. Nevertheless, microbial biodegradation of petroleum pollutants is a major process and is the reason that oceans are not covered with oil today. As an example of the ability of microorganisms to degrade petroleum pollutants, measurements indicate that after the 1978 wreck of the supertanker Amoco Cadiz off the coast of France, microorganisms biodegraded 10 tons of oil per day in the affected area. Microbial biodegradation represented the major process responsible for the ecological recovery of the oiled coastal region (Atlas, 1995).

The susceptibility of petroleum hydrocarbons to biodegradation is determined by the structure and molecular weight of the hydrocarbon molecule. n-Alkanes of intermediate chain length ($C_{10} - C_{24}$) are degraded most rapidly. Short chain alkanes (less than C_9) are toxic to many microorganisms but they generally evaporate rapidly from oil slicks. As alkane chain length increases, so does resistance to biodegradation. Branching, in general, reduces the rate of biodegradation because tertiary and quaternary carbon atoms interfere with degradation mechanisms or can block degradation altogether. Aromatic compounds, especially of the condensed polynuclear type, are degraded more slowly than alkanes. Alicyclic compounds are frequently unable to serve as the sole carbon sources for microbial growth unless they can be degraded via cometabolism by two or more co-operating microbial strains with complementary metabolic capabilities (Atlas, 1995).

2.10. Diesel as a pollutant

Diesel is largely comprised of simple un-branched *n*-alkanes, with only around 4% of poly-aromatic compounds (Heath *et al.*, 1993.). Although metabolism of *n*-alkanes from C_2 to C_{12} is possible (Chakrabarty, 1973) these may however act as solvents, permeabilising cells by partial solubilisation of membrane phospholipids (Sikkema & Poolman, 1993) and are therefore toxic to many microorganisms. The initial enzymes required for alkane metabolism are mono-oxygenases. Meta-cleavage dioxygenases are key enzymes in the degradation of aromatic compounds (Daly *et al.*, 1997). Polycyclic aromatic hydrocarbons (PAHs) such as naphthalene and phenanthrene are readily biodegradable; however, PAHs with more than five rings may be recalcitrant (Allard & Neilson, 1997). As these enzymes consume oxygen, it must be available in sufficient quantities to prevent limitation of hydrocarbon degradation. One approach to the enhancement of oxygen transfer in

constructed windrows or biopiles is the addition of bulking agents such as woodchips, sawdust, leaves or shredded rubber tyres to improve the porosity of soils (Cookson, 1995).

2.10.1. The bioremediation mechanism

The petroleum hydrocarbons belong to the family of organic compounds called alkanes. The aerobic biological mechanism of petroleum hydrocarbon metabolism in both long chain hydrocarbons occurs monoterminally to the corresponding alcohol, aldehyde and monobasic fatty acid. The primary alcohol derived from petroleum hydrocarbons is oxidised to the corresponding aldehyde by alcohol dehydrogenase, and the aldehyde is oxidised to a fatty acid by aldehyde dehydrogenase. The end by-products of these reactions are fatty acids, carbon dioxide, and water (Ranart Environs, 2003).

2.11. Pathway of Petroleum Hydrocarbon Oxidation

$H_3C - (CH_2)\ nCH_3$

$\downarrow \rightarrow O_2$

$H_3C - (CH_2)\ nCH_2OH$

(Primary Fatty Acid Alcohol)

$\downarrow$

$H_3C - (CH_2)\ n - CHO$

(Fatty Aldehyde)

$\downarrow$

$H_3C - (CH_2)\ n - COOH$

(Monocarboxylic Fatty Acid)

$\downarrow \rightarrow O_2$

$HOH_2C - (CH_2)\ n - COOH$

(Hydroxy Fatty Acid)

$\downarrow$

$HOOC - (CHn)\ n - COOH$

(Dicarboxylic Fatty Acid)

(Marine Environment Protection, 1999).

Aerobic biodegradation of aliphatic hydrocarbons with bacterial strains depends on biological (enzymatic activity ; steric hindrance-diffusion into the cells) and physico chemical parameters (solubility; emulsion effect; surface tension). As the most chemicals, intimate contact between the microbial cell surface and hydrocarbons appears necessary for high degradation rates. The most common pathway of alkane biodegradation is oxidation

at the terminal methyl group. The alkane is oxidised first to alcohol and then to the corresponding fatty acid. After formation of a carboxyl group the oxidation proceeds by successive removal of two carbon units through β-oxidation, which is universal to most living systems. Under β-oxidation, the beta methylene group is oxidised to a ketone group followed by the removal of a two-carbon fragment from the coumpound (Weiner, 2000).

2.12. Case history (on oil bioremediation in water)

The Alaska Oil Spill Bioremediation Project was to demonstrate the feasibility of oil bioremediation as a secondary cleanup tool on selected beaches in Prince William Sound, and to further the understanding of the microbial ecology of oil biodegradation on shorelines. It was shown that the addition of oleophilic slow-release/granular and nutrient solution fertilisers to oil-contaminated beaches in Prince William Sound increased oil biodegradation rates greater than four-fold over removal rates on untreated oiled beaches. The application of fertilizer solutions proved to be the most efficient system for exposing oil-degrading microorganisms to nutrients (Pritchard *et al.* 1992).

The Exxon Valdez spill formed the basis for a major study on bioremediation through fertiliser application and was the largest application of this emerging technology. Inipol (an oleophilic microemulsion with urea as a nitrogen source, laureth phosphate as a phosphate source, and oleic acid as a carbon source) and Customblen (a slow-release fertiliser composed of calcium phosphate, ammonium phosphate, and ammonium nitrate within a polymerised vegetable oil coating) were used. Within approximately 2 to 3 weeks, oil on the surfaces of cobble shorelines treated with Inipol and Customblen was degraded so that these shorelines were visibly cleaner than non-bioremediated shorelines. Monitoring of the oil-degrading microbial populations and measuring the rates of oil

degradation activities by a joint Exxon, United States Environmental Protection Agency (USEPA), and State of Alaska Department of Conservation team showed that a five fold increase in rates of oil biodegradation typically followed fertiliser application. The addition of fertilisers caused no eutrophicaltion, no acute toxicity to sensitive marine test species, and did not cause the release of undegraded oil residues from the beaches. Because of its effectiveness, bioremediation became the major treatment method for removing oil pollutants from the impacted shorelines of Prince William Sound. The success of the field demonstration program introduces the consideration of bioremediation as a key component (but not the sole component) in any cleanup strategy developed for future oil spills (Atlas & Bartha, 1998).

Data on the rate and extent of microbial degradation of oil was crucial to the acceptance of bioremediation as a cleanup technology. This enhanced biodegradation was evidenced by changes in several constituent hydrocarbon groups resulting in the disappearance of oil residues. Supporting studies demonstrated that bioremediation of oil is a reasonable and environmentally sound secondary cleanup procedure. It appears to work in both surface and subsurface beach material. Although there was an overall lack of general oil biodegradation at Disk Island, studies during the summer of 1990 at Elrington Island showed that a pulse application of nutrients provides sustained accelerated biodegradation of oil over a three to four week period. This pulse application phenomenon has significant potential for addressing future oil spills since it is as effective as a continuous long-term application. In addition, the use of sampling baskets containing homogenized beach material was a reliable method to do direct sampling of beach material (Pritchard *et al.*, 1992).

2.13. Natural cleaning

Observed timescales of the natural cleaning process of oil spillages range from a few days to 20 or more years. Given that in extreme cases, thick deposits of oil may remain after about 20 years, it is reasonable to extrapolate that natural cleaning may take several decades in some very sheltered environments. Natural cleaning timescales are affected by a number of factors e.g. oil type (viscosity movements into and out of sediment shores) and volume of oil (heavy loadings may lead to greater retention times in sediments), microbial flora, physical factors (eg. pH, temperature, and oxygen availability) of the environment (Baker *et al.*, 1993).

2.14. Problems encountered while conducting a bioremediation project.

The bioremediation of contaminated land is affected by a large number of variables, some of which can be easily measured, while others are more difficult to measure. The key factors include:

- Type of contamination (gasoline, diesel, heavy, crude, chlorinated, hydrocarbons etc.)
- Depth of contamination
- Intensity of contamination
- Temperature
- Soil type
- Hydrogeochemistry of site
- Availability of moisture and air
- pH level
- Presence of bactericides, such as some heavy metals.

- Bacterial predators
- Other chemicals (Oil cleaning bioproducts, 2003)

In view of these many variables, it is clear that even with a good site diagnosis it is not easy to make an exact prediction of the time necessary to achieve any given degree of bioremediation.Within the range of 10°C to 45°C, the rate of microbial activity typically doubles for every 10°C increase in temperature (Atlas & Bartha, 1998). Temperature will also influence the physical nature of hydrocarbons. For example, short chain alkanes will be more readily volatilised at higher temperatures (van Deuren *et al.*, 1997). Water availability in contaminated soils may limit microbial activity and growth. However, excessive water may result in closure of soil pores and therefore limit oxygen transfer. During treatment, water content is typically retained at 50-80% of soil water holding capacity (Cookson, 1995). The optimum pH range for hydrocarbon degradation in soil has been commonly reported as being between 6.5-8 (Morgan and Watkinson, 1989). Dibble and Bartha, (1979), concluded that pH 7.7-7.8 was optimal for hydrocarbon degradation and suggested that lower values may result in partial inhibition of degradation.

Microbial degradation represents the major route responsible for the ecological recovery of polycyclic aromatic hydrocarbons (PAHs) contaminated sites, however the success of bioremediation projects has been limited by the failure to remove high-molecular-weight PAHs. The recalcitrance of high-molecular-weight PAHs to microbial degradation has led to research focussed on evaluating a wide phylogenic spectrum of microorganisms for their degradative ability. This has resulted in identification of a diverse group of bacteria and fungi that partially degrade, cometabolically oxidize, or mineralise some high-molecular-weight PAHs to detoxified products. It is important to note that for effective and fast

degradation, microorganisms should have co-operative metabolic activities of mixed microbial populations (Boonchan, *et. al.*, 2000).

2.15. Eventual fate of oil remaining in the environment

Degradation of oil (as distinct from physical dispersion) occurs through both chemical and biological oxidation processes. The complete degradation of some hydrocarbons may involve a combination of oxidation and biodegradation. Chemical reactions are usually catalysed by light (photo-oxidation) and lead to a variety of oxygen containing intermediate compounds including alcohols, ethers, dialkyl peroxides, and carbonyl compounds. Factors affecting chemical oxidation include light intensity and duration, aeration, and oil thickness. Microbial degradation of PAHs is an important process involved in the eventual disappearance of oil from the environment. For microbial degradation of PAHs to take place effectively, the following requirements must be fulfilled:

1. Appropriate species of microorganism must be present. Over 200 species of bacteria and fungi are capable of degrading hydrocarbon compounds.
2. The temperature must be suitable for microbial activity.
3. There must be an adequate supply of oxygen. Because degradation is oxidative it proceeds slowly at low oxygen concentrations.
4. There must be an adequate supply of nutrients, notably nitrogen and phosphorus compounds (Atlas, 1995).

2.16. Soil and water microorganisms

Several areas of soil microbiology have been prominent for years. Microbiologists have long been interested in an ecosystem that contains a vast number of dissimilar species and

morphological types as well as a variety of beneficial and detrimental interactions among them. New areas of concern have arisen in recent years and one of these is the role of indigenous heterotrophs in detoxification. Soils receive herbicides, insecticides, fungicides, municipal and industrial wastes, and a variety of other toxic substances, and many of these are destroyed before the concentration of the toxicants rises to a point where they are of ecological concern. By thus acting as agents of biodegradation, microorganisms in the soil are a significant means of ridding natural environments of potentially hazardous chemicals and wastes. It has also been evident that the indigenous communities frequently fail to degrade or detoxify many toxic substances. These failings are evident in the increasing pollution of ground and surface waters in many regions and in the persistence of certain types of pesticides, for example, DDT (Atlas, 1995).

Bacteria are numerically the dominant group of microorganisms in the soil. However, because their cells are small, the total biomass of bacteria frequently is less than that of fungi. However, in terms of metabolic activity, certain processes in well-aerated soils are dominated by bacteria, and under anaerobic conditions, bacteria are chiefly responsible for biochemical changes underground. Because of the wide range of physiological and nutritional types of bacteria, no one culture medium and no single method are considered adequate for defining and estimating the size of the bacterial community of the soil. Plate counts on agar media give large numbers of bacteria, frequently from 10^6 to 10^8 or more per gram of soil. The size of the community varies with the location and the environmental conditions that prevail (Atlas, 1995)

2.17. The Nitrifying Bacteria.

Key genera: *Nitrosomonas*

Nitrobacter

These bacteria are able to grow chemolithotrophycally at the expense of reduced inorganic nitrogen compounds and are called nitrifying bacteria. No chemolithotroph is known that will carry out the complete oxidation of ammonia to nitrate; thus, nitrification of ammonia in nature results from the sequential action of two separate group of organisms, the ammonia oxidising bacteria, the nitrosofyers and the nitrite-oxidising bacteria; the true nitrifying (nitrate-producing) bacteria. Nitrosifying bacteria typically have genus names beginning in "Nitroso," while true nitrifyers usually begin with "Nitro,". *Nitrosomonas* and *Nitrobacter* are major genera of nitrifying bacteria. Historically, the nitrifying bacteria were the first organisms to be shown to grow chemolithotrophically; Winogradsky showed that they were able to produce organic matter and cell mass when provided with CO_2 as sole carbon source (Madigan *et. al.*, 2000).

Many species of nitrifying bacteria have remarkably complex internal membrane systems in many respects similar to the internal membranes found in their phylogenetic close relatives, the purple anoxyphototrophs and the methane-oxidising (methanotrophic) bacteria. The membranes are the location of a key enzyme in NH_3 to hydroxylamine; the latter is further oxidized to NO_2^- by the nitrosifying bacteria. The NO_2^- generated in the reaction is oxidized to NO_3^- by the nitrifying bacteria (Madigan *et al.*, 2000).

The nitrifying bacteria are widespread in soil and water. They are present in highest numbers in habitats where considerable amounts of ammonia are present, such as sites

where extensive protein decomposition occurs (ammonification) and in sewage treatment facilities. Nitrifying bacteria develop especially well in lakes and streams that receive inputs of sewage or other waste waters because these are frequently high in ammonia (Madigan *et al.*, 2000). Enrichment cultures of nitrifying bacteria are readily established by using mineral salt media containing ammonia or nitrite as electron donor and bicarbonate (HCO_3) as sole carbon source. Many nitrifying bacteria, especially the ammonia oxidizers, are inhibited by the traces of organic material present in most agar preparations. Most of the nitrifying bacteria are obligate chemolitotrophs. Species of *Nitrobacter* are an exception and are able to grow chemoorganotrophically on acetate or pyruvate as sole carbon and energy source. However, although the group is somewhat heterogenous morphologically, they are fairly tightly related phylogenetically and are either alpha or beta (Madigan *et al.*, 2000).

2.18. Free living Aerobic Nitrogen-fixing bacteria

Key genera

- *Azobacter*
- *Azomonas*

A variety of organisms that inhabit primarily the soil are capable of fixing N_2 aerobically. The genus *Azobacter* comprises large, gram negative, obligately aerobic rods capable of fixing N_2 nonsymbiotically. The first species of this genus was discovered by the Dutch microbiologist M.W. Beijerinck early in the twenthieth century, using an aerobic enrichment culture technique with a medium containing N_2 (air) but devoid of a combined

nitrogen source. Most free-living nitrogen fixing bacteria are alpha or gamma (Madigan *et al.*, 2000).

2.19. The Unified Soil Classification method.

The Unified Soil Classification classifies soils on the basis of the factors texture, and liquid limits. The system is comprised of fifteen soil groups, each identified by a two-letter-symbol. Soils are classified in terms of particle size, coarse-grained soils being sands and gravels, while fine-grained soils are silts and clays. Gravel is defined as having a particulate grain size ranging from 76.2 mm to 4.76 mm, and sand from 4.76 mm to No.200 sieve size, while clay and silt have a component grain size less than No.200 sieve, which is about the minimum individual grain size recognisable by unaided human eye. The Unified Soil Classification enables the researcher to make preliminary assessment of the suitability of the soil for the project concerned (Roberts, 1981).

Rationale

The settling method is based on Stokes Law which states that denser (larger, usually) particles sink farther than less dense (smaller) particles when suspended in a liquid. There are 2 critical assumptions: (1) the particles all have the same density and (2) the particles are spherical. Actually, neither of these assumptions can be perfectly satisfied (Day, 1965).

The pipette method measures the actual percent by weight of each particle size class in the sample. The hydrometer method uses the density of the soil/water mixture. The more particles that are in suspension at any one time, the more dense the mixture, the higher the hydrometer will float in the soil water mixture. As larger mineral particles fall from suspension, the density of the soil/water mixture decreases. As the density decreases, the

hydrometer sinks farther into the mixture. The stem of the hydrometer is worked in grams of sediment remaining in suspension (Day, 1965).

In each method, after a certain number of seconds have elapsed from the time the soil/water mixture is thoroughly mixed (usually 40 seconds), all particles of one size will have fallen below a certain level in the suspension. After 6 hrs and 52mins, all the silt (0,005-0.002mm) will have fallen below this level, and only clay (less than 0,002mm) remains in the suspension. The hydrometer reading (corrected) at 6 hours and 52 minutes will be a measure of the amount of clay remaining in suspension. From this hydrometer reading, and the initial total weight of the sample, one will be able to calculate the proportion of the sample that is clay. The proportion of the silt is the difference between the calculated percent clay and remaining silt and clay % after the sands were removed (Day, 1965).

2.20. *Aim and objectives*

2.20.1 Aim

The overall aim of the study was to investigate the biodegradability of diesel that contaminate soil by using bioaugmentation.

2.20.2 Objectives

- To determine the level of diesel contaminant that remained in the soil using the GCMS.
- Determination of optimum temperature condition for the growth of bioremedial microbes isolated from the bioremediation product from Eco-Sol in the laboratory.
- Investigation of the soil for the presence of common soil organisms.

Chapter 3

Methodology

3.1. Site description

The site was identified at Oilco east of Empangeni in Northern Kwa-Zulu Natal in the Republic of South Africa, a company affiliated to Shell. A diesel tank of the volume of 500 liters leaked and contaminated the soil to depth of ± 1.2 m which is ± 5m wide and 2m in length.

3.2. Plot setup

The experimental plot on the field was measured and divided into 3 plots namely:

a) Plot 1 which was 2 x 2 meters wide, was the noncontaminated part on the same site, which acted as a control.

b) Plot 2 was the contaminated site that was treated with the commercially available product of Eco-Sol, which comprised of bacteria, nutrient and cellulose/wood shavings. 5kg of the product was sprinkled at the depth of 100cm (level 1) from the surface covering the area of ± 10 m^2 and then about 10 liters of water was also sprayed over the same area. The same procedure was employed at levels 2 (70cm below surface), 3 (40cm below the surface) and lastly level 4 which was the surface. The samples of the soil samples were also collected at each level of the soil horizon before the application of the product.

c) To reach all the levels of the plot and to cover the area with the soil, a pre-cleaned spade which was sterilized with 70 % ethanol was used to dig the area.

d) Plot 3 was the contaminated site, which was treated with mixed bacterial cultures of 10 ml nutrient broth suspension of *Bacillus cereus*, 10 ml *of Serratia marscescens*, 10 ml

of *Mycobacterium spp.*, 10 ml of *Pseudomonas putida*, 10 ml *Rhodococcus spp.* and 10 ml suspension of *Micrococcus spp.*, which were prepared in the University of Zululand's laboratory in the Department of Biochemistry and Microbiology, South Africa, and no nutrients or woodshavings were added. The cultures were initially isolated from the hydrocarbon contaminated soil at the university and were kept live by constantly being subcultured and stored in the refrigerator at the university's Department of Biochemistry and Microbiology. The cultures were then cultured on nutrient agar plates that were covered on the surface with 0, 1 ml of diesel and then allowed to diffuse over a period of 10 minutes They were then incubated at 37°C over a period of 2 weeks and then purified by culturing on freshly prepared nutrient agar-diesel plates. Because of the pristine location of the site, there were no other sources of hydrocarbons, eliminating the concerns of advective sources.

3.3. Sample collection

Samples were taken at 15-day intervals starting from day 0. Samples were taken from each of the plots at different depths before soil treatment and other samples were also taken at different depths after soil treatment over a period of 75 days.Samples were obtained by drilling with a stainless steel 40-mm percussion gauge auger.The auger and tools were washed, dried and rinsed with 70% ethanol between sampling.

3.4. Sampling method

About 300-g soil sample was removed from auger and placed in precleaned, sterile Schott bottles covered with alluminium foil and then the cap replaced. The samples were immediately labeled and placed in a cooler box with ice. The samples were taken at 4 different depths i.e. surface, 40cm, 70cm, and 100cm below the surface. The samples were

collected at 15 day intervals starting from day 1 which was the 04th of March 2003, and this was the day before the samples were treated with the product up to the 17th of May 2003. 150 g of the soil samples were placed in a cooler box and sent to Durban for analysis of hydrocarbons present using the GCMS at the Technology Services International by Dr. Van Rossum.

3.5. Chemical analysis of the soil.

Analyses for all samples were carried out on Hewlett-Parckard 5890 series 2 GC system coupled to a mass spectrophotometer VG TRIO 2000. Positive electron impact at 70 eV was used. The ion source was maintained at 200°C. Data was acquired in the full scan detection mode from 45 to 350 amu at the rate of 1 Scan sec-1. A solvent delay time of 4 min. was used. Sample introduction was performed using a standard split/splitless-type injector in the splitless injection mode. Splitless time was 5 min for SPME fiber desorptions. The injection port temperature was maintained at 250°C. Separation was performed on a 30m x 0,25 mm x 0,25µm. SPB-1701 capillary column. The head pressure was set at 60 kPa. The column oven was initially held at 100°C for 2 min., programmed to 200 °C at a rate of 10°C min-1, then to 250°C at 20°C min-1 (held for 5 min.). Helium was used as a carrier gas at a 0,9µl min.-1 flow rate (set at 100°C) (Frombert *et al.*, 1996).

3.6. Soil classification

The soil was classified for the quality of each of the main sand, silt and clay fractions in samples of the soil from each horizon of the soil profile. The reason for this classification is that high clay content soils retard the passage of water and air and may be anaerobic. For this purpose the Particle Size Determination (PSD) method was used (Day, 1965).

A 2mm (No.10) sieve was used to separate the gravel (particles coarser than 2mm) from the grains less than 2mm in diameter and the percent sand was isolated by wet sieving through a set of nested sieves. The silts and clays in each sample were determined by using a pipette that measures weight percent of sample or a hydrometer that measure the density of a solution of silt and clay suspended in water (Day, 1965).

3.6.1. P.S.D (Particle Size Determination) Method

All soil samples taken at different depths of the contaminated soil in day 0 (before soil treatment) were dried in oven at 100°C. The samples were gently broken up and passed through a 2mm sieve with the use of a wood mortar and pestle. 20g of each one of the samples were weighed using the analytical balance. The sample was then transferred to a beaker and 10 ml of 30% H_2O_2 was added to it. When the reaction diminished, approximately 50 ml of distilled water was added and brought to a boil for 15-20 minutes. The reaction was observed carefully to prevent boil over. It was then removed from the heat source and cooled. 20 ml of sodium hexametaphosphate (i.e. Calgon) was added and the caps were then put on the shaker. It was ensured that the bottles on the shaker were counterbalanced. The samples were left on the shaker overnight. For each sample a 62, 5 mm sieve was then placed over a large funnel and set in a 1000-ml cylinder. The number of each cylinder was then recorded on the data sheet. The samples were then removed from the shaker and gently poured through the sieve ensuring that none of the sample was lost by spillage. All silt and clay was thoroughly washed through the sieve using distilled water. The entire sand fraction (very fine - very coarse) was then in the sieve. All sand was carefully transferred to a 50-ml beaker, dried and weighed. The cylinders were then containing only silt and clay fractions of the sample and were filled to the 1000 ml mark with distilled water. Seven beakers for each sample were obtained and their numbers

recorded and tare weights recorded on the data sheet. They were used for pipette "pulls" of the different size fraction...vcs silt, cs silt, med. silt, fn. Silt, vf silt, cs clay, vf clay. The temperature of the water in the cylinder was recorded and then the settling time chart consulted to determine the time and depth at which "pulls" were to be made for the various size fractions. The samples were then agitated vigorously for 20 seconds. The time count for the first settling time of the sample was begun immediately after ceasing stirring of each sample. At the required time the fraction was "pulled" at a depth of 10 cm using a 20ml pipette (the depths used were according to the instructions on the settling time chart). The sediment sample from the pipette was dispensed into a 50ml beaker designated for that size fraction. The pipette was washed into beaker with distilled water. The samples were placed in drying oven. When the samples had dried, they were placed in the dessicator to cool and weighed immediately. Steps 12-16 were repeated for remaining size fraction (See table for results), (Day, 1965).

3.7. Determination of optimum temperature conditions for bioremedial microbes in the laboratory.

0.1ml of mixed culture was inoculated into 9.8 ml of a freshly prepared mineral salt medium and 0.1ml degenerated diesel as the sole carbon source was added to give a final volume of 10ml. The mixture was then incubated at 28° C in a shaking incubator with revolutionary speed of 190 revolutions/minute for 1 week. After a week 0.1 ml of the bacterial consortium was used to prepare 10 dilutions from 10^{-1} to 10^{-10}. 0.1 ml of each dilution was then placed at 10 nutrient agar plates and the spread plate technique performed for each of the 10 plates/dilution (Basson, 1987). Due to patent regulations of the product, the names of the microorganisms that were isolated from the product cannot be disclosed. The plates were then placed at the following incubation temperatures: 0° C, 20° C, 25° C,

30° C, 35°, 40°C and 45°C for 24 hours to identify the optimum temperature at which bioremedial microbes perfomed best. The optimum biodegradation temperature was then identified by selecting the plates that showed maximum growth of bioremedial microbes. The temperature that was selected assisted in identifying the best time to start the project for successful treatment of the soil (Chan *et al.*, 1993).

3.8. Investigation of the presence of different groups of soil organisms:

This was done for all samples from day 0 to day 6.

The following groups of organisms were observed:

- Facultative bacterial counts using the spread plate method (Basson, 1987)
- Nitrofyers – spread plate method on mineral nitrite medium (culture medium 2b, Ballows *et al.*, 1992).
- Nitrosofyers – spread plate method on mineral salt medium (culture medium 2^b, Ballows *et al.*, 1992).
- Free living nitrogen fixing bacteria (Semi solid) LGI medium a, Ballows *et al.*, 1992).

All plates were incubated at 28°C for 48hrs.

Chapter 4.

4.1 Results

4.1.1 Table1: The results of the soil texture analysis.

GRAIN SIZE DISTRIBUTION %

	GR	CS	MS	FS	SLT	CL
Surf.	10.1	5.6	30.0	9.0	45.2	0.2
40 cm	5.9	3.6	20.3	19.0	49.0	2.2
70 cm	0.9	2.7	22.8	21.6	50.0	3.5
100 cm	0.1	1.0	22.6	24.5	51.5	2.2
n/c	7.8	1.0	15.2	17.1	55.5	3.3
n/t	9.1	7.8	16.9	19.4	44.5	2.4

GR – gravel (> 2.0 mm), CS – coarse sand (2.0-0.5 mm), MS – medium sand (0.5-0.2 mm), FS – fine sand (0.2-0.02 mm), SLT – silt (0.02-0.002 mm), CL – clay (< 0.002 mm), Surf. – Surface, n/c – control, n/t – broth treated part.

4.1.2. Determination of optimum temperature for the growth of bioremedial microbes isolated from the bioremediation product.

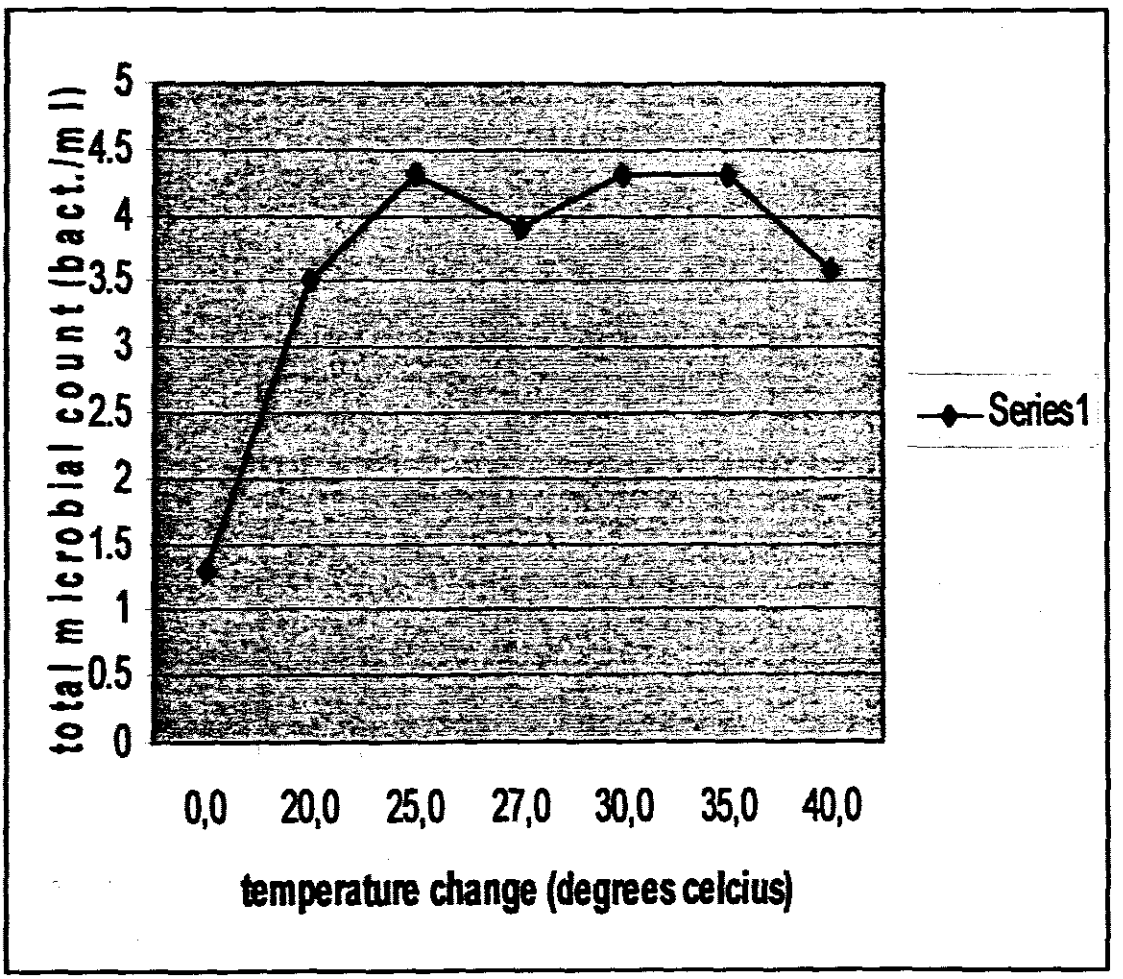

Figure 1. Log total microbial counts vs. temperature.

- **bact./ml – bacteria per milliliter**

4.1.3. Bacterial enumeration of soil and water samples.

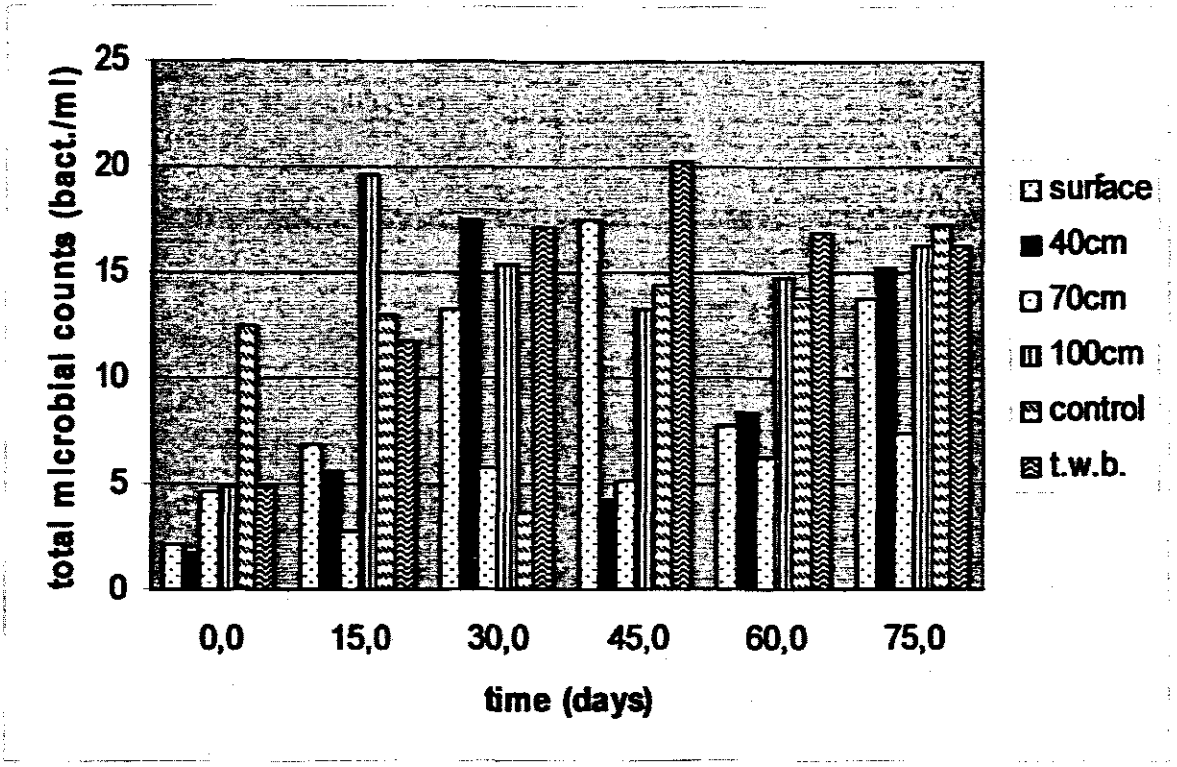

Figure 2. Graph of log total counts (soil samples) vs. change in time (days)

t.w.b. -Sample treated with bacteria only.

As time progressed, the total counts of microorganisms isolated from different levels of the contaminated soil horizon seemed to increase as shown in figure 2. This was mostly significant in the deeper layers of the soil.

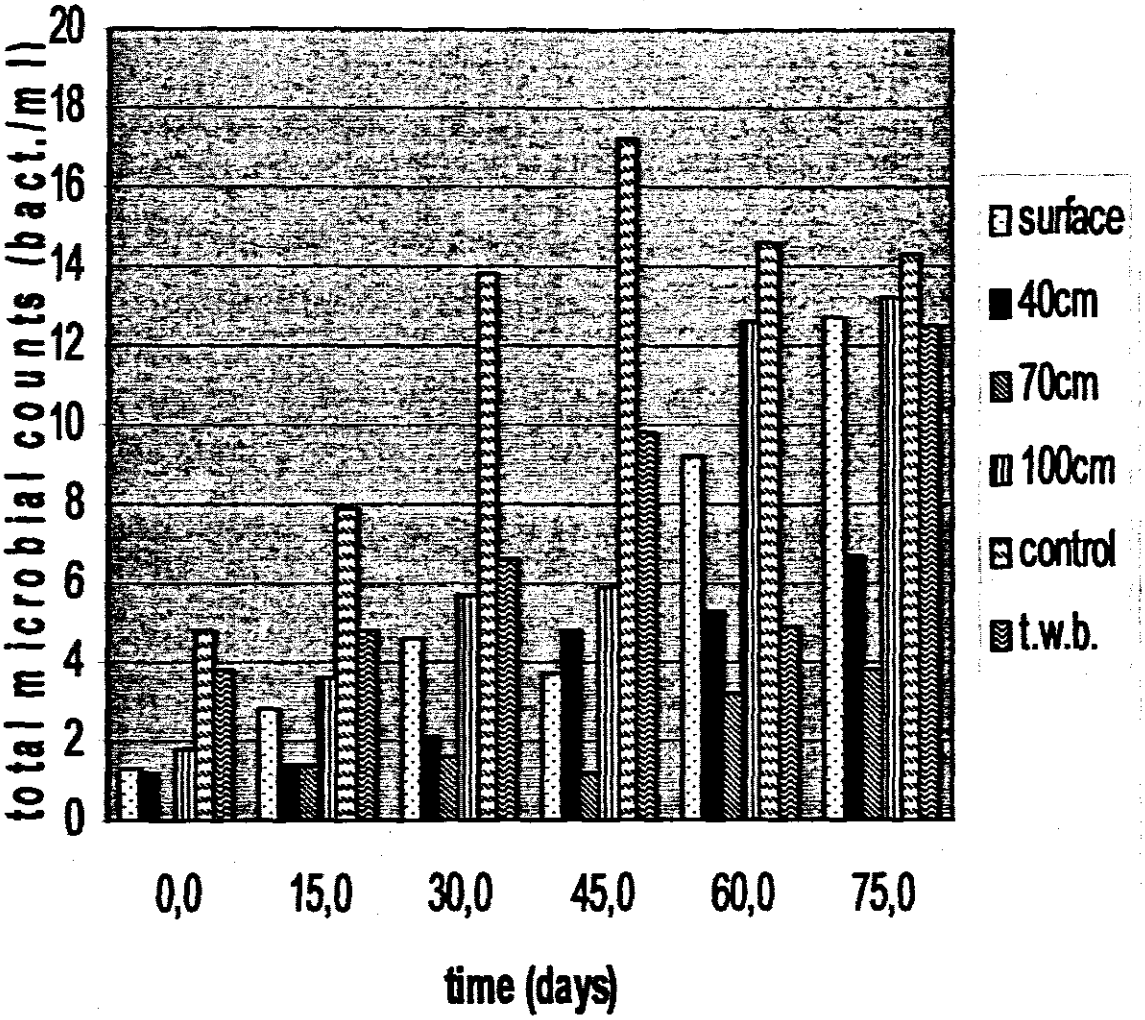

Figure 3. Graph of log counts (nitrofyers) vs. time (days)

There was a significant increase of the nitrofyers in a descending order from the surface, 40cm, 70cm and then a great increase at the deepest layer which was 100cm.

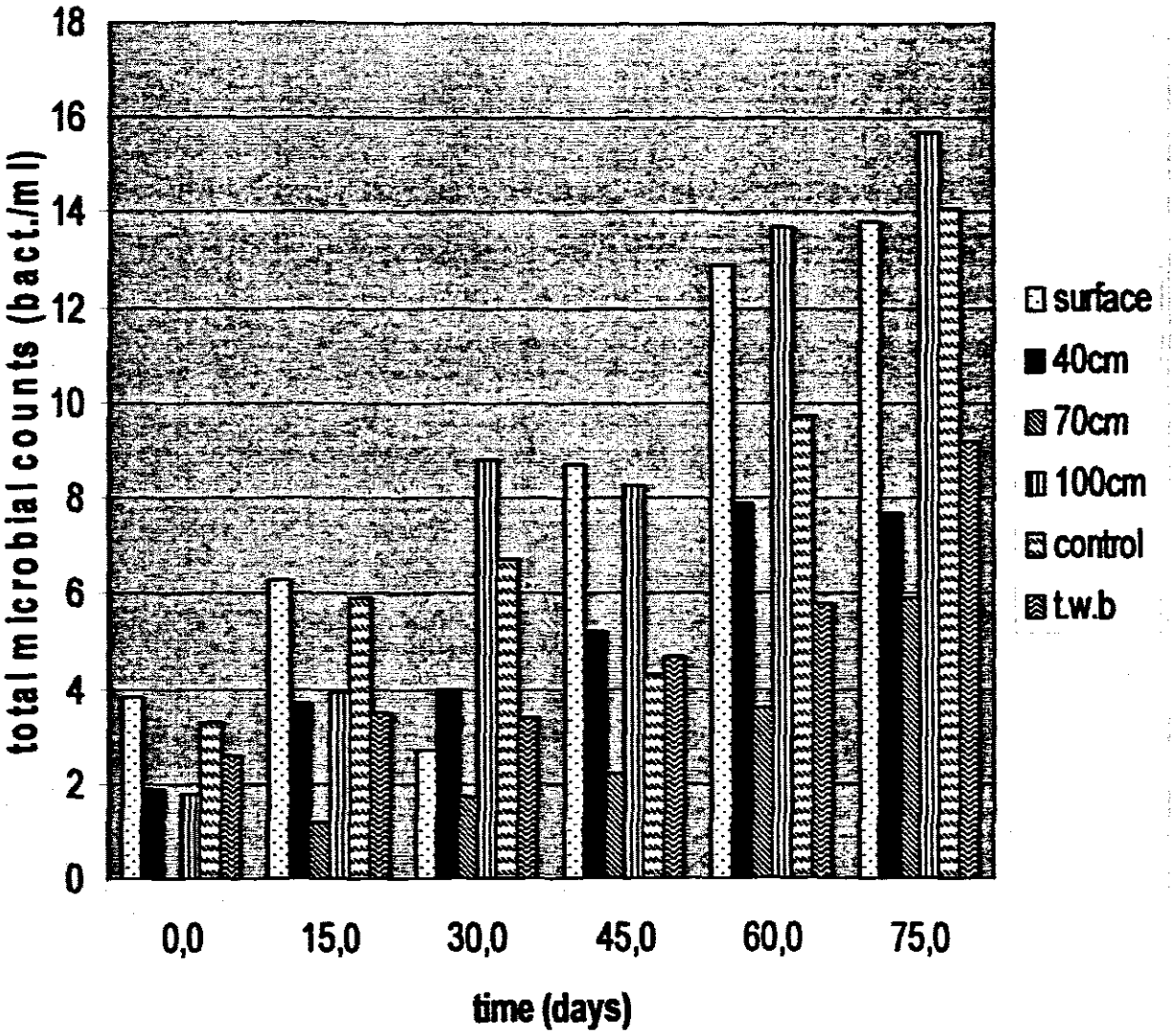

Figure 4. Graph of log of total counts (nitrosofyers) vs. time (days)

The nitrosofyers seemed to show the same trend as the nitrofyers and this could be attributed to the fact that they are phylogenetically related strains.

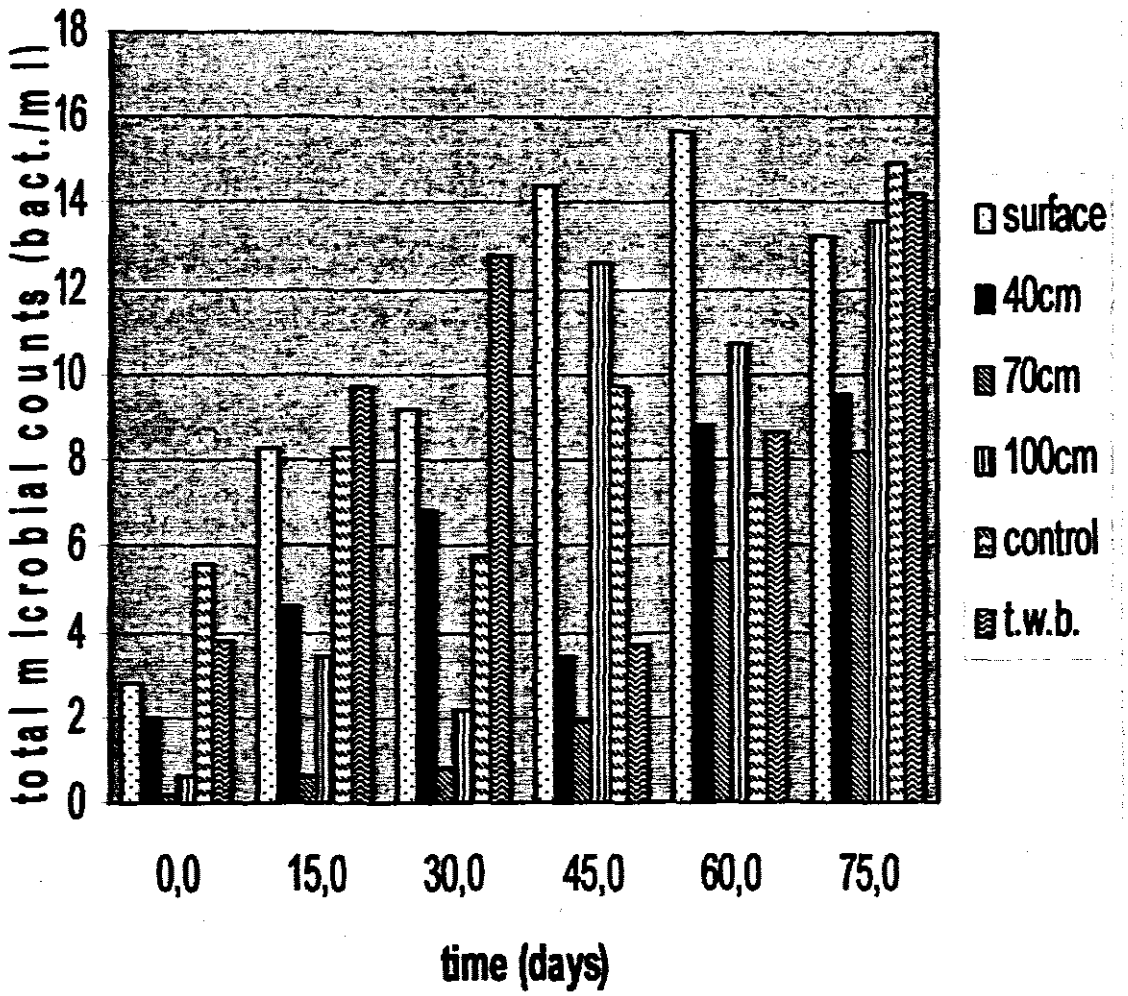

Figure 5. Graph of log of counts (Nitrogen fixing bacteria) vs. time (days)

The nitrogen fixing bacteria also seemed to follow the same order of growth as the nitrifying bacteria shown in Figure 4 and Figure 5.

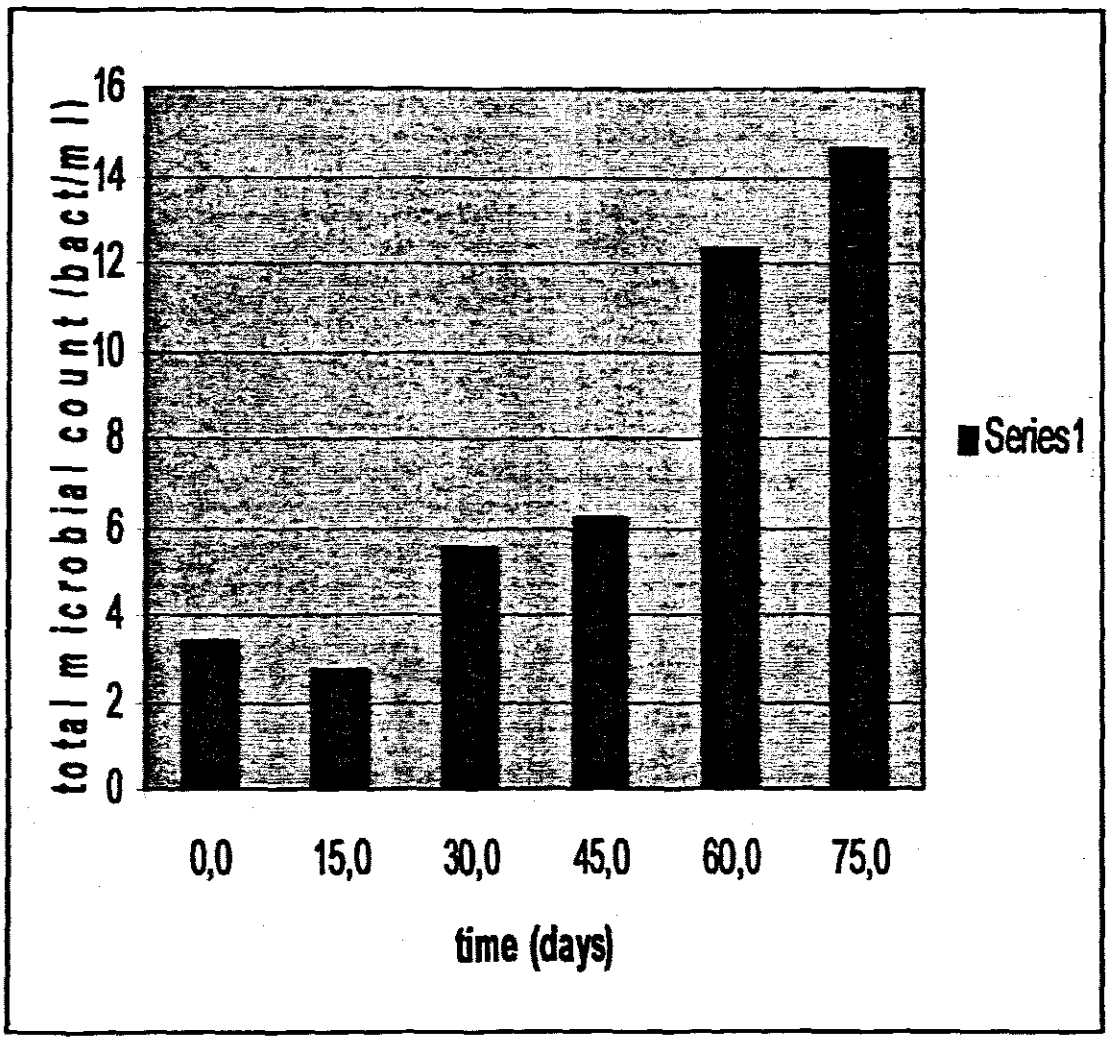

Figure 6. Graph of log total counts (water samples) vs. time (days)

There was a significant increase in total counts of microbes found in the ground water as time progressed.

4.1.4. Hydrocarbon content enumeration of soil and water samples.

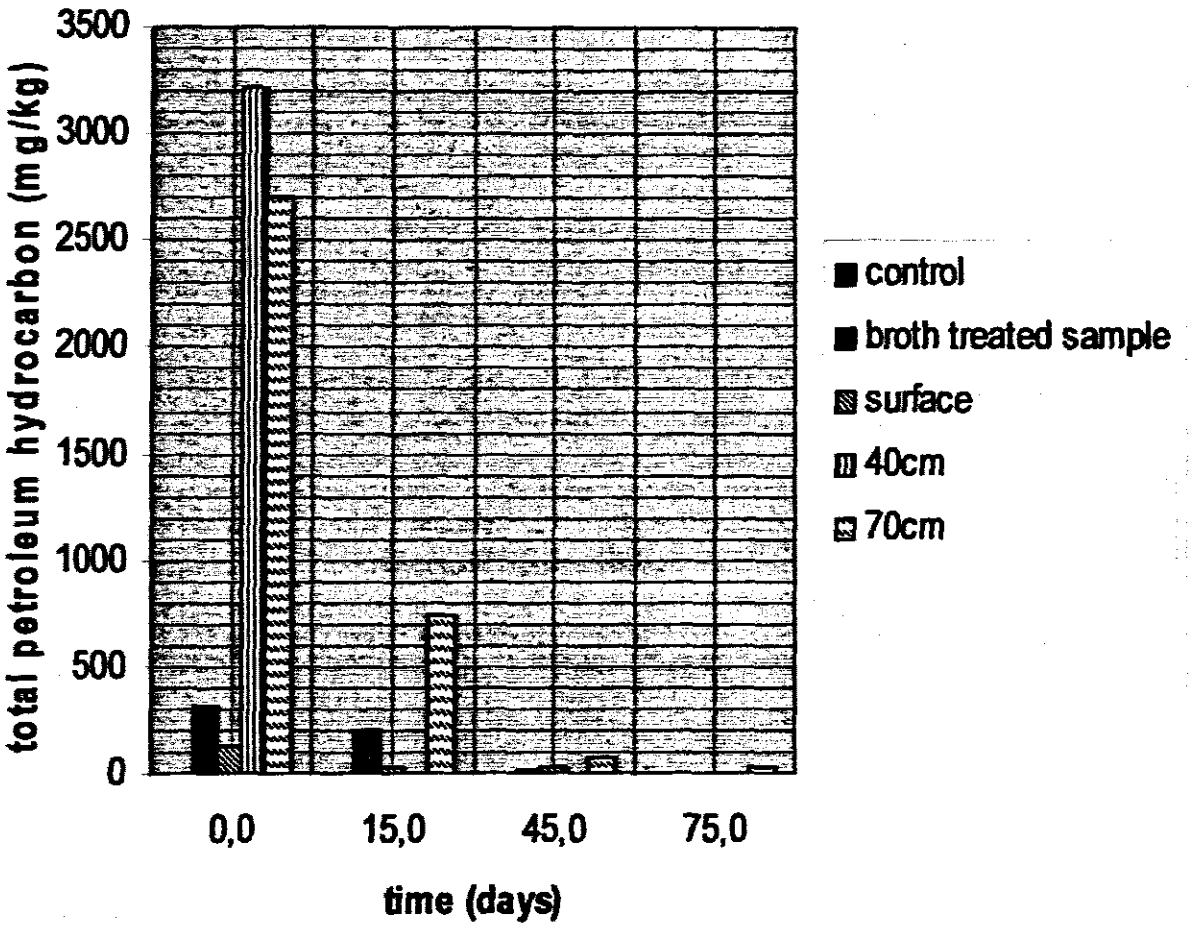

Figure 7. Graph of total hydrocarbon vs. time (days)

There was a significant decrease in hydrocarbon content thoughout the course of the study and this was a good indication of the success of the project.

Chapter 5

5.1 Discussion

The results that were obtained showed great success of the bioremediation project conducted at OILCOR. This was evident from the results that were obtained as can be viewed on the results section. It is also very important to note that the bacterial consortia that were isolated from the University laboratories could effectively remove the contaminated within a shorter period as compared to Eco-Sol isolates and this could be due to the fact that the depths of contaminations were not the same and also the intensity of contamination was not the same and therefore there was a great correlation between the availability of nutrients, contaminant type and the soil type. The moisture content of the soil also contributed a lot to the rate of bioremediation and this was evidenced on the days at which the temperature was too high or too low where the rate of bioremediation was slower than the one at which the temperature was almost the same as the optimum temperature that was identified at the University of Zululand laboratories.

The soil type is one of the main factors when determining whether or not in situ treatment is possible. Effective treatment of subsoils requires continual access to nutrients by microbes to promote growth. Tight soils, those with high clay content are more likely to plug up and restrict the free flow of nutrients to the microbes. On the other hand, soils with high sand content allow nutrients and oxygen to flow and are therefore more tractable to bioremediation (Senn, 1999)

The purpose of the method used was to determine the quantity of each of the main sand, silt, and clay fractions in samples of soil from each horizon of the soil profile. A 2-m (No.10) sieve was used to separate gravel (particles coarser than 2-mm) from the grains

less than 2-mm in diameter and the percent sand was isolated by wet sieving through a set of nested sieves. The silts and clays in each sample were determined by using a (1) pipette that measures weight percent of sample or (2) a hydrometer that measure the density of a solution of silt and clay suspended in water (Day, 1965). The soil was classified using the particle size determination method in order to make preliminary assessment of the suitability of the soil for the project. The regional contaminated soil comprised of the sandy loam type of soil, which was identified as not being a problem in limiting the rate of bioremediation.

The soil was classified using the particle size determination method in order to make preliminary assessment of the suitability of the soil for the project. After identification of the soil types from each horizon of the soil profile using the P.S.D method, the regional contaminated soil comprised of the sandy loam type of soil. According to Senn, 1999 this particular soil type is not a limiting factor for bioremediation as it allows the free flow of oxygen and nutrients which makes them more tractable to bioremediation.

Temperature is one of the most important factors that influence growth of cells. Cells grow within a well-defined temperature growth range. A minimum temperature below which cells are metabolically inactive and a maximum temperature above which cells do not grow define this growth range. Within this range of extremes is an optimal growth temperature at which cells exhibit their highest rates of growth and reproduction (Atlas, 1995).

A series of dilutions of bacterial consortia were prepared to the 4^{th} dilution and then inoculated on nutrient agar plates with 0.1ml of degenerated diesel and then placed at different temperatures. Four plates were prepared for each incubation temperature, from

the 1st dilution to the 4th dilution. The 1st four placed were placed at room temperature, the 2nd four at 0° C, the 3rd four at 20° C, the 4th four at 25° C the 5th four at 30° C, the 6th four at 35° C and the last four at 40°C for 48hrs.

The trend shown in figure 1 indicated that with the increase in temperature from 20 degrees celcius to 35 degrees celcius there was a huge growth of microorganisms at these temperatures and this assisted in identification of the optimum temperature suitable for growth of bioremedial microbes. Below and above the prementioned temperatures, there was a decrease in microbial growth.The study was conducted during the summer period of the year (as noted in the tables for weather conditions from the period 04/03/2003 to 17/05/2003), and at this time of the year in the area of the study, daily maximum temperatures can reach 33°C and from the results it was observed that the optimum growth temperature was between the region of 25°C and 35°C which is about the normal temperature conditions in this part of the country meaning that this was the right time to do the project since temperature conditions were favourable for microbial degradation of diesel. Through comparing the optimum temperature and the weather conditions it was observed that the conditions were favourable enough to start the project at during that period of the year.

To better understand the structure and activity of microbial communities in response to contaminant loading and its subsequent effect on the biological component, the culture independent methods were applied as a primary characterization to directly examine the response of the bacterial community *in situ* to pollutant loads in each of the treatment components described. The study established that the distribution and the physiological status strongly correlated with key aspects of the chemical composition of the pollutant

(diesel), indicating a potential relationship between the functionality of these defined groups and process chemistry (Whiteley & Bailey, 2000)

The availability of oxygen is commonly the limiting factor in biodegradation of petroleum hydrocarbons and other contaminants in the subsurface. Active contaminant degrading microbial populations are commonly present in the subsurface, as are sufficient quantities of nutrients (e.g., nitrogen and phosphorus) (Calabrese and Kostrecki, 1991). However, the absence of sufficient oxygen supply causes the subsurface environment to become anoxic, and causes microbes to function in less efficient anaerobic manner. When oxygen is added to the subsurface establishing oxic conditions, facultative heterotrophs (facultative – microbes capable of both aerobic and anaerobic metabolism and heterotrophs – derive their energy from oxidation of organic carbon compounds) will convert from anaerobic to aerobic metabolism, and more efficiently and quickly degrade the petroleum hydrocarbons. To enhance oxygen transfer in the soil or subsurface we added woodchips as bulking agents to promote growth of microbes in the subsurface, as suggested by Calabrese and Kostrecki, 1991.

From the results of the total counts in Table 3, it was observed that at the control site there was a large number of microbial populations, and this could be associated to the fact that there was not much competition for resources or predation by protozoans etc. since the site was free of diesel contaminants. Bacterial population density generally decreases with depth as a function of the availability of organic carbon and molecular oxygen, parameters which typically decrease with depth, but in the study at 100cm there seemed to be more bacteria than 40cm below the surface. This could be due to the addition of woodchips and nutrients at the site, the soil type which permitted easy flow of water , nutrients and oxygen

at that depth and the availability of hydrocarbons at that depth was low therefore normal soil organisms were high at that depth.

At the depth of 70 cm below the surface the population density of microbes was least and this could be associated with the highest levels of contamination at that depth and this therefore lead to inhibition of microbial growth due to toxic nature of persisting hydrocarbons at that depth i.e. (C10 – C12). At 40 cm the population density of microbes was a fraction higher and this could be associated with the fact that it is nearer to the surface. The microbes therefore had easy access to oxygen and nutrients. The temperature conditions at 40cm are higher than that below and therefore the hydrocarbon degraders had outgrown the normal soil microbes and therefore the population size increased. At the surface the average counts were obtained and this had resulted from the fact that there were less hydrocarbons at this position and therefore normal soil organisms had reinhabited the site after the hydrocarbon degraders had less substrate (hydrocarbon) to degrade.

At the site where the soil was partially contaminated and treated with bacteria only, the total counts were higher. This could be associated with the short depth of sampling which was about 0, 3 m and also the total hydrocarbons at this site were too low, therefore bioremediation took less time and the normal soil organisms reinhabited their site.

Initially the counts of the nitrofyers showed that their presence in the soil was low. This could be as a result of the absence of the nutrients in the soil for microbial growth. This resulted in nitrifying bacteria struggling to survive at all the depths. There was also a competition for the substrate in the soil. The nitrosofyers and the nitrofyers are phylogenetic close relatives and also the absence of or low populations of nitrosofyers

means even lesser numbers of nitrofyers since the nitrofyers use the final product of nitrosofyers for their survival. Towards the end of the study period the populations of the nitrifying bacteria increased since bioremediation was reaching its completion. The bioremedial microbes were decreased as a result of the fact that the substrate was depleted.

The nitrogen fixers were low in their numbers at the beginning of the study period and as the days progressed after the soil had been treated with bioremedial microbes the population of nitrogen fixers increased since the soil was getting rehabilitated and this could be viewed quite clearly in the last 30 days of the study period. The total counts of the microbial samples taken from the 1st day, i.e. before the soil was treated and those taken after the soil was treated showed that the microbial populations increased as the days proceeded during the study period and this had resulted from the fact that as toxins that were leaching into the ground water levels were getting reduced in the soil solution, the normal soil and water microoganisms had reinhabited the site over the bioremediation or rehabilitation period.

As more attention is given to managing environmental liability using risk-based approaches, it is clear that there is a need to quantify more than just total petroleum hydrocarbons (TPH). For example, most risk based approaches for petroleum contaminated sites require a quantitative understanding of the levels and distribution of polycylic aromatic hydrocarbon (PAH) concentrations present in site media as well as the TPH concentrations. Current practice is to use chromatographic methods with flame ionization detection to screen for TPH and then use this information to decide which samples need additional higher resolution analyses. Depending on the site and the site's history, the sample extract is re-analyzed using GC/MS methods or, for older sites, that the

site has to be re-sampled and the re-acquired site media be re-submitted to the laboratory for additional analysis.

Diesel is largely comprised of unbranched *n*-alkanes with only around 4 % of polyaromatic compounds. (Heath *et al*, 1993).When evaluating risks associated with petroleum products in the terrestrial environment, benzene, toluene, ethylbenzene, and xylene (BTEX) and polyaromatic hydrocarbons generate the greatest concern because of their associated toxic, carcinogenic and mutagenic properties. BTEX is generally only found in trace quantities in diesel fuel because of its flash-point specifications. Alkanes are nonpolar molecules (no dipoles) with only induced dipoles which give rise to London forces. With an increase in size, the number of electrons increase, the induced dipole increases, the London forces become stronger, boiling points and melting points increase. Therefore at room temperature the first four compounds in the series are gases, C_5 to C_{16} are liquids and C_{17} and higher are solids (Weiner, 2000).

At the surface of the rehabilitated site C14, C15 and C16 were found, and within 45 days C14 and C15 were completely degraded but C16 could only by completely degraded in a 75 day period. This resulted from the fact that as the carbon chain length increases resistance to biodegradation also increases thus increasing the time period of bioremediation. The other reason for low concentration of the hydrocarbons at the surface is that some other organic compounds evaporate at the surface and also the conditions are favorable enough for bioremediation to take place. At 40cm below the surface of the contaminated site the contamination was that of C10 – C16 and within a period of 15 days after the soil was treated, C10-C14 showed complete degradation and the last two which is

C15 and C16, took a longer time to degrade completely and this could be associated with the fact that these had much longer chain lengths.

The depth of 70cm showed the highest levels of contamination as viewed on Table13. This could be associated with the fact that as conditions in the soil got more anoxic as a result of increasing depth , bioremediation was slower at this depth and C11 and naphtalene could be viewed to be completely degraded within the 45 day period. Following these two was C12-C15 which were degraded to completion over the period of study (75 days), C16 however could not be completely degraded within the period of study but it's levels were reduced to significant amounts in the soil with change in time as observed from the Table and figure.The soil samples of the partially contaminated soil, which was treated with bacteria only showed that there were less contaminants on the site and as a result complete degradation of the present hydrocarbons could be achieved within a 15 day period for both C12 and C13 which are short chain alkanes and therefore bioremediation was faster at this site.

Chapter 6

6.1. CONCLUSION

Bioremediation when used within a risk –based framework, can offer a cost-effective and sustainable remediation strategy. However, there is a danger that the increase in commercial 'solve all'approaches may jeopardize clients confidence that bioremediation is a credible alternative to other remediation strategies. This is in part, due to lack of reporting of in depth/peer reviewed field trials and also an increase in the use of bioremediation products without sufficient initial investigation and monitoring or validation.

From the results that were obtained over the period of study which was limited to 75 days due to constructions and developments made on the site at OILCO, the conclusion that can be drawn is that there is a great correlation between the soil type, depth of contamination, components of the contaminants and also the concentration of contaminants. There was an observable reduction of contaminants to almost complete reduction. The only problem was that of the depth of 75cm where bioremediation was a bit slower and this could be related to the fact that there were many contaminants found deeper as compared to other depths, and this could also be attributed to the fact that deeper down the surface conditions become more anaerobic and microbes struggle to perform at their best ability. However rapid bioremediation occurred in the bioaugmented site (Plot 2), at the site that was treated with laboratory prepared bioremedial microbes only (Plot 3), with the partially contaminated soil as their initial inoculum had completeley remediated the soil within the 1st 15 days after soil treatment and therefore no difference in rates of bioremediation at the nonaugmented site (Plot 3) and the augmented site (Plot 2) could be discerned. This could have resulted from the differences intensity of contamination at both sites, which was very

low at the nonaugmented site. There was an increase in total microbial counts for the 1st three sampling days of the subsurface levels and then on the 4th day they decreased and then increased, this was due to bioremedial microbes multiplying as they consume their substrate, which is diesel and then died as the substrate was getting almost completely degraded.

Groundwater becomes unusable when contaminated with petroleum products based on the drinking water standards. Groundwater users are therefore at risk when ground water becomes contaminated (Noonan and Curtis 1990). It was imperative in the study that there need to be a great focus on such a research since it is cheap and safe and also bioremediation as a cleanup tool was observed to be highly effective since its application on the contaminated site reduced almost all of the contaminants.

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Appendix A

Media preparation

Diesel mineral salt medium

(a) Basal medium ingredients solution.

Solution A

$(NH_4)SO_4$, 1,0g

K_2HPO_4, 1,0g

KH_2PO_4, 0,05g

Distilled H_2O, 700ml

Solution B

$CaCl_2$, 0,01g

$FeSO_4..7H_2O$, 0,005g

Distilled H_2O, 100,0ml

Solution C

$MgSO_4.7H_2O$, 0,2g

$MnCl_2.4H_2O$, 0,010g

Distilled H_20, 100ml

(b) Diesel – 0,1ml/40ml medium a in 250ml sterile stoppered Erlenmeyer flask.

Nutrient agar plate preparation

20g of nutrient agar suspended in 1L of freshly distilled of completely demineralised water and allowed to stand for 15 minutes.

Boil in a pressure cooker to dissolve completely. Sterilize in the autoclave (15min. at 121°C).

Pour into sterile plates and allow to solidify and then store at the storing temperature or use immediately (Basson, 1987).

Medium for nitrofyers

Distilled water 1000,0 ml

$NaNO_3$ 2,000mg

$MgSO_4.7H_2O$ 50,0mg

$CaCO_3$ 3,0mg

KH_2PO_4 150,0mg

$FeSO_4.7H_2O$ 0,15mg

$(NH_4)_6Mo_7O_{24}.4H_2O$ 50,0 micrograms

NaCl 500mg

(Basson, 1987)

Medium for nitrosofyers

Distilled water 1000,0 ml

$(NH_4)_2SO_4$ 130,0mg

$MgSO_4.7H_2O$ 200,0mg

$CaCl_2.2H_2O$ 20,0mg

K_2HPO_4 87,0mg

Chealated iron 1,0g

$Na_2MoO_4.2H_2O$ 100,0 micrograms

$MnCl_2.4H_2O$ 200,0micrograms

$CoCl_2.6H_2O$ 2,0micrograms

$CuSO_4.5H_2O$ 20,0micrograms

$ZnSO_4.7H_20$ 100micrograms

Phenol red 0.5% 1.0ml

(Basson, 1987)

Nitrogen-fixing bacteria

Ingredients/liter

(Semi-solid) LGI medium (a)

Sucrose 5,0g

K_2HPO_4 0,2g

KH_2PO_4 0,6g

$MgSO_4.7H_2O$ 0,2g

$CaCl_2.2H_2O$ 0,02g

$Na_2MoO_4.2H_2O$ 0,002g

Bomothymol blue olution; 0,5% in 0,2N KOH 2ml

FeEDTA, 1,64 % 4ml

KOH – to adjust pH to: 6,0

Vitamin solution(d) 1,0ml

Agar 1,75g

(Basson, 1987)

Vitamin solution

Biotin-10mg

Pyridoxyl-HCL – 20mg

H_2O – 100ml

All ingredients should be added to the medium in a stated order.

Appendix B

Raw data

4.2. Optimum temperature identification

Table 2. The total bacterial counts for samples incubated at 7 different temperatures.

Temperature	Total bacterial count (*bacteria/ml*)	Log total bacterial count
1.*Room temperature(27° C)*	8000	3.9
2. 0° C	20	1.3
3. 20° C	3200	3.5
4. 25°C	18497	4.3
5. 30° C	20000	4.3
6. 35°C	19559	4.3
7. 40° C	4400	3.6

4.3. Bacterial enumeration of soil and water samples.

Table 3. log of total microbial counts of soil samples.

ing day	Surface	40 cm	70 cm	100 cm	control	T.w.b
	2.1	1.8	4.6	4.9	12.5	4.9
	6.9	5.6	2.8	19.6	13.0	11.7
	13.2	17.5	5.7	15.4	3.6	17.1
	17.5	4.2	5.1	13.3	14.4	20.3
	7.8	8.4	6.3	14.7	13.8	16.9
	13.8	15.3	7.4	16.3	17.2	16.3

Table 4 Log of nitrofyers of soil samples.

day	Surface	40 cm	70 cm	100 cm	Control	t.w.b.
	1.3	*1.2*	*0*	*1.8*	*4.8*	*3.8*
		1.4	*1.4*	*3.6*	*7.9*	*4.8*
	4.6	*2.1*	*1.6*	*5.7*	*13.8*	*6.6*
	3.7	*4.8*	*1.2*	*5.9*	*17.2*	*9.8*
	9.2	*5.3*	*3.2*	*12.6*	*14.6*	*4.9*
	12.7	*6.7*	*3.8*	*13.2*	*14.3*	

Table 5. The log of nitrosofyers of soil samples.

ıoline day	Surface	40 cm	70 cm	100 cm	Control	t.w.b.
	3.8	*1.9*	*0.0*	*1.8*	*3.3*	---
5	*6.3*	*3.7*	*1.2*	*3.9*	*5.9*	*3.5*
0	*2.7*	*4.0*	*1.7*	*8.8*	*6.7*	*3.4*
5	*8.7*	*5.2*	*2.2*	*8.3*	*4.3*	*4.7*
	12.9	*7.9*	*3.6*	*13.7*	*9.7*	*5.8*
5	*13.8*	*7.7*	*5.9*	*15.7*	*14.1*	*9.2*

Table 6. Log of free living nitrogen fixing bacteria of soil samples

Sampling day	Surface	40 cm	70 cm	100 cm	Control	t.w.b.
1. 0	*2.8*	*2.0*	*0.2*	*0.6*	*5.6*	*3.8*
2. 15	*8.3*	*4.6*	*0.6*	*3.4*	*8.3*	*9.7*
---	*9.2*	*6.8*	*0.8*	*2.2*	*5.8*	*12.8*
4. 45	*14.4*	*3.4*	*1.9*	*12.6*	---	---
5. 60	*15.7*	*8.8*	*5.7*	*10.7*	*7.2*	*8.6*
6. 75	*13.2*	*9.5*	*8.2*	*13.6*	*14.9*	*14.2*

Table 7. Log of total microbial count of water samples

Sampling day	Microbial count
1. 0	*3.4*
2. 15	*2.7*
3. 30	*5.5*
4. 45	*6.2*
5. 60	*12.3*
6. 75	*14.6*

4.4. Hydrocarbon content enumeration of soil and water samples.

Table 8: ***Results of*** **BTEX analysis** ***of soil samples that were taken*** **before soil treatment.**

Position	N/C Control (1)	Wtb-B/T (1)	Surface B/T 40 cm (1)	B/T 40 cm (1)	B/T 100 cm (1)	B/T 70 cm (1)
	Day 0	Day 0	Day 0	Day 0	Day 0	Day 0
Ethanol	0	0	0	0	0	0
Benzene	0	0	0	0	0	0
Tame	0	0	0	0	0	0
Toluene	0	0	0	0	0	0
Ethylbenzene	0	0	0	0	0	0
0-Xylene	0	0	0	0	0	0
M+p-Xylene	0	0	0	0	0	0
Naphtalene	0	0	0	0	0	4.97
Alkanes						
Carbon 9	0	0	0	0	0	0
Carbon 10	0	0	0	2.26	0	1.05
Carbon 11	0	0	0	17.75	0	9.87
Carbon 12	0	1.28	0	49.6	0	36.59
Carbon 13	0	1.36	0.66	111.55	0	79.15
Carbon 14	0	0	2.19	101.17	0	84.71
Carbon 15	0	0	2.2	101.41	0	88.86
Carbon >16	0	0	35.89	517.9	0	401.8
Total Hydrocarb		12.22	122.13	3218.29	113.89	2696.46
Comment		Oil	Diesel	Mineral diesel	Mineral diesel	Degenera -ted diesel

- ***N/C – non contaminated***
- ***(1) – 1st sample***
- ***wtb – will be treated with broth cultures***
- ***B/T – before treatment***
- ***DRO – Diesel Range Organics***

- ***GRO – Gasoline Range Organics***

Table 9. Results *of* BTEX analysis *of soil samples.*

Position	Surface (2)	40 cm (2)	70 cm (2)	TWB (4)	Surface (4)	40 cm (4)	70 cm (4)	TWB (4)
	Day 15	Day 15	Day 15	Day 15	Day 45	Day 45	Day 45	Day 45
Ethanol	0	0	0	0	0	0	0	0
Benzene	0	0	0	0	0	0	0	0
Tame	0	0	0	0	0	0	0	0
Toluene	0	0	0	0	0	0	0	0
Ethylbenz.	0	0	0	0	0	0	0	0
0-Xylene	0	0	0	0	0	0	0	0
M+p-Xylene	0	0	0	0	0	0	0	0
Naphtalene	0	0	3.32	0	0	0	0	0
Alkanes								
Carbon 9	0	0	0	0	0	0	0	0
Carbon 10	0	0	0.92	0	0	0	0.21	0
Carbon 11	0	0	5.47	0	0	0	1.03	0
Carbon 12	0	0	18.96	0	0	0	3.22	0
Carbon 13	0	0	28.77	0	1.28	0	7.92	0
Carbon 14	0	0	36.92	0	1.36	0	11.67	0
Carbon 15	1.33	0	42.11	3.81	0	1.46	8.42	0
Carbon >16	12.3	0	93.29	16.36	0	6.9	42.7	2.13
Tot. Hydr.	24.2	0	742.39	204.35	28.67	0	73.35	306.82
Comment	M/diesel		M/diesel	D/diesel	Oil		M/diesel	D/diesel

- ***Twb – treated with broth cultures***
- ***(4) – 4th sample***
- ***(2) – 2nd sample***
- ***Ethylbenz. – ethylbenzene***
- ***Tot hydr. – total hydrocarbons***
- ***M/Diesel – mineral diesel***
- ***D/Diesel – degenerated diesel***

Table 10. Table of results of BTEX analysis of soil samples.

Position	Surface (6) Day 75	TWB (6) Day 75	40 cm (6) Day 75	70 cm (6) Day 75	Control (6) Day 75
Ethanol	0	0	0	0	0
Benzene	0	0	0	0	0
Tame	0	0	0	0	0
Toluene	0	0	0	0	0
Ethylbenze.	0	0	0	0	0
0-Xylene	0	0	0	0	0
M+p-Xylene	0	0	0	0	0
Naphthale.	0	0	0	0	0
Alkanes					
Carbon 9	0	0	0	0	0
Carbon 10	0	0	0	0	0
Carbon 11	0	0	0	0	0
Carbon 12	0	0	0	0	0
Carbon 13	0	0	0	0.14	0
Carbon 14	0	0	0	1.27	0
Carbon 15	0	0	0	0	0
Carbon >16	0.02	0	0	13.12	0
Tot. Hydr.	0	0	0	22.96	0

- (6) – 6th sample
- Hyd – hydrocarbon

Table 11. Log of samples with significant change in hydrocarbon content at the surface of the rehabilitated site.

	C-13	*C-14*	*C-15*	*C-16*
Day 0	0	0.34	0.34	1.55
Day 15	0	0	0.12	1.08
Day 45	0	0	0	0.32
Day 75	0	0	0	0

Table 12. Log of samples with significant change in hydrocarbon content taken from depth of 40 cm.

	C-10	*C-11*	*C-12*	*C-13*	*C-14*	*C-15*	*C-16*
Day-0	0.35	1.24	1.70	2.05	2.01	2.01	2.71
Day-15	0	0	0	0	0	0.58	1.21
Day-45	0	0	0	0	0	0.16	0.84
Day-75	0	0	0	0	0	0	0

Table 13. Log of samples with significant change in hydrocarbon content taken from the depth of 70cm of the rehabilitated site.

	Napt	*C-10*	*C-11*	*C-12*	*C-13*	*C-14*	*C-15*	*C-16*
ay 0	0.70	0.02	0.99	1.56	1.90	1.93	1.95	2.60
ay 15	0.52	0	0.74	1.28	1.46	1.57	1.62	1.97
ay 45	0	0	0.01	0.5	0.90	1.07	0.93	1.63
ay 75	0	0	0	0	0	0.10	0	1.12

Napht. - *Naphtalene*

Table 14. Log of samples with significant change in hydrocarbon content taken from the surface of the partially contaminated soil that was treated with bacteria.

	C-12	*C-13*
Day-0	0.11	0.13
Day-15	0	0
Day-45	0	0
Day-75	0	0

Table 15: Weather conditions from 04/03/2003 to 18/03/2003

Weather conditions for the 1st 15 days.

Date	temperature	weather
04/03/2003	26°C	Mild
05/03/2003	28°C	Warm
06/03/2003	27°C	Mild
07/03/2003	31°C	Hot
08/03/2003	31°C	Hot
09/03/2003	29°C	Hot
10/03/2003	30°C	Hot
11/03/2003	33°C	Very hot
12/03/2003	31°C	Hot
13/03/2003	32°C	Hot
14/03/2003	28°C	Warm
15/03/2003	27°C	Light rain
16/03/2003	30°C	Cloudy & hot
17/03/2003	29°C	Hot
18/03/2003	25°C	Rainy

Table 16: Weather conditions from 19/03/2003 to 02/04/2003

Weather conditions for the 2nd 15 days.

Date	Temperature	weather
19/03/2003	26°C	Rainy
20/03/2003	25°C	Rainy
21/03/2003	23°C	Light rain
22/03/2003	27°C	Hot
23/03/2003	27°C	Hot
24/03/2003	28°C	Hot
25/03/2003	29°C	Hot
26/03/2003	27°C	Hot
27/03/2003	29°C	Cloudy
28/03/2003	28°C	Humid
29/03/2003	28°C	Hot
30/03/2003	25°C	Warm
31/03/2003	30°C	Hot
01/04/2003	27°C	Rainy
02/04/2003	28°C	Rainy

Table 17: Weather conditions from 03/04/2003 to 17/04/2003

Weather conditions for the 3rd 15 days.

Date	temperature	weather
03/04/2003	29°C	Hot
04/04/2003	24°C	Mild
05/04/2003	25°C	Mild
06/04/2003	25°C	Mild
07/04/2003	27°C	Hot
08/04/2003	27°C	Hot
09/04/2003	27°C	Rainy
10/04/2003	29°C	Rainy
11/04/2003	28°C	Light rain
12/04/2003	33°C	Very Hot
13/04/2003	26°C	Mild
14/04/2003	25°C	Mild
15/04/2003	29°C	Hot
16/04/2003	28°C	Hot
17/04/2003	24°C	Cool

Table 18: Weather conditions from 18/04/2003 to 02/05/2003

Weather conditions for the 4th 15 days.

Date	temperature	weather
18/04/2003	25°C	Cool
19/04/2003	25°C	Cool
20/04/2003	26°C	Mild
21/04/2003	23°C	Cool
22/04/2003	24°C	Mild
23/04/2003	23°C	Cool
24/04/2003	26°C	Warm
25/04/2003	29°C	Hot
26/04/2003	27°C	Hot
27/04/2003	25°C	Mild
28/04/2003	26°C	Mild
29/04/2003	22°C	Cool
30/04/2003	21°C	Cool
01/05/2003	21°C	Cool
02/05/2003	23°C	Warm

Table 19: Weather conditions from 03/05/2003 to 17/05/2003

Weather conditions for the 5th 15 days.

Date	temperature	weather
03/05/2003	19°C	Cold
04/05/2003	20°C	Cold
05/05/2003	21°C	Cool
06/05/2003	20°C	Cold
07/05/2003	22°C	Light rain
08/05/2003	25°C	Mild
09/05/2003	23°C	Cool
10/05/2003	27°C	Hot
11/05/2003	28°C	Hot
12/05/2003	30°C	Hot
13/05/2003	29°C	Hot
14/05/2003	22°C	Cool
15/05/2003	19°C	Cold
16/05/2003	19°C	Cold
17/05/2003	23°C	Mild