BIOREMEDIATION OF PETROLEUM HYDROCARBON CONTAMINATED SOIL USING INDIGENOUS CULTURES

ZHONGYUN MA

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BIOREMEDIATION OF PETROLEUM HYDROCARBON CONTAMINATED SOIL USING INDIGENOUS CULTURES

By

© Zhongyun Ma, B. Eng.

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ABSTRACT

This research consisted of studying the biodegradation potential of Total Petroleum Hydrocarbons (TPH) in a diesel contaminated soil using indigenous cultures. From a site investigation conducted on a polluted site in Argentia (Newfoundland, Canada), typical soil profiles and contaminants were determined for helping in setting up an Environmental Test Facility. The potential for bioremediation of soils in that area was studied in the laboratory. Four kinds of cultures were isolated from the petroleum hydrocarbon (diesel) contaminated soil, enriched in the laboratory and injected into the soil as a seed to increase the population of cultures. The contaminated soils were incubated in closed reactors at temperatures ranging from 25 to 5 °C and pH values from 6 to 8. The addition of mineral salts as nutrients was also included. Surfactants were used as additional chemicals to enhance the rate of bioremediation.

The degradation of TPH was evaluated by concentration monitoring (Gas Chromatography) and bacteria counting. Temperature effects study showed that biotreatability markedly decreased with decreasing temperature. The optimal rate of bioactivity was obtained in a neutral or slight acid condition, and surfactant Triton X-100 showed an enhancement of degradation of petroleum hydrocarbon, while the surfactant Tween 60 did not improve degradation. Nutrient requirement was also clearly identified. Under optimal condition, the TPH removal efficiency reached 50% of the initial value. It was also observed that bacteria seeding is possible and echances the remediation rate.

Surfactants were used to wash diesel out of the contaminated soils in column tests. Distilled water, 0.5% (w/w) aqueous solution of surfactant Tween 60 and 0.5% (w/w) aqueous solution of surfactant Triton X-100 were used as leaching solutions. Corresponding TPH removals were obtained as 5.3%, 21.7%, and 67.8% respectively, demonstrating potential efficiency of combining physical and biological remediation methods.

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A	Cross-sectional area of a compacted soil specimen
BI	Bacteria Injection
BOD	Biochemical Oxygen Demand
BTEX	Benzene, Toluene, Ethylbenzene, Xylene
C	Concentration in TPH for the prepared standard read from the calibration curve
cfu	colony form unit
CMC	Critical Micelle Concentration
COD	Chemical Oxygen Demand
conl	control sample
°C	degree Celcius
GC/FID	Gas Chromatography/Flame Ionization Detector
GC/MS	Gas Chromatography/Mass Spectrometer
h_i	average water head difference between inflow and outfolw during Δt_i
PH	Petroleum Hydrocarbon
L	Length of the sample
LOI	Loss On Ignition
k	hydraulic conductivity
MS	Mineral Salt
NAPL	None Aqueous Phase Liquid
NBI	Non Bacteria Injection

NN	No Nutrients
PAH	Polycyclic Aromatic Hydrocarbon
ppm	part per million
T	Temperature
TPH	Total Petroleum Hydrocarbon
Tri	Triton X-100 (surfactant)
TSA	Trypticase Soya Agar
Twn	Tween 60 (surfactant)
Δt_i	individual time interval
UST	Underground Storage Tank
V	Volume of solvent
V_i	The effluent volume collected during the time interval Δt_i
W	Weight of dry soil

Chapter 1

Introduction

1.1 Petroleum Hydrocarbons in the Environment

Petroleum hydrocarbons are widespread in our environment as fuel and chemical compounds. The uncontrolled release of petroleum hydrocarbons negatively impacts many of our soil and water resources. The contamination can result from leaking Underground Shorage Tanks (UST), petroleum refineries and bulk storage facilities, broken oil pipelines, spills of petroleum products in chemical plants and transportation processes (Sherman and Stroo, 1989). The risks of explosion and fire are also serious threats to the environment.

The U.S. Environmental Protection Agency (EPA) has reported that there were about 1.6 million of USTs and 37,000 hazardous tanks in 1992. Approximately 320,000 USTs are leaking, and 1,000 tanks are confirmed as new release each week (Cole, 1994). Approximately 200,000 USTs are in use in Canada. It leads to a considerable amount of petroleum hydrocarbon leaks and contamination in soil and groundwater (Scheibenbogen et al., 1994). As reported by Gruiz and Kriston (1995) an amount of 6,000,000 tons petroleum waste enter the environment each year causing serious environmental problems.

Even if the problems associated with fuel storage and distribution are solved, contamination incidental to production and commercial usage would continue to threaten groundwater supplies. Many manufacturing processes necessarily produce water and sludges that are contaminated with hydrocarbons. At a typical oil refinery facility, more than 23 different waste streams have been identified, several of which have been classified as

hazardous waste (Sims, 1990).

1.2 Remediation of Petroleum Hydrocarbons

Since the contamination of soil and groundwater by uncontrolled releases of petroleum products has become a significant problem, a number of technologies have been tested to remediate the polluted sites. In U.S. about 16,000 sites are treated each year by the states and responsible parties according to Cole (1994). Treatment processes have incorporated physical, chemical or biological methods, or a combination of them.

Remedial action on a contaminated site can involve in situ or ex situ action. The remediation methods include excavation and landfill disposal or incineration. However, these methods are expensive, and only transfer the contamination from one place to another. Bioremediation has been claimed to be an inexpensive, natural method of cleanup of petroleum contaminated soil or water. Both in situ and ex situ treatment of bioremediation have been shown to be feasible. In situ biological treatment involves the stimulation of native microbial community to levels that effectively degrade contaminants. Treatment using in situ biological methods can prove to be efficient and cost effective for the cleanup of contaminated soils and groundwater.

1.3 Objectives of This Study

This study consisted of two experimental tasks. The first project involved sampling and working on an actual site in Argentia, Newfoundland, Canada, a former site of an U.S. naval facility. More than a hundred of underground storage tanks were used on that site

leading to extended contamination by petroleum hydrocarbons such as gasoline and diesel. The involvement of the author in the first step of the establishment of a testing facility leads to question the actual feasibility of bioremediation on that site. The second part of this work was performed in laboratory using closed system reactors to biodegradate petroleum hydrocarbon contaminated soils from the Argentia site. All experiments intended to assess the effectiveness of bioremediation using native bacterial cultures.

The objectives of the site study were to

1. Collect soil samples from Argentia contaminated areas,
2. Characterize the physical properties of the soil recovered and identify the petroleum hydrocarbon contaminants,
3. Help to set up a testing facility to simulate typical soil profiles and the contaminants distribution on the Argentia site, and
4. Monitor bioremediation processes by testing water samples.

The objectives of the laboratory study were to

1. Characterize the physical properties of the soil used in the testing facility,
2. Test potential of bioremediation of petroleum hydrocarbon contaminated soil,
3. Set up close system reactors to evaluate the biodegradation of diesel contaminated soil,
4. Evaluate the effects of the temperature, pH, nutrients, population of bacteria and use of surfactants on the degradation of the diesel contaminated soil, and
5. Evaluate the leaching of diesel by typical surfactants in a column test.

The content of this thesis has been organized in six chapters that are presented as follows:

- Chapter 1 is the present introduction: the petroleum hydrocarbons in the environment and possible remediation methods. In this section, the pathes through which petroleum hydrocarbons enter the environment are introduced, and some data given indicating how much amount of petroleum hydrocarbons can be accidentally released into the environment. Inaddition, several remediation methods are presented.

- Chapter 2 is a review of fundamental principles of bioremediation, which includes a description of the microorganisms in soil, their roles in bioremediation and the definition of intrinsic and engineered bioremediation. Groups of petroleum hydrocarbon ware defined and general bioremedediation methods are presented. The factors affecting on bioremediation are discussed. General information of the pathway of hydrocarbon degradation and a description of surfactant properties are also introduced.

- Chapter 3 summarizes available information on the Argentia site and the site investigation results, showing the soil profile and the concentration of petroleum hydrocarbons. The Environmental Testing Facility is described to show the simulation process that has been under taken.

- Chapter 4 presents the laboratory experiments, which were conducted using closed system reactors and columns. Materials used in experiments, including soil, petroleum

hydrocarbon, nutrients, and surfactants are presented. Experimental methods are also introduced.

- Chapter 5 contains the results obtained from closed reactors and column tests and discusses the factors affecting bioremediation and removal of TPH by surfactants.

- Finally in Chapter 6, some conclusions and recommendations are presented .

Chapter 2

Literature Review

The literature review prepared for this study consists of two parts. Section 2.1 to 2.4 are devoted to bioremediation, its applications, limitations, and general information on the biodegradation pathway. Section 2.5 deals with surfactants. This reflects the experimental work undertaken according to the initial objectives of combining both techniques.

2.1 Fundamental Principles of Bioremediation

2.1.1 Microorganisms in Soil

" The most important principle of bioremediation is that microorganisms can be used to destroy hazardous contaminants or transform them to less harmful forms" (US National Research Council, 1993).

Since ZoBell (1946) reported that nearly 100 species of bacteria, representing 30 microbial genera, had hydrocarbon oxidizing properties , many species and genera have been found to have this ability (Texas Research Institute, 1982a) and to be widely distributed in soils. In the present study, we will concentrate on microorganisms that are present in the soil, thus excluding artificial import of extraneous species.

Microorganisms in the soil include bacteria, fungi , algae and protozoa. The bacteria are most abundant in the soil and can be heterotrophic or autotrophic in their metabolism. Heterotrophic bacteria use one or more organic compounds as a source of carbon for survival and growth, while autotrophic use inorganic material as a source of nutrients and CO_2 as the

sole source of carbon for growth and obtain their energy from light (Pelczar et al, 1986). Heterotrophic bacteria are the most important organisms in the transformation of organic compounds, and the purpose of engineered bioremediation is to enhance their activity (JRB and Associates, 1984).

Bacteria are classified into two groups, Gram-passive and Gram-negative, depending on their cell wall structure and composition. Gram-passive bacteria have a thick peptidoglycan cell wall and when stained by Gram staining technique introduced by Christian Gram in 1884 to distinguish between Gram-negative and Gram-passive bacteria by using series of staining reagents, they appear dark blue or violet. The Gram-negative bacteria have a more complex cell wall than those of Gram-passive bacteria with the presence of an outer membrane surrounding a thin layer of peptidoglycan. After Gram Stain, they appear as pink coloured (Killham,1994).

Microorganisms can release enzymes in soil. Enzymes have the ability to catalyze the oxidation of a variety of different hydrocarbons indicated by their broad substrate specificities (Gibson and Yeh, 1973). The enzyme activity of soil is the sum of the activity of all accumulated enzymes. The native enzyme activity is the result of many processes which lead to partial incorporation of locally produced enzymes into the soil environment. In other words, these enzymes are immobilized at the surface of the soil particles (McLaren, 1975).

2.1.2 Role of Microorganisms in Bioremediation

Bioremediation is a process which uses microorganisms and their biodegradative capacity to remove contaminants from the soil. In particular, native soil microorganisms play a key role in soil bioremediation. They perform as biogeochemical agents to transform complex organic compounds into simple inorganic compounds or into their constituent elements. This process is termed mineralization. The microorganisms (bacteria) are adsorbed to soil particles by the mechanism of ionic exchange. In general soil particles have a negative charge , and soil and bacteria can hold together by a ionic bond involving polyvalent cations (Killham ,1994).

Microorganisms can destroy contaminants based on microbial metabolism which is the life process of the microbial cell by which the nutritional and functional activities of an organism are maintained (Pelczar et al, 1986). They can take the contaminants for their own growth and build up new cells. Generally, soil microorganisms carry out two tasks: they take a source of carbon, which is a new cell constituent, from an organic contaminant, and they use electrons provided by contaminants to obtain energy.

2.1.3 Intrinsic and Engineered Bioremediation

Two classes of bioremediation technologies have been developed. One is termed **intrinsic**, which uses naturally occurring microorganisms to degrade contaminants and do not need engineered interventions at the site. Intrinsic remediation relies on the activity of indigenous microorganisms. The second group of technologies involves **engineered** intervention, usually to enhance the rate of bioremediation by introducing engineered modified processes such as adding microorganisms and supplying nutrients. The principle

of engineered remediation is to change environmental conditions for accelerating microorganisms activity. Therefore, the degradation of the contaminants in engineered processes can be conducted according to tighter schedule thus reducing risks and costs.

An intrinsic bioremediation case study was documented on Vancouver Island, B.C. in 1973. Approximately 180 tons of fuel oil was spilled. Cretney et al. (1978) reported that biodegradation accounted for almost complete removal of *n*-alkanes during the first year after the spill. Pristane and phytane were biodegraded more slowly, but were almost completely gone after 4 years. The non *n*-alkane components of the C_{28} to C_{30} range of appeared to be the most resistant to degradation of all the components resolvedly by gas chromatography.

An engineered bioremediation was conducted in a New Jersey wheat field which had been contaminated with approximately 1.9 million litres of kerosene over 1.5 hectares. A remediation program consisting of liming, fertilizing and frequent tilling was initiated, and the decrease of hydrocarbon contaminants was monitored for a 2-year period . During that period, the hydrocarbon content of the surface soil decreased to an insignificant level and the field returned to a near-normal productive state (Dibble and Bartha , 1979).

2. 2 Petroleum Hydrocarbon Bioremediation

2.2.1 Definition of Petroleum Hydrocarbons

Petroleum hydrocarbons are a mixture of hydrocarbons obtained from reservoirs of crude petroleum. The petroleum hydrocarbons include aliphatic hydrocarbons and aromatic hydrocarbons. The most common petroleum hydrocarbons contaminating environment are the gasoline, diesel and fuel oils.

Petroleum hydrocarbons are between C_6 and C_{25} (Parr et al, 1994). Gasoline is a light fraction in the range from C_6 to C_{10} (Parr et al, 1994) with a boiling temperature ranging from 23°C to 204°C (PEDCO Environmental Inc., 1978). Diesel fuel is in the middle distillate group (C_6 to C_{22}) with boiling temperature between 202° and 320°C (Holmes and Thomsom, 1982). Most diesel hydrocarbons are between the C_{10} and C_{18}. Fuel oil and lubricants are heavier cuts in petroleum products and similar in composition and characteristics to middle distillates. These types of fuels are relatively viscous and insoluble in water and are relatively immobile in the subsurface (Petrov, 1987). Petroleum products have basically similar chemical and physical properties. For the purpose of remediation of contaminants, the most important physical properties are volatility, solubility in water, specific gravity, and kinematic viscosity (Cole, 1994).

2.2.2 General treatment methods for petroleum hydrocarbons

Several remediation methods have been developed for cleaning up petroleum hydrocarbon contaminated soil. A traditional method is to excavate the polluted soil and landfill it under controlled condition. This method is not really remediation and is not acceptable for large areas or volumes since landfilling has become cost prohibitive. Another method for remediation is soil venting. It removes volatile hydrocarbons from the vadose (unsaturated) zone. It usually treats raw gasoline contamination. As an alternative method, bioremediation can be used to clean contaminants either in-situ or ex-situ (Cole, 1994). During the biotreatment process, the hydrocarbons are degraded by naturally occurring (indigenous) soil microorganisms to carbon dioxide, water, and biomass (Huesemann, 1994).

This process of breaking complex molecule down to simple molecule by microorganisms is called biodegradation. Bioremediation treatment technologies include:

(1) bioaugmentation defined as a treatment technology in which bacteria are added to contaminated medium. This technique is used in bioreactors and ex-situ systems;

(2) biostimulation, which is a treatment process that simulates the indigenous microbial populations in soil or ground water. The treatment can be done in-situ or ex-situ;

(3) bioreactor treatment, which is a process conducted in containers or reactors and frequently used to treat liquids or slurries contamination;

(4) bioventing treatment, which is a method to draw oxygen through the soil to stimulate microbial growth and activity;

(5) landfarming, which is used to treat solid-phase contamination. It can be done in situ or in a treatment cell (Baker and Herson, 1994 a).

Among other advantages the bioremediation processes can be done at the contaminated site with minimal transport and handling, which reduce the costs and environmental potential hazards.

Bioremediation is limited only by the lack of understanding of the microbial ecology and physiology of polluted sites and interactions between the microbial community and the physical and geochemical environment in which contaminants are degraded (Major, 1991).

2.2.3 In-situ and on site bioremediation

Bioremediation as a treatment technique can be used in-situ or on site. In-situ bioremediation means that contaminants are treated without excavation or removal from

the site. Advantages of in-situ remediation are a relatively low cost, little change in the soil structure and results that may meet regulatory clean up guidelines (Gruiz and Kriston, 1995) In-situ remediation therefore is a possible method when it is too expensive to excavate and transport the contaminated soil from the site (Wardell, 1995).

In-situ bioremediation requires that the soil matrix has the ability to supply oxygen, nutrients and contaminant-degrading organisms. The process is conducted through injection wells at the head or within the plume of contaminated groundwater in order to enhance the biodegradation rate at which the indigenous organisms grow and metabolize the contaminants (Canter and Knox, 1985).

On site remediation methods imply the excavation of the contaminated soils and the construction of a lined biotreatment cell on site. On site treatment allows a better control of remediation parameters such as temperature, moisture content, nutrient concentration, and oxygen availability. But the excavation of the contaminated soil increases the cost of the operation.

2.2.4 Laboratory Treatability studies

Laboratory studies are necessary for assessing the biodegradation potential of a site prior to initiating the process at full-scale. Laboratory treatability studies are conducted in various ways. Generally, three kinds of tests are used: (1) pan studies which simulate solid-phase bioactivity; (2) flask studies that perform liquid-phase and slurry-phase biological process; (3) column studies which represent in situ bioremediation (Nelson et al, 1994).

Laboratory tests can be used to select optimal conditions for bioremediation. Several conditions are usually tested including unmodified microorganisms, nutrient amended microorganisms, and biologically inhibited conditions. These tests can measure the rate of change on the microbial populations. They provide data on the rate and extent of conversion of contaminants.

A laboratory scale biotreatment of diesel contaminated soil was conducted in a bioreactor by Britto and his coworkers (1994). Soil was contaminated by 1500mg of diesel per kg of wet soil. A continuous type reactor was operated and nutrients, moisture, and oxygen were monitored at all time. The reactor was operated for 70 days in a steady condition, and diesel fuel was measured in terms of TPH. Over the treatment process period, 80% of TPH was removed.

2.3 Factors Affecting Bioremediation

Bioremediation generally occurs when the microorganisms use the pollutant as a carbon source. Hence, degradation is accompanied by microorganism growth. An efficient degradation is dependant on the presence of other required nutrients, including nitrogen, phosphorus, and so on. Suitable environmental conditions, with respect to pH, temperature, moisture content, and redox potential are also required.

2.3.1 Microbial factors

Many microorganisms are able to degrade petroleum hydrocarbons. They are present in contaminated soil and water, most of them are aerobic organisms and can make use of

organic contaminants for their growth. Since individual organisms can metabolize only a limited range of hydrocarbon substrate, it is necessary to assemble several bacteria with a broad catabolic potential which has the ability to dissimilate or break down complex organic molecules and release energy, in order to degrade the complex mixture of hydrocarbons that may affect a contaminated site. Keuning and Jager (1994) used pure and mixed Pseudomonas cultures to degrade chlorobenzene, toluene, xylene, and ethanol. The results showed that a mixed culture made of three strains demonstrated more stable growth behaviour and degraded contaminants to much lower concentrations than pure cultures.

Natural soil microorganisms may not have the metabolic capability to readily degrade certain compounds, and seeding of microorganisms into the soil has been performed to enhance the process of bioremediation (bioaugmentation). Generally, natural soil microorganisms have been previously isolated and enriched as a "seed". They are added during in situ treatment thus increasing the biomass and reducing the time necessary for remediation (Hinchee et al., 1994).

As indicated before many hydrocarbon-degrading bacteria can be found in soils and some of the common ones are listed in Table 2-1.

Table 2-1. Common Hydrocarbon Degrading Bacteria Found in Soils

Bacteria	
Achromobacter	Micrococcus
Acinetobacter	Mycobacterium
Alcaligenes	Nocardia
Arthrobacter	Proteus
Bacillus	Pseudomonas
Brevibacterium	Sarcina
Chromobacterium	Serratia
Corynebacterium	Spirillum
Cytophaga	Streptomyces
Erwinia	Vibrio
Flavobacterium	Xanthomonas

(After Killham, 1994)

2.3.2 Hydrocarbon variety and concentrations

Hydrocarbon variety and concentrations are factors that affect biodegradation. Hydrocarbons with a low molecular weight are relatively easy to biodegrade. Branched hydrocarbons degrade more slowly than the corresponding straight-chain hydrocarbons. Generally, when molecule size increases, the rate of biodegradation decreases, and monoaromatic compounds are more rapidly degraded than the two-, three-, four- and five-ring compounds. Comparatively lighter mixtures such as gasoline can be readily biodegraded to low levels. Heavier products such as number 6 fuel oil, a heavy fuel oil with a range C_{18}-C_{25} (Baker and Herson, 1994b), or coal tar which contains many heavy molecular compounds, degrade much more slowly than gasoline.

The concentration of hydrocarbon can affect the bioactivity and be toxic to the microorganisms (US. Environmental Protection Agency, 1985). High concentration of hydrocarbons can be inhibitory to microorganisms, thus slowing down the remediation rate. At highly elevated concentration, contaminant can become toxic for microorganisms. (Alexander, 1985).

2.3.3 Soil structure

Soil structure controls the transmission of water, oxygen, and nutrients to the area of bioactivity. Generally, fine particles such as clay and silt transmit these substances slowly. Permeable soils, such as sands and gravels, are more favourable to nutrient transport and relatively rapid clean up can be achieved. Characteristics of the soils, such as composition, particle size distribution, percent moisture content, percent organic and cation exchange capacity (Skladany and Baker, 1994), may also be important for the remediation of contaminants.

2.3.4 Nutrients

Most microorganisms existing in the subsurface are part of an ecosystem that has low organic carbon content. The heterophic microorganisms found in soils possess the ability to degrade petroleum products (Odu, 1978, Pinholt, 1979), but they require nutrients to grow. Nitrogen and phosphorous are the most common nutrients for bacteria. Jamison et al. (1975) reported that addition of nitrogen and phosphorus enhanced in situ gasoline degradation. Other nutrients required for bacteria metabolism are potassium, magnesium, calcium,

sulphur, sodium, manganese, iron, and trace metals. The essentials for biological growth and sources are listed in Table 2-2.

Table 2-2 Essential Elements for Biological Growth

Element	Source
Carbon	Air and water
Hydrogen	
Oxygen	
Nitrogen	Soil, inorganic fertilizers, or in waste
Phosphorus	
Potassium	
Sulfur	
Calcium	Soil liming materials, or in waste
Magnesium	
Iron	Soil, soil amendments, or in waste
Manganese	
Boron	
Molybdenum	
Copper	
Zinc	
Chlorine	
Sodium	
Cobalt	
Silicon	

(After Fry et al , 1992)

Westlake et al. (1978) examined the in situ degradation of oil in a soil of the boreal region of the Northwest Territories of Canada. Where fertilizer containing nitrogen and phosphorus was applied to the soil, there was a rapid increase in bacterial numbers. This was followed by a rapid disappearance of n-alkane and isoprenoids and a continuous loss of weight of saturated compounds in the recovered oil. This study indicated that addition of nitrogen and phosphorus containing fertilizers can be used as nutrients to stimulate microbial degradation of petroleum hydrocarbons.

2.3.5 Oxygen

In hydrocarbon aerobic bioremediation, oxygen availability is a critical factor (Floodgate, 1973). Bacteria activity proceeds more rapidly if sufficient oxygen is provided. During aerobic biodegradation, molecular oxygen is reduced to water while petroleum hydrocarbon is oxidized to create energy, cell mass, and carbon dioxide.

The supply of oxygen to the scene of microbial activity is controlled by soil saturation and conduction. Dineen et al (1990) reported that the requirement of oxygen to degrade hydrocarbon is 3.1 g of oxygen for 1.0 g of hydrocarbon. The largest amount of oxygen required is approximately 200,000 ppm in a well aerated soil and 8 ppm in a saturated soil. Brown and his coworkers (1984) developed several projects where oxygen supply was identified as a critical point if the processes are to be generally applicable. This demonstration led to use hydrogen peroxide as an oxygen carrier (Brown et al., 1984). Increasing oxygen availability by treating the soil with dilute hydrogen peroxide, H_2O_2, at a concentration up to 1000 mg/l (Texas Research Institute,1982a) has been successfully tried.

Dlathman et al. (1991) evaluated the utilization of hydrogen peroxide for enhanced biological treatment of petroleum hydrocarbon contaminated soil in laboratory. JP-5, diesel fuel, and lubricating oil were used as model petroleum hydrocarbons. The concentration of the H_2O_2 was approximately 500 mg/l. Biotreatment was monitored by bacteria population density and concentration of petroleum hydrocarbons. Results showed enhanced removal of the petroleum hydrocarbon after comparing control sample and test sample.

Soil venting is a method that provides oxygen to the contaminated area by introducing air into the vadose zone in order to increase the activity of native bacteria and allow them to degrade the contaminants. Dineen et al (1990) reported on three bioventing projects in southern California. They treated the vadose zone with ammonia and air resulting in a one to two orders of magnitude increase in the microbial counts and in the amount of degraded hydrocarbons.

2.3.6 Temperature

Soil temperature is another factor which can affect microbiological activity and the rate of the contaminant decomposition (Sims and Bass, 1984). Generally, a high temperature induces a high rate of biological degradation processes in the soil (JRB and Associates Inc.,1982). Very low rates of hydrocarbon utilization were found by Gunkel (1967) at low temperature because low temperature leads to a slow rate of microbial growth. The rate of degradation can double for every 10^0C rise in temperature (Thibault and Elliot, 1979). ZoBell (1969) found that hydrocarbon degradation was over an order of magnitude faster at 25°C than at 5°C. Most soil microorganisms have an optimal growth for temperature in the

range of 20 to 35°C (Parr et al.,1983). The majority of organisms that degrade petroleum products are active in this range. Enrichments of thermophilic microorganisms have an optimal temperature for degradative activity comprised between 50° and 60°C .

2.3.7 Soil moisture

Microorganisms require water for microbial growth and for diffusion of nutrients and by-products during the degradation process (JRB and Associates Inc., 1984). If the soil is too dry, many microorganisms will die. If water content of the soil is too high, oxygen transfer to microorganisms will be resisted by the flooded soil and the rate of the hydrocarbon degradation will be reduced. The optimum soil water content for bioremediation is dependent on the soil type. Generally, the optimum activity occurs when the soil moisture is 50-80% of the field capacity, also termed the water holding capacity which is defined as "the amount of the water remaining within the soil after gravitational water has drained away" (Baker, 1994) or the percentage of water in a soil when it was saturated (JRB and Associates, Inc.,1984). When moisture content is lower than 10% of the holding capacity, the bioactivity becomes marginal (Testa and Winegardner, 1991).

2.3.8 pH value

Biological activity in the soil can be affected by the pH. Some microorganisms can survive in a wide range of pH, but others are sensitive to small variations. The bacteria grow better in pH values between 6.5 and 8.5 (Dibble and Bartha,1979). Bioremediation is

therefore favoured by near neutral pH values (6-8). Soil pH can be adjusted if necessary to enhance microbial activity.

2.4 General Information on Degradation Pathway of Petroleum Hydrocarbons

Petroleum hydrocarbon is a complex mixture of hydrocarbons. Several studies have been performed to determine the metabolic pathways for degradation of these compounds. In this section, general information on the degradation pathways is reviewed.

2.4.1 Degradation pathway of aliphatic hydrocarbons

2.4.1.1 Oxidation of hydrocarbons

The n-alkanes are generally considered to be the most readily degraded compounds in a petroleum mixture. Biodegradation of n-alkanes with molecule weight up to C_{44} has been demonstrated (Haines, 1974). Three steps are involved in degradation of aliphatic hydrocarbons (Gaudy, Jr and Gaudy, 1980). The initial step is an oxidation reaction that involves molecular oxygen, and oxidation is catalyzed by an enzyme. The terminal methyl group is first oxidized to a primary alcohol. The alcohol then undergoes successive oxidation to form an aldehyde which is then converted to a fatty acid. The conversion of the alcohol

to an aldehyde and then to a fatty acid are mediated by the enzymes as shown in Figure 2-1. Further oxidation of the fatty acid proceeds via β-oxidation.

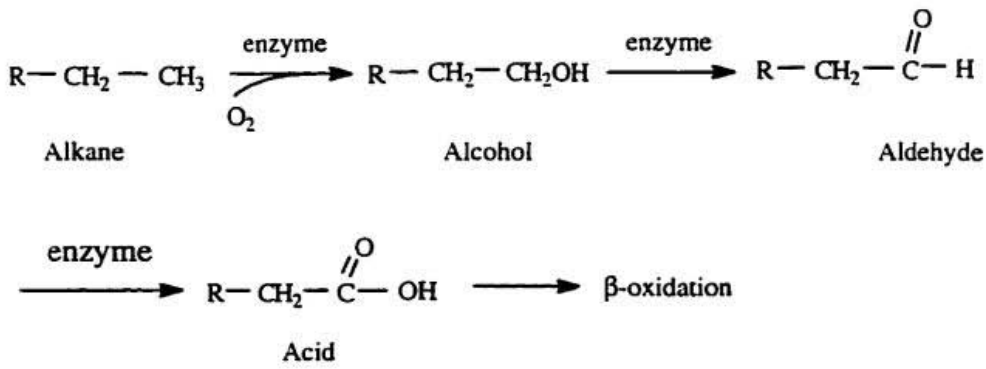


Figure 2-1 Degradation of Aliphatic Hydrocarbon (After Gaudy, Jr and Gaudy, 1980)

2.4.1.2 β-oxidation reaction

β-oxidation is an oxidation process in which the beta carbon (second carbon from the carboxyl carbon) is oxidized. The first step involved in the β-oxidation reaction is the conversion of fatty acid into acyl-CoA with an enzyme catalysing the reaction. The acyl-CoA is converted into an unsaturated acyl-CoA by the enzyme. The unsaturated acyl-CoA is then converted into β-hydroxyacyl-CoA and then to β-ketoacyl-CoA with the mediation of the enzymes. The product is now cleaved into acetyl-CoA and fatty acid acyl-CoA by the enzyme thiolase. The fatty acid acyl-CoA which is shorter than the original fatty acid and by two carbon atoms now goes through the same series of reaction, loosing the next two carbon atoms as acetyl-CoA. Repetition of this reaction sequence converts a fatty acid with an even number of carbon atoms totally to acetyl-CoA which enters the Tricarboxylic Acid Cycle

(TCA) (Figure 2-2).

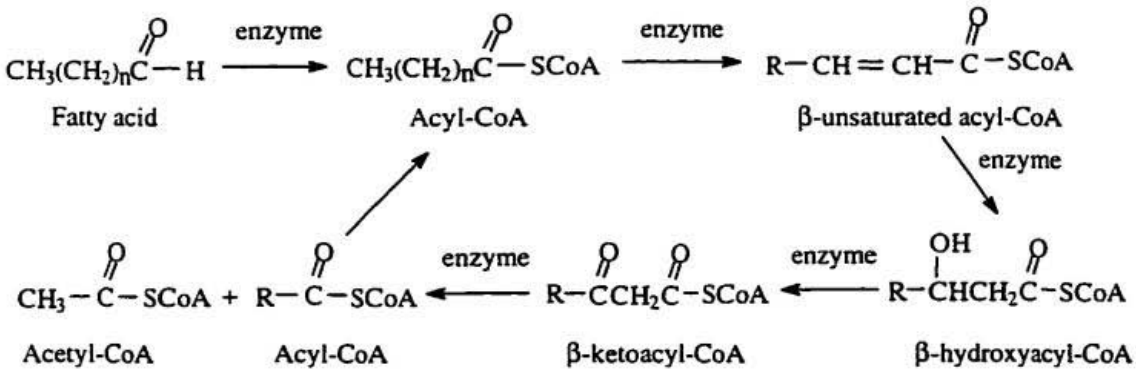


Figure 2-2 Degradation of Fatty Acid by β-oxidation (After Gaudy, Jr and Gaudy, 1980)

2.4.1.3 The TCA cycle

The TCA cycle begins when the two-carbon compound acetyl CoA condenses with the four-carbon compound oxaloacetic acid to form citric acid, a six-carbon organic acid, which is converted into isocitric acid . One carbon atom is then removed as a CO_2 from the isocitric acid to form a α-ketoglutaric acid which undergoes oxidation decarboxylation by removal of one more carbon as a CO_2 , yielding a succinyl-CoA. These two reactions are catalyzed by the enzymes. The succinyl-CoA undergoes a series of reactions, first yielding succinic acid, fumaric acid, then malic acid, and finally oxaloacetic acid. The enzymes catalyze these conversions. The oxaloacetic acid passes through the process again with the next molecule of acetyl-CoA.

For each acetyl-CoA entering the TCA cycle, two molecules of CO_2 are released, one by the decarboxylation of isocitric acid, and the other by the decarboxylation of α-ketoglutaric acid. The net result of the passage of the acetyl-CoA through the TCA cycle is the complete oxidation of acetyl-CoA to CO_2 with production of four molecules of hydrogen (Figure 2-3).

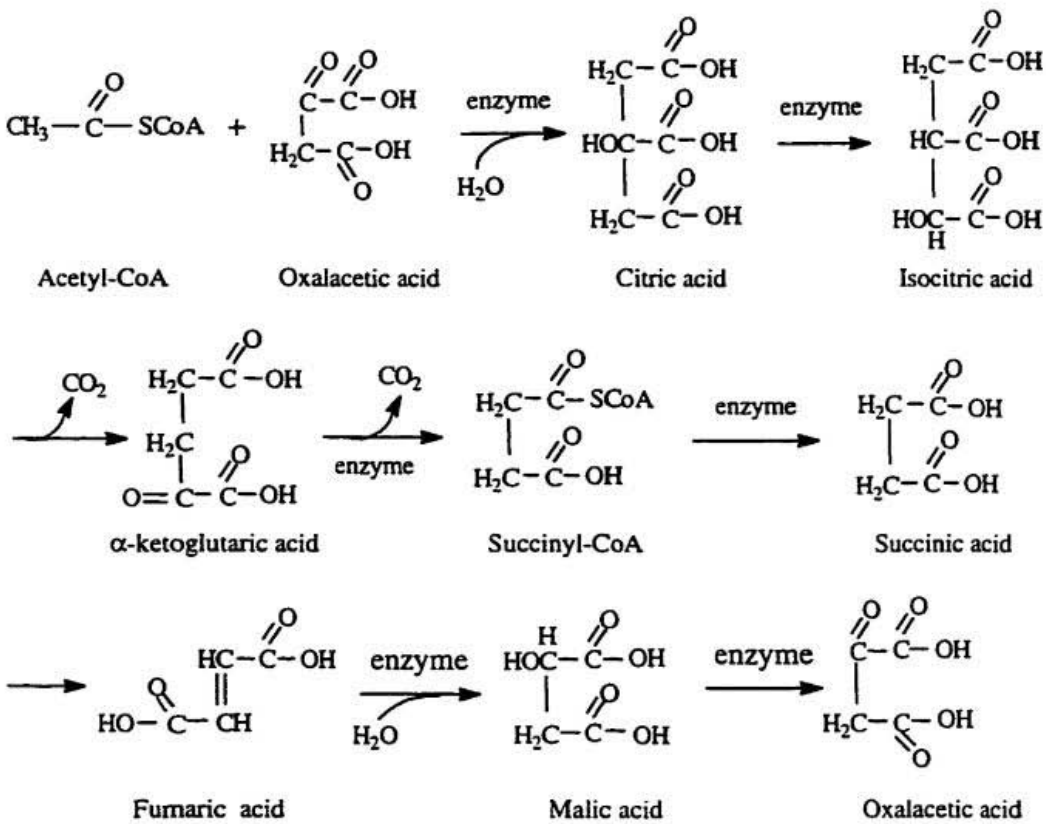


Figure 2-3 The Tricaroxylic Acid (TCA) Cycle (After Gaudy, Jr and Gaudy, 1980)

2.4.2 Degradation pathway of aromatic hydrocarbons

Most aromatic hydrocarbons, such as benzene and its derivatives are initially degraded by cleavage of the aromatic ring to form a straight-chain acid. The cleavage is carried out by dioxygenases and involves the incorporation of molecular oxygen into the ring structure. The resulting compound dihydro-dihydrobenzene is then converted to catechol by the enzyme dehydrogenase and then cleaved between the two closed hydroxyalted carbon atoms by the enzyme to form muconic acid which is further metabolized into β-ketoadipic acid. The enzyme is involved in the reaction to active β-ketoadipic acid and further into succinic acid and acetyl-CoA which are intermediates in the TCA cycle. The degradation path is shown in Figure 2-4.

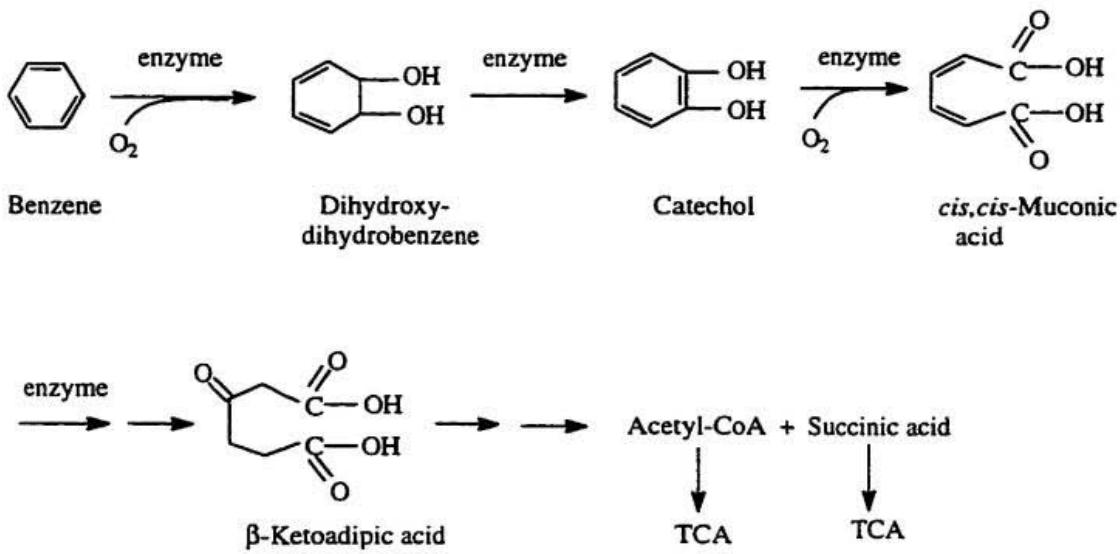


Figure 2-4 Degradation of Typical Aromatic Hydrocarbon

(After Gaudy, Jr and Gaudy, 1980)

Some Polycyclic Aromatic Hydrocarbons (PAH) can be degraded by bacteria such as Pseudomonas species. The degradation pathway of naphthalene (bicyclic aromatic hydrocarbon), anthracene and phenanthrene (tricyclic aromatic hydrocarbonand) were reported by Schlegel (1993). Unlike benzene, they are initially degraded into salicylate instead of catechol. The salicyalte is then converted to form catechol by the enzyme. Like the degradation of catechol, the carboxy-muconic acid is converted into β-ketoadipic acid which is further metabolized in a manner similar to the β-ketoadipic acid produced from catechol degradation .

2.5 Surfactant Definition and Properties

2.5.1 Classification of surfactants

Surfactants, surface active agents, are amphiphilic molecules which consist of two distinct structural parts. One is polar, and another is nonpolar. The polar part of the molecule has an affinity for water and other polar substances, while the nonpolar part is hydrophobic (Edwards et al., 1991).

Surfactants are classified based on the charge of the hydrophillic group. Functional groups in the hydrophillic end can impart a charge to this part of the molecule. An anionic surfactant carries a negative charge at its hydrophillic end while a cationic surfactant carries a positive charge. When negative and positive charges are present the surfactant is defined as zwitterionic, or if no polarization occurs, it is termed nonionic surfactant (West and Harwell, 1992). Four examples of these types of surfactants are listed in Table 2-3.

Table 2-3 Four types of surfactants (After West and Harwell, 1992)

Surfactant examples	Ionic type	Molecular structure
Sodium dodecylsulfate	Anionic	$C\ H_3(CH_2)_{11}OSO_3^-\ Na^+$
Benzyltrimethylammonium	Cationic	$[(CH_3)_3N\text{-}CH_2\text{-}C_6H_5]^+Br^-$
Triton-100	Nonionic	$C_8H_{17}C_6H_4(OCH_2CH_2)_x\text{-}OH$
B-N-alkyl aminopropionic acid	Zwitterion	$R^+\ NH_2CH_2COO^-$

2.5.2 Effects of surfactant on petroleum hydrocarbons biodegradation

Surfactants can affect hydrocarbon solubilization and mobilization, and influence the success of bioremediation, since the physical state of a hydrocarbon can determine its rate of biodegradation. Surfactants can increase the bioavailability and improve microbial utilization rates.

Solubilization of an organic contaminant by a surfactant depends on a process called micelle formation. As a result of its amphiphilic nature, a surfactant molecule may dissolve in water as a monomer, adsorb at an interface or be incorporated with other surfactant molecules as part of a micelle. When the surfactant concentration is less than a specific concentration, surfactant molecules exist predominantly in monomeric form. The surfactant concentration at which monomers begin to assemble in colloidal

aggregates (Figure 2-5) is termed the critical micelle concentration (CMC). Aggregates of micellar surfactant molecules create a hydrophobic less polar core into which contaminant are accommodated. Therefore, the solubilization of contaminants is markedly increased (Yeom and Ghosh, 1993).

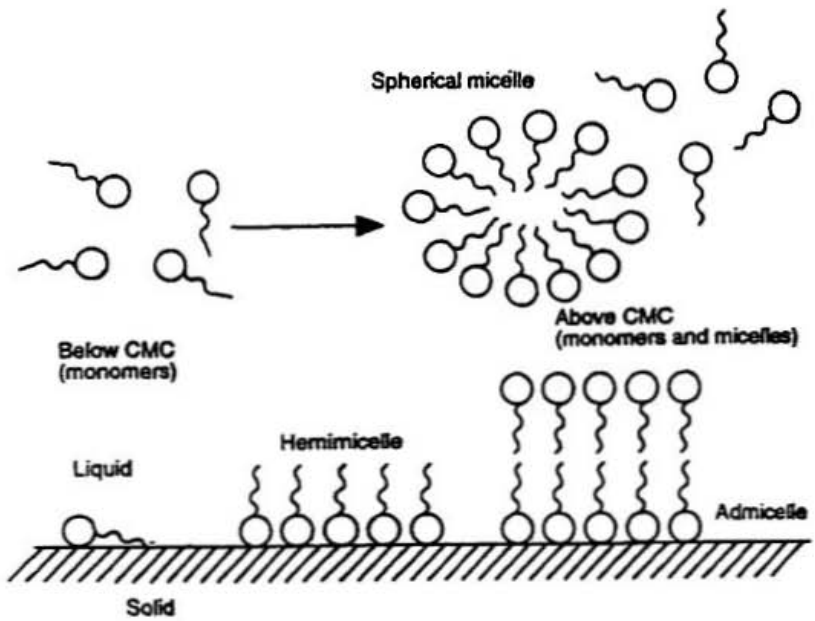


Figure 2-5 Surfactant micellization (After West and Harwell, 1992)

Surfactant can mobilize trapped contaminants in a soil matrix (Bury and Miller, 1993), depending on the surface tension reduction. Low surface tension increases the wetting of the soil particles and provides better contact between surfactant and contaminants (Texas Research Institute, 1982b and Ellis et al, 1986).

Many of the surfactants which have been subject of study involving petroleum hydrocarbon solubilization and biodegradation are nonionic surfactants. Studies have showed the beneficial effects of surfactant on hydrocarbon biodegradation in laboratory experiments involving soil or sediment solids. Rittmann and Johnson (1989) pointed out that cultured oil-degrading bacteria and surfactant added to lubricating oil-contaminated soils greatly increased the initial oil degradation rates and the removal extent. The primary reason for the degradation enhancement in the surfactant system was attributed to the increased interfacial area which made the substrate more bioavailable.

Rittmann and Johnson (1989) reported that nonionic surfactants, such as alkylphenolethoxylates, alkylethoxylates, are effective to degrade oil because they reduce interfacial tension between water and the hydrocarbons. Liu et al (1991) examined the enhanced solubilization of phenanthrene, anthracene and pyrene by anionic and nonionic surfactant in soil-water suspensions.

Surfactants can be chemical surfactants or biosurfactants which are produced by some microorganisms when grown on a specific substrate. These particular microorganisms enhance the bioavailability of both organic and inorganic compounds through producing biosurfactants (Champion et al, 1994).

Many oil-degrading microorganisms produce emulsifying agents. Naturally occurring

biosurfactants such as Sophorose Lipids and Rhamnolipid, seem to be effective in the degradation of hydrocarbons (Van Dake et al., 1993). Oberbremer et al (1990) examined the effect of the addition of a biosurfactant, the sophorose lipid, on hydrocarbon degradation in a soil. They found that the hydrocarbons degradation rate could be doubled by addition of this biosurfactant. Ishihara et al (1995) used a microbial consortium SM8 to degrade 50 to 60% of the saturated hydrocarbons and 30 to 40% of the aromatic hydrocarbons of crude soil in 30 days in batch culture. Lindoerfer et al. (1992) demonstrated that biologically produced surfactants will enhance rates of petroleum hydrocarbon biodegradation significantly. They showed that treating crude oil-contaminated soil with a mixture of a glycolipid biosurfactant and a chemical surfactant could produce a threefold increase in the overall rate of hydrocarbon biodegradation.

However, surfactant may affect soil biology. Laboratory evidence of inhibitory effects of surfactant under different conditions on Poly Aromatic Hydrocarbon (PAH)-degrading microorganisms was reported by Laha and Luthy (1991). They found that nonioic surfactant alcohol ethoxylates at concentration of CMC inhibited the mineralization of the phenantherene, and did not enhance the rate of mineralization of the phenanthrene at a sub-CMC concentration in soil-water systems. Soil microorganism activity and vitality have been negatively influenced by some types and concentrations of surfactant (Litz et al., 1987).

Chapter 3

The Argentia Site and the Environmental Testing Facility

3.1 Site Presentation

The Argentia is the former site of a United States Naval Facility shown in Figure 3-1. It is located on the western coast of the Avalon Peninsula of Newfoundland, Canada, approximately 130 km West of St. John's and 150 km South of Clarenville. It was constructed in 1941 in two areas. The Northside, which is approximately 432 hectares in size, contained all the facility's hangars, fuelling areas, supply and office buildings, and two large underground tanks farms for the storage and distribution of petroleum fuel products. The Southside was the residential area. The Argentia has been closed in 1994, and the land turned over to the Canadian Government (Argentia Remediation Group, 1995).

There are a total of 167 tank locations and pipeline installations identified on the Northside and Southside. Some tanks and pipelines are leaking and caused contamination of the soil and ground water in that areas. An environmental risk assessment has been undertaken by the Argentia Remediation Group (Argentia Remediation Group, 1995). The objective of this study was to investigate a typical contaminated area, i.e. the Northside Bulk Fuel Farm, and to obtain soils and contaminants information to assist in developing a soil profile for the Environmental Testing Facility to be described hereafter.

The Testing Facility was setup in the Southside to provide an experimental tool for

testing and assessing innovative remediation techniques in particular biological methods. The facility is the first commercial test site in North America and will serve companies in testing their environmental cleanup equipments and techniques.

3.2 Site Investigation

3.2.1 Soil sample collection

Research started by investigating a typical area of the Argentia contaminated site in order to know what amount and what types of contaminants were left underground. A backhoe was used to dig a pit at the Northside Bulk Fuel Farm of the Argentia as shown on Figure 3-1 refered to as (PIT). Down to a depth of 40 cm, the soil was not contaminated. A shovel was used to collect around 20 kg of soil , which was placed in a strong plastic bag for soil physical property tests.

The pit was further dug down to 1.2 m depth, and a nuclear densimeter was used to measure the density and moisture content of the soil in place. At 2.0 m depth, brown gravel and coarse and sticky soil were encounted, and strong fuel smell could be felt. To avoid damage to the densimeter, no measurement was done below that depth. All measurements and the soil description are given in Table 3-1. At each depth, soil samples were collected, placed in glass jars and sealed immediately. Soil samples were kept at 4 ^{0}C in a refrigerator to prepare for extraction of contaminants. Physical properties of the soil were measured and are summarised in Table 3-2. The grain size distribution for the soil collected at 40 cm is shown in Figure 3-2 using ASTM standard D422-63 (ASTM, 1996a). Soil consisted of 62.1% gravel, 26.8% sand, 8.6% silt, and 2.5% clay.

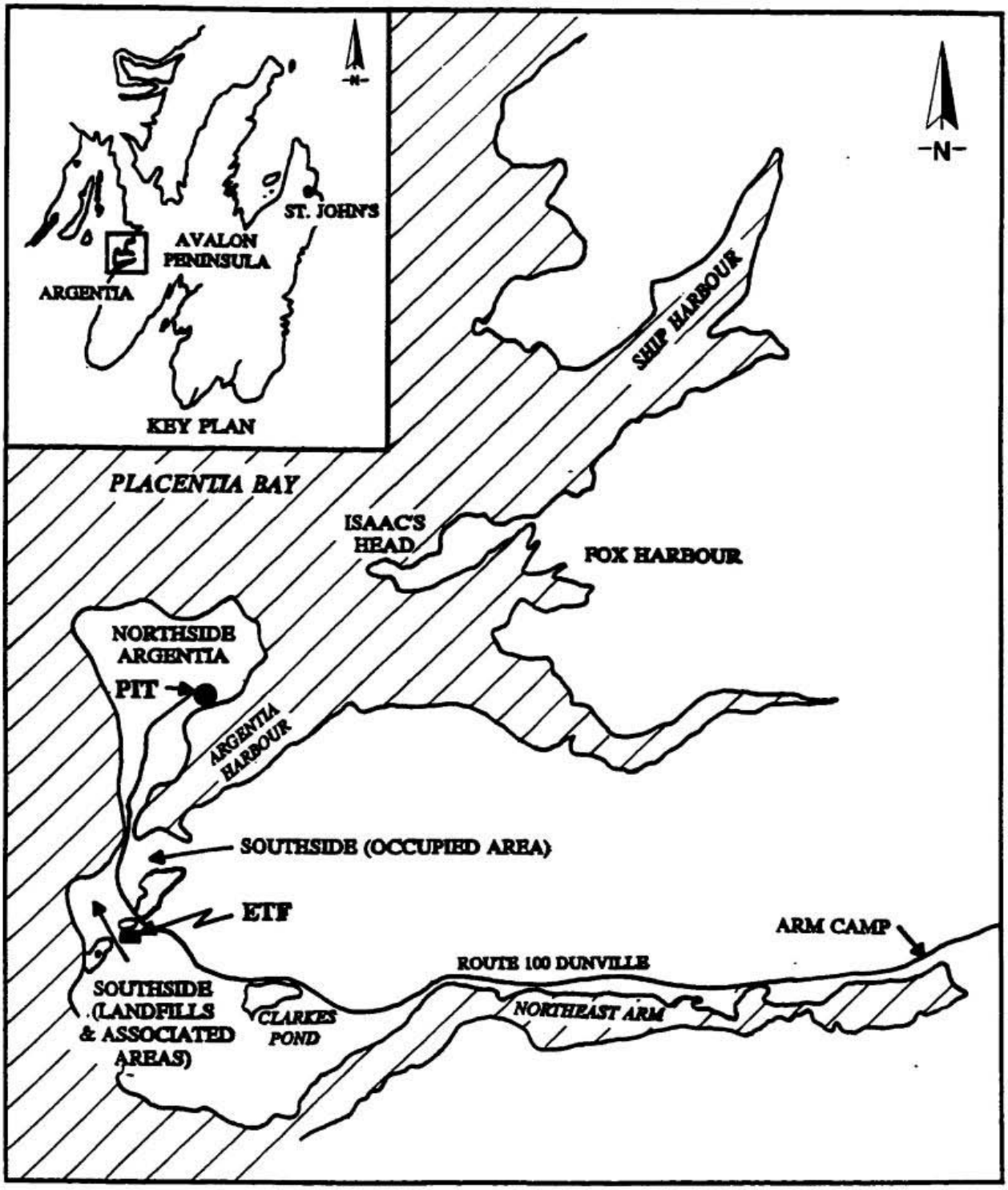


Figure 3-1 Location of The Argentia Site (After Argentia Remediation Group, 1995)

Table 3-1 Soil Description and in Place Properties

Depth (m)	Dry Density ($kg/m^{3)}$	Wet Density (kg/m^3)	Moisture Content (%)	Description of soil
1.2	1918	2020	5.4	Dark coarse gravel
1.8	2045	2202	7.7	Dark coarse gravel , smell like fuel
2.0	not measured	not measured	not measured	Brown coarse gravel, strong fuel smell , with sticky layers
2.5	not measured	not measured	not measured	Grey coarse gravel, strong fuel smell
3.5	not measured	not measured	not measured	Grey coarse gravel, fuel smell, reached ground water level

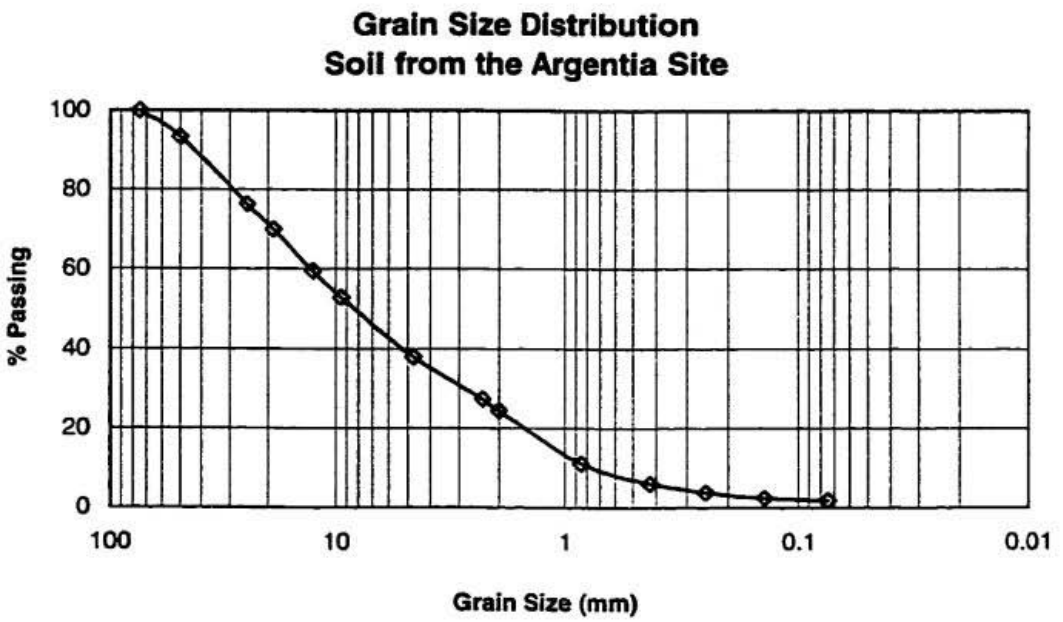


Figure 3-2 Grain Size Distribution of Soil collected at Argentia (PIT, depth of 40 cm)

Table 3-2 Summary of typical Argentia Soil Properties at Depth of 40 cm

Soil Properties	Values
Liquid Limit (CAN/BNQ 2501-092) of Fines	22
Plastic Limit (ASTM D424-59) of Fines	15
Plasticity Index of Fines	7
Shrinkage Limit (ASTM D424-59) of Fines	14
Specific Gravity (ASTM D854-59)	2.69
Optimum Water Content (% dry wt.)	11.1
Maximum Dry Density (g/cm^3)	2.15
pH (1:1 Soil -Water)	6.5

3.2.2 Extraction of hydrocarbons

Following soil sampling, contaminants were extracted, using Soxhlet extraction method 3540 (Test Methods for Evaluating soil waste, SW-864, 1982). Particles larger than sieve # 8 US (2.36 mm) were removed. 20-30 g of the remaining soil was blended with an equal weight of anhydrous sodium sulfate in a glass beaker for 20 minutes. The mixture was placed in a cellular extraction thimble. 300 ml of the extraction agent (methylene chloride) was prepared in a flask with two boiling stones.

Soil sample was extracted for 24 hours at three cycles per hour. Following the extraction, the extract was concentrated in volume to around 1 ml in a rotary evaporator at a temperature of 30°C. The concentrated extract was pipetted in a 2 ml vial and blown down to a constant weight using nitrogen. The vial was then sealed and stored in a refrigerator.

The amounts of extracted products from soil samples at different depths are listed in Table 3-3.

Table 3-3 Extraction of Contaminants from Samples at Different Depths

Sample No	Depth (m)	Extracted Contaminants (mg/kg of dry soil)
1	1.2	450.5
2	1.8	629.3
3	2.0	5694.7
4	2.5	17031.3
5	3.5	796.4

3.2.3 Separation of hydrocarbons

The extract obtained from depth 2.5 m was used to evaluate the typical composition of saturated and aromatic hydrocarbons in a glass adsorption chromatography column. The precleaned adsorbents (silica gel and alumina) are activated by heating them in an oven at 80°C. Six grams of the silica gel and six grams of alumina were measured, and partially deactivated by adding 0.45 gram of distilled water to silica and 0.45 gram to the alumina. The column was first cleaned with acetone followed by hexane and C_2Cl_2 and drained out, then silica was mixed in a slurry with C_2Cl_2 and poured into the glass column. Several rinses of hexane were done to get all the silica gel into the column. With the silica in the lower section, the excess C_2Cl_2 was drained slightly above the silica top. Alumina was loaded into the column using the same procedure.

After the column was packed, the extract was taken out from the refrigerator and 50 mg of it was pipped into a 1 ml vial filled with hexane that was in turn placed in a 5 ml beaker. The beaker was then placed in a ultrasonic bath for 3 minutes. The content of the 1ml vial was injected in the column by a syringe, which was rinsed with hexane. 30 ml of hexane was added to the column content, and effluent was collected by a flask labelled "saturated hydrocarbon". 30 ml of 8/2 of hexane/dichloromethane was then added to the column, and the effluent was collected in another flask labelled "aromatic hydrocarbon".

The solvent in the flask was evaporated using the rotary evaporator, and the content in the flask was pipetted into a 1ml preweighed vial, then dried using N_2 to a constant weight which was recorded. Using this procedure, the extract under study showed a composition of 90.8% of saturated hydrocarbon and 9.2% of aromatic hydrocarbon. The vial was sealed and stored in the refrigerator for Gas Chromatography (GC) analysis.

3.2.4 Analysis of contaminants

A Gas Chromatography/Flame Ionization Detector has been used to identify individual hydrocarbons by using the EPA test method 8015 (Test Methods for Evaluating soil waste, SW-864, 1982). The schematic of the procedure is showed in Figure 3-3.

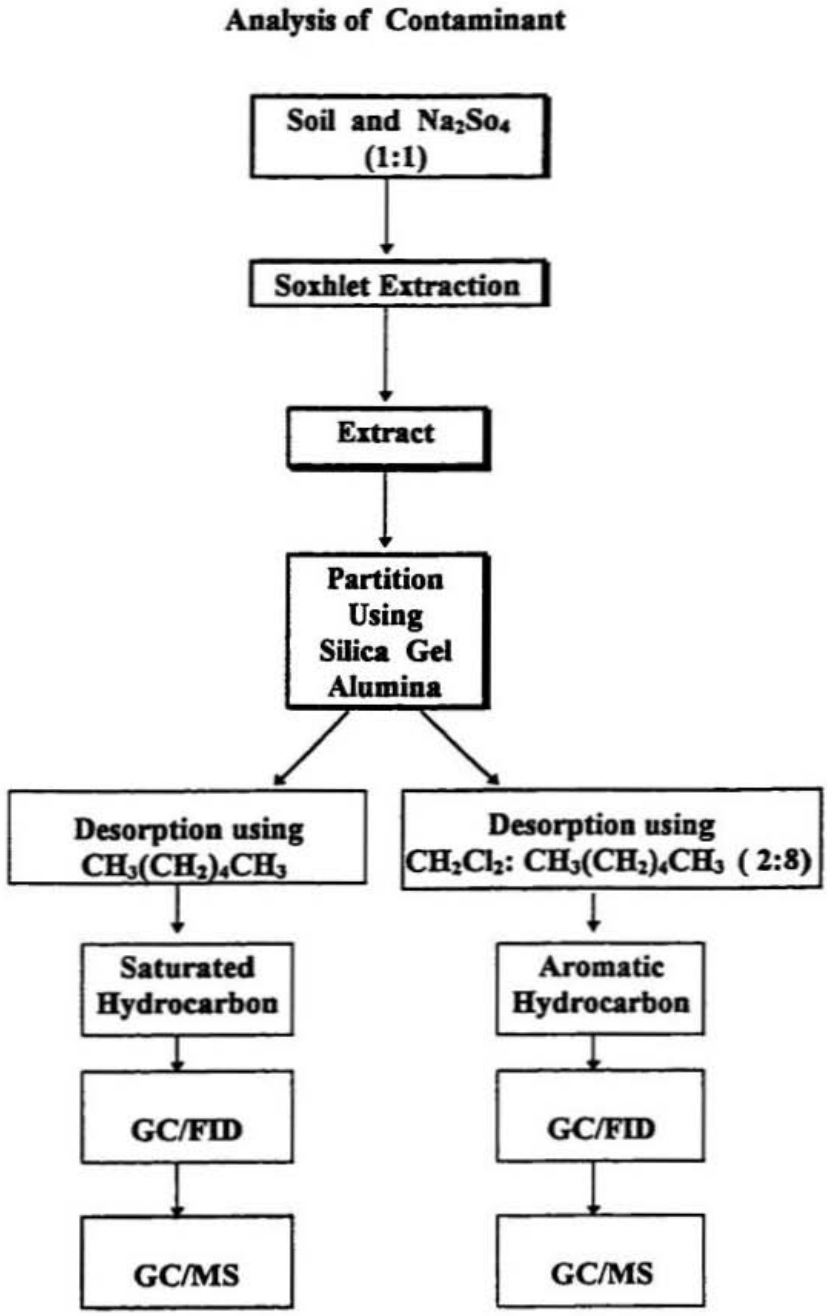


Figure 3-3 Schematic of Extraction, Partition and Analysis of Hydrocarbons

Table 3-4 Hydrocarbon Analysis of a typical Argentia Contaminated Soil Extract from PIT at depth of 2.5 m

Hydrocarbon	No of Carbon	Mole Fraction
Hexanes	C6	<0.0001
Heptanes	C7	<0.0001
Octanes	C8	0.0004
Nonanes	C9	0.0074
Decanes	C10	0.0222
Uncecanes	C11	0.0477
Dodecanes	C12	0.0688
Tridecanes	C13	0.1001
Tetradecanes	C14	0.1009
Pentadecanes	C15	0.1444
Hexadecanes	C16	0.1078
Hepatadecanes	C17	0.1256
Octadecanes	C18	0.0849
Nonadecanes	C19	0.0646
Eicosanes	C20	0.0451
Heneicosanes	C21	0.0363
Docosanes	C22	0.0212
Triacosanes	C23	0.0131
Tetracosanes	C24	0.0068
Pentacosanes	C25	0.0020
Hexacosanes	C26	0.0004
Heptacosanes	C27	0.0002
Octacosanes	C28	0.0001
Nonacosanes	C29	<0.0001
Tricontanes plus	C30+	<0.0001
Total		1.0000

3.3 The Environmental Testing Facility

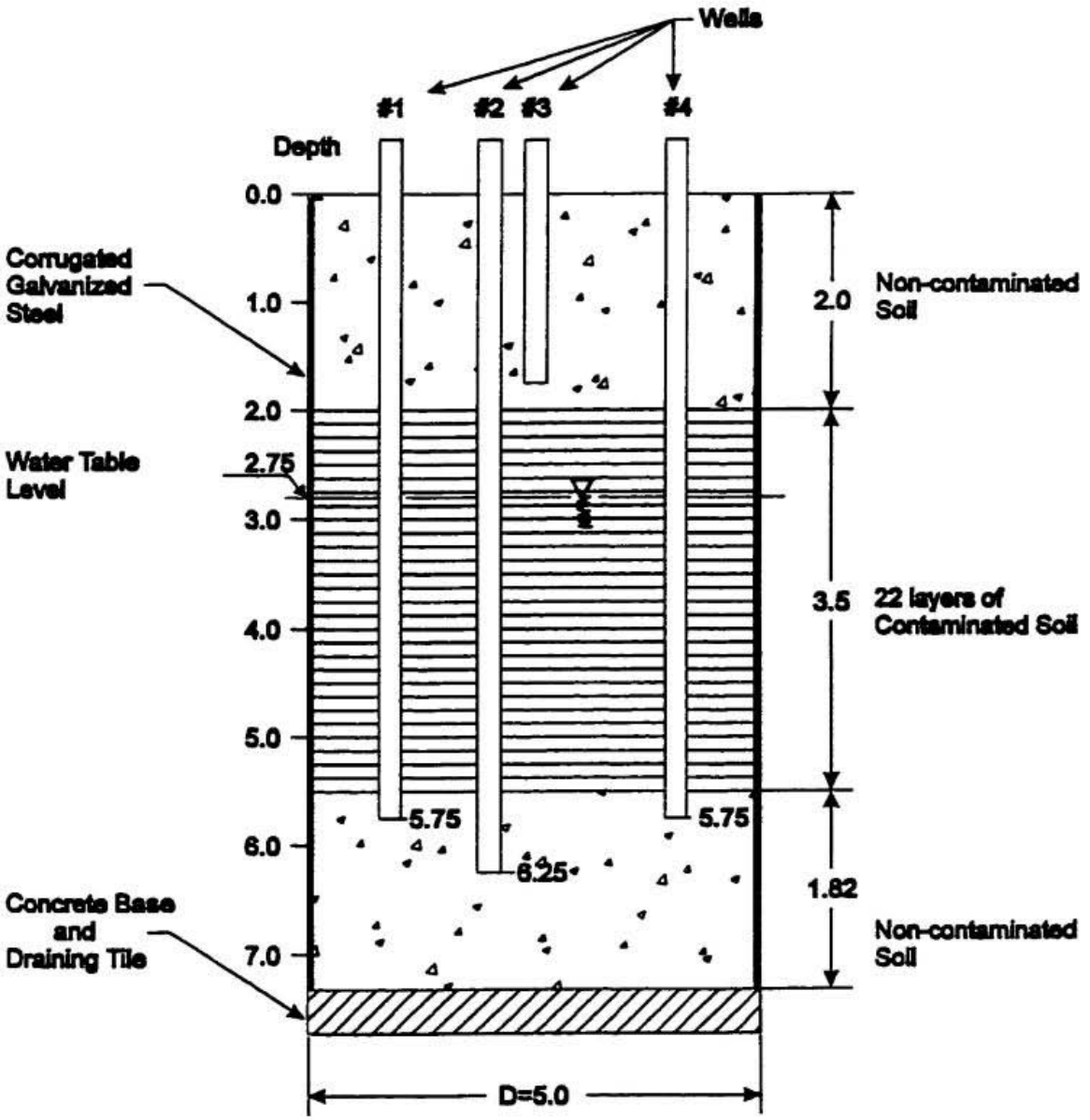
On the Argentia site, the area referred as ETF in Figure 3-1 was chosen for the construction of a biotechnology testing facility. A circular treatment cell made of galvanized corrugated steel, 5 m in diameter and 7 m in height, was installed on the concrete base as shown in Figure 3-4. Drainage tiles were placed on the floor of the treatment cell to promote drainage of excess water from the cell. Four 50 mm diameter pipes made of polyvinyl chloride were installed vertically within the treatment cell. They serve as wells to control the water table level and inject nutrient, bacteria and air during the bioremediation process. Temperature can be monitored and a heater is used to adjust the temperature of the soil and ground water. A vapour extraction unit is available to vacuum the exhaust gas. A large plastic tank was set up adjacent to the treatment cell to provide water to be used in the cell.

The cell was filled with soil in order to simulate typical conditions that exist on the Argentia contaminated site. Three layers of soil were prepared and compacted. First, non contaminated soil, which was transferred from an external commercial pit, was compacted between the base of the cell (depth of 7.32 m) to a depth of 5.5 m. On top of this compacted soil, artificial contaminated soil was placed between depths of 5.5 m and 2.0 m. Diesel fuel was used as a model contaminant with a rough concentration of 7000 mg/kg (ppm) of soil. It was spreaded on the soil with a watering can and plowed by a rake to thoroughly mix diesel fuel with soil. To make the soil contamination homogeneous, the contaminated soil was compacted layer by layer, and total a 22 layers were placed. For each layer, the mixture

of diesel and soil was compacted using walking-behind gasoline powered tamper. The density and moisture content were measured with a nuclear densimeter and the results are shown in Table 3-5. The concentrations and distribution of contaminant in the contaminated layers were determined (Loss on Ignition test) and the results are presented in Figure 3-5. Finally, non contaminated soil was placed between the depth of 2.0 m and the ground level. The cell was then covered. Ground water table was set at the depth of 2.75 m.

Table 3-5 Density and Moisture Content of Contaminated soil (depth 5.5 to 2.0 m)

Layer	Depth	Wet density (kg/m^3)		Dry density (kg/m^3)		Moisture content (%)	
No	(m)	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
1	5.5~5.30	1747.5	1699.4	1561.5	1484.6	11.9	14.5
2	5.10	1731.5	1635.3	1555.1	1497.4	11.3	9.2
3	4.90	1859.7	1856.5	1723.4	1704.2	7.9	8.9
4	4.78	1827.4	1763.5	1705.8	1619.2	7.1	8.9
5	4.63	1859.7	1763.5	1707.4	1635.3	8.9	7.8
6	4.50	1792.4	1747.5	1657.7	1696.2	8.1	3.0
7	4.25	1715.4	1571.1	1496.2	1439.7	14.8	9.1
8	4.16	1875.7	1795.6	1739.5	1643.3	7.8	9.3
9	4.00	1619.2	1699.4	1510.2	1507.0	7.2	12.8
10	3.88	1699.4	1633.7	1540.7	1208.8	10.3	35.1
11	3.71	2183.6	1891.8	2121.0	1813.2	2.9	4.3
12	3.53	1939.9	1996.0	1507.0	1539.1	28.7	29.7
13	3.40	1872.5	2084.2	1808.4	1731.5	3.5	20.4
14	3.28	2212.4	2028.0	2100.2	2005.6	5.3	1.1
15	3.15	2020.0	2388.8	1603.2	2316.6	20.0	3.1
16	3.00	2401.6	2020.0	2371.1	1931.9	1.3	4.6
17	2.83	1442.9	1771.5	1390.0	1354.7	3.8	30.8
18	2.68	1635.3	1386.8	1603.2	1138.3	2.0	21.8
19	2.53	2396.8	2396.8	1983.2	2196.4	20.9	9.1
20	2.36	2408.0	2404.8	2079.4	2262.1	15.8	6.3
21	2.20	2403.2	2403.2	2254.1	2148.3	6.6	11.9
22	2.00	2396.8	2404.8	2182.0	2340.7	9.9	2.7



All dimensions in meter

Figure 3-4 Schematic Environmental Testing Facility
(After C-CORE manual Draft)

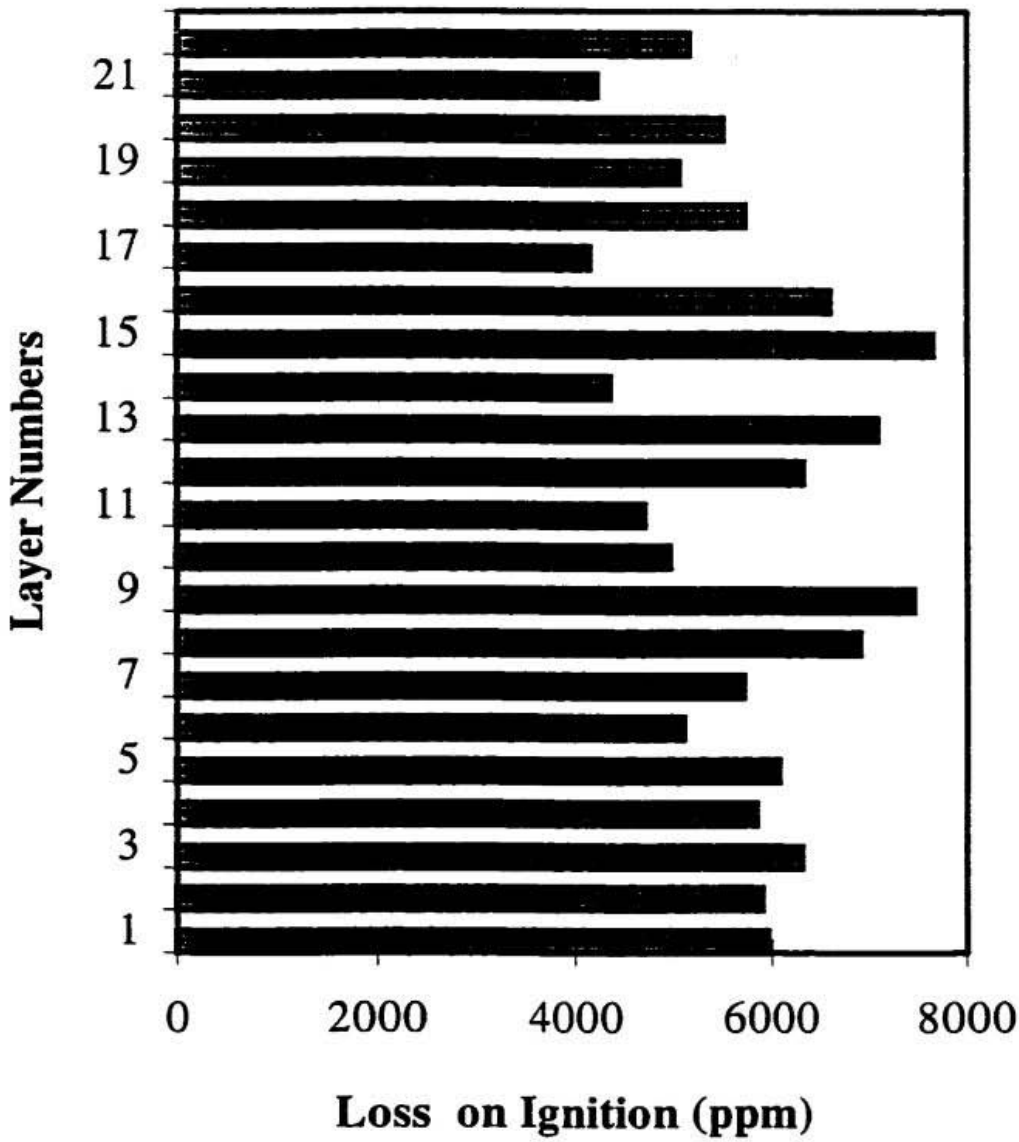


Figure 3-5 Concentration and Distribution of Contaminant

(ETF soil between depth of 5.5 m and 2.0 m)

3.4 Typical Protocol for in Situ Remediation of Diesel Contaminated Soil

3.4.1 Operation of the treatment cell

The treatment cell started operation in December, 1996. A company specialized in bioremediation went on the site and conducted a trial test. Nutrients and bacteria were injected into the treatment cell through one of the well placed in the centre of the cell. Hence nutrients and bacteria reached the simulated ground water in the cell. Air was also circulated to supply oxygen which is a very important factor to accelerate the rate of biodegradation. The temperature was monitored . Because of proprietary concerns and limitation, details of the exact procedures cannot be made available in the present study.

3.4.2 Water sampling and measurement

Water from the treatment cell was collected in one of the peripheral well in order to know the growth of bacteria during biodegradation process. Water samples were tested for several parameters such as dissolved oxygen, temperature, pH, salinity correction and oxygen as % of total saturation. This was done immediately on the test site using a portable Dissolved Oxygen Meter (Ati Orion, 1996). Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD_5) were measured in the laboratory within 24 hours. Both COD and BOD values give an indication of the organic content in the contaminated water. The difinitions and testing procedures are given in Eaton, et al (1995). Typical results are shown in Table 3-6.

Table 3-6 Example of Parameters Measured During All Operations

Sampling Date	23-12-96	16-01-97
Temperature °C	5.5	1.8
Dissolved Oxygen (mg/L)	13.30	12.33
Salinity Correction (mg/L)	8.47	7.72
Oxygen as % of Total Saturation	106.3	88.1
pH Value	7.09	7.19
COD (mg/L)	96.60	77.28
BOD_5 (mg/L)	9.33	4.91

After review of test results, it was found that the site temperature was too low to carry out the bioremediation process. The Field-Testing Facility was shut down to wait for warmer weather. The facility was restarted in June, but complete site results could not be obtained for this study.

Chapter 4

Laboratory Experiments: Materials and Methods

4.1 Materials

4.1.1 Soil

The soils used in the bioremediation experiments were collected from the Argentia Testing Facility tank where they had been contaminated by diesel fuel with a concentration of about 7000 ppm. They had a strong fuel smell and treatment operations were therefore carried out under a fume hood. Soils were first sieved through a sieve #4 US (4.75 mm) to remove gravel size, debris and chunks. Then the soils were thoroughly mixed in a plastic bag to make them more homogeneous and they were placed in refrigerated storage at 4^{0}C. For the experiments , selected amounts of soil were taken and put into a glass jar.

The soils were characterized according to grain size distribution, pH value, and physical properties. The grain size distribution is shown in Figure 4-1 and the sieving procedure followed the ASTM D422-63 (ASTM, 1996a). The truncated material is a well graded sand with little fines (1.9% < #200) US sieve. The soil properties are summarized in Table 4-1.

Figure 4-1 Grain Size Distribution (Soil for Bioremediation Experiments)

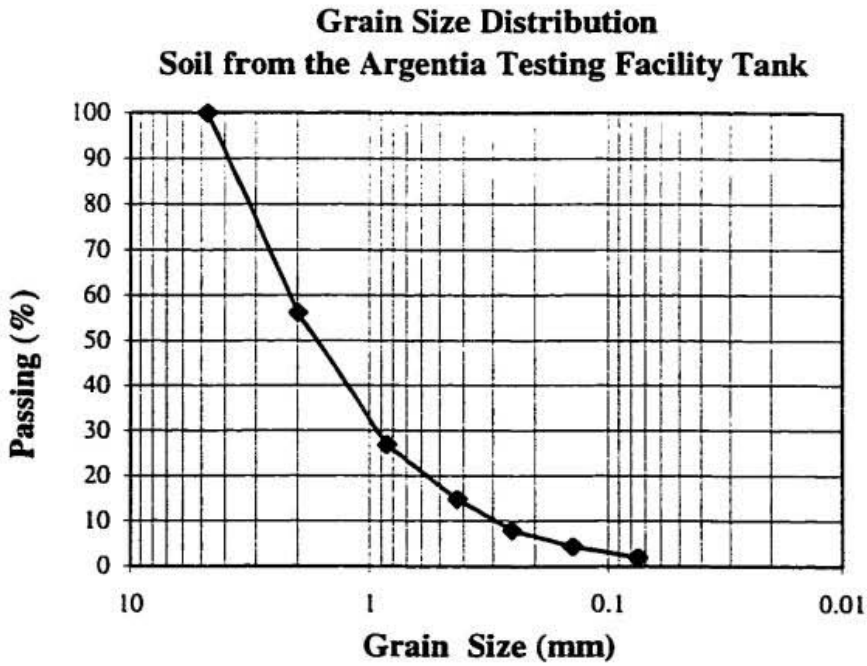


Table 4-1 Properties of Soils Used in Argentia Test Facility

Soil Properties	Values
Liquid Limit (CAN/BNQ 2501-092) of	21
Plastic Limit (ASTM D424-59) of Fines	15
Plasticity Index of Fines	6
Shrinkage Limit (ASTM D42 4-59) of	14
Specific Gravity (ASTM D854-59)	2.75
Optimum Water Content (% dry wt.)	11.7
Maximum Dry Density (g/cm^3)	2.12
pH (1:1 Soil -Water)	6.2

4.1.2 Petroleum hydrocarbon

Diesel fuel was selected as a model petroleum hydrocarbon because it is commonly used as fuel and is less volatile than gasoline. Diesel fuel is a mixture of hydrocarbons in the range of C_6-C_{22}. The Total Petroleum Hydrocarbon value (TPH) can be used to evaluate the total hydrocarbon concentration. Benzene, Toluene, Ethylbenzene, and Xylene (BTEX) concentrations are relatively low in diesel fuel and generally can not be detected. Some chemical and physical properties of diesel fuel are listed in Table 4-2

Table 4-2 Typical Chemical and Physical Properties of Diesel Fuel

(After Custance et al., 1992)

Diesel Fuel Properties	Value
Density (g/cm^3)	0.84
Aqueous solubility (mg/l)	0.20
Vapour pressure (mmHg)	0.03
Diffusion coefficient in air (cm^2/s)	4.63×10^{-2}
Henry's law constant (atm-m^3/mol)	4.2×10^{-2}
[organic carbon:water] Partition coefficient	$10^{3.04}$

4.1.3 Nutrients

Nutrients, especially nitrogen and phosphorous, must be added if the microbial populations present in the soil are expected to consume the total petroleum hydrocarbons (TPH) contaminants at a reasonable rate. The mineral salts (MS) that were used as nutrients are

listed in Table 4-3. Mineral salts were dissolved in distilled water. After adjusting the pH to 6.8 with HCl, sterilization was carried out in an autoclave at 121°C and 15 lbs per square inch for 30 minutes.

Table 4-3 Composition of Mineral Salts Aqueous Solution used for Nutrients

Mineral salts	Concentration (mg/litre of distilled water)
K_2HPO_4	800
KH_2PO_4	200
$MgSO_4 \cdot 7\ H_2O$	100
NaCl	500
$NaNO_3$	500
$(NH_4)_2SO_4$	500
$FeSO_4 \cdot 7\ H_2O$	10
$CaCl_2$	20

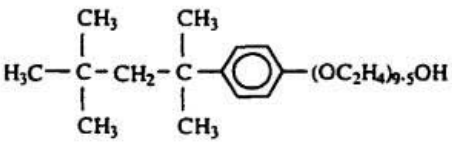
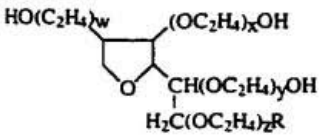
4.1.4 Surfactants

Surfactants used in this study are commercial nonionic surfactants, Triton X-100 and Tween 60, which were obtained from Aldrich Chemical Co., Inc, (Milwaukee, WI, U.S.A.). Their chemical structure is shown in Table 4-4. The critical micelle concentration of Triton X-100 is reported to be approximately 130 mg/l (Kile and Chiou, ,1989). The Tween 60 (Ethoxyethylated sorbitan ester) was selected for its apparent nontoxicity. It is used as food and pharmaceutical emulsifier. It is presumed that such surfactant would not pose

contamination problem if applied as an additional chemical for environmental remediation.

At room temperature, the Tween 60 exists as a waxy solid. It is soluble in warm water gently heated using a water bath or a magnetic stirring heating plate. Triton X-100 is viscous liquid and is water soluble.

Table 4-4 Surfactants used in the experiments

Surfactant	Structure	Class
Triton X-100		Alkylphenyl-ethoxylates
Tween 60		Ethoxylated sorbitan ester

4.2 Bacterial Cultures

4.2.1 Isolation of colonies from contaminated soil

One gram of contaminated soil was placed in a test tube and serially diluted using a 9 ml physiological saline solutions (0.85% NaCl). Then 0.2 ml of the 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions were spreaded on plates that were previously prepared from trypticase soya agar (TSA). The plates were put in an incubator at 25°C under aerobic conditions for 48

hours. Specific colonies were isolated and subcultured onto TSA plates and incubated for another 48 hours. Plates were then put in a refrigerator at 4°C for further study and identification. Four types of colonies were obtained from contaminated soil. Test tubes and spatulas were sterilized and all manipulations were carried out in a biological containment cabinet to minimize the possibility of introducing contaminants.

4.2.2 Identification of colonies by Gram Stain technique

Bacterial cells are difficult to observe because they are nearly transparent. However, most bacteria can be stained by dyes to increase the contrast between the cells and the background. The Gram Stain technique consists of five steps.

(1) one smear of each colony was prepared on a glass slide;

(2) smear was stained with crystal violet solution for one minute, then washed off with Gram's iodine;

(3) Gram's iodine solution was left on the smear for one minute, then washed with water and drained;

(4) smear was decolorized with alcohol (95%) until free colour (approximately 30 seconds), and slide was washed with water and drained;

(5) smear was flooded with safranine for 30 seconds, then washed and bloated dry by placing the slide between two clean pages of paper. The shapes of the cells were then observed through a microscope. The characteristics of colonies were detected and are listed in Table 4- 5.

Table 4-5 Colony Characteristics

Colony	Colony characteristic	Cell shape	Gram stain	Cell colour after stain
AG-1	Large circular, cream colour	Rod	Negative	Pink
AG-2	Medium circular, cream colour	Small rod	Negative	Pink
AG-3	Pinpoint colony, cream colour	Irregular rod	Negative	Pink
AG-4	Spreading colony, cream colour	Branched threadlike filament	Positive	Purple

4.2.3 Enrichment of bacterial cultures

The enrichment of the cultures was carried out in a 125 ml autoclaved flask. One tiny bit of each colony was taken from a TSA plate and suspended in the flask containing 40 ml mineral salts with 0.4 ml of diesel fuel as the sole carbon source. Each colony was also inoculated into separate 125 ml flasks containing 40 ml of mineral salt solution without any diesel. These flasks were used as controls. Flasks were incubated at 25°C in a Psycrotherm Controlled Environment Incubator Shaker (manufactured by New Brunswick Scientific Co. Inc) for two weeks at 150 revolutions per minute (rpm). A visible increase of turbidity in a flask was used as an indication of an isolated colony's ability to grow using diesel fuel as the carbon source.

4.2.4 Microbial enumeration

The enumeration of microbial populations was performed by the plate counting technique. One of the common methods is the spread plate method which is a simple and rapid way to count viable microbial cells in soil (Carter, 1993, and Moskovits et al., 1995). This procedure can be conducted by preparing a serial dilution (e.g., 1:10 - $1:10^{10}$) of a soil sample, spreading an aliquot of dilution on the surface of TSA plate, and incubating the TSA plate under appropriate conditions. The detailed procedure used in this study is as follows.

First, 99 ml of physiological saline solution and 1g of contaminated soil were placed in a 250 ml sterilized flask to make a 1:100 dilution. Then 1ml of suspension was transferred to a 15 ml sterilized test tube with 9 ml physiological saline inside to make a dilution $1:10^3$. The same procedure was repeated until the required dilution was reached. The prepared dilutions were spreaded on TSA plates and incubated under aerobic conditions at 25°C for 48 hours. An average number of colonies, corresponding to dilutions giving between 30 and 300 per plate, was computed by the following equation. It is expressed as number of colony form unit (cfu).

$$No\ of\ cfu/g\ of\ soil = \frac{Average\ number\ of\ colonies\ X\ dilution\ factor}{Initial\ weight\ of\ soil} \qquad (4\text{-}1)$$

4.2.5 Potential for Bioremediation

The potential for bioremediation can be assessed in two ways. The principle developed in this study is to test weather or not an isolated colony can grow when diesel fuel is used as the sole carbon source. If bacteria can grow under this condition, it means that

the colonies can use the diesel fuel for their metabolism and biodegradation is likely to happen.

In a first experiment, the growth potential of each colony was determined in a flask by providing nutrients and diesel fuel. No additional carbon source was added to the flask which was placed in an incubator shaker using the procedures mentioned as above.

A second test was conducted by using a plate which was only made of mineral salts and agar. Each colony was spreaded on the surface of the plate. Diesel vapours were used to provide the only carbon source in the experiment. A bandage was cut into small piece and sterilized by autoclaving. Diesel fuel was pipped on the surface of a piece of bandage and put in the lid of the plate. Plate was placed upside down, so that the bacteria could obtain carbon from the diesel fuel vapour. Plates were put in an incubator at 25°C until visible growth was observed.

4.3 Analysis of Total Petroleum Hydrocarbons

4.3.1 Extraction of petroleum hydrocarbons

Diesel contaminated soil was extracted by soxhlet extraction using Soxtec HT2, Tecator Co., Sewden. The extraction principles are the same as mentioned in Chapter 3 section 3.2.2, but the operation method is sightly different. Five grams of soils were placed into a thimble. The same amount of anhydrous sodium sulfate was used as a drying agent. 100 ml of methylene dichloride and two boiling stones were placed into the extraction cup. The temperature was adjusted so as to give a condensing drop rate of 3-5 drops per second according to the method suggested by Tecator Co. (Tecator Co. manual, 1996). Following

one hour extraction, the soil was rinsed for another half hour, and the extract was purged by air until less 1ml of residual methylene dichloride was left. The extract was transferred to a 2 ml vial for subsequent GC analysis.

4.3.2 Analytical Methods

4.3.2.1 Instrumental parameters

A Gas Chromatography/Flame Ionization Detector (Hewlett-Packard, model 6890) was used to detect and quantify the total petroleum hydrocarbons in soil and aqueous effluent by EPA method 8015 (Test Methods for Evaluating soil waste, SW-864, 1982). The capillary column (30m x 320μm x 0.25μm) was packed with 5% Phenyl Methyl Siloxane. The oven temperature was set at 60°C for 2 minutes, programmed at 10°C/min to 300°C and then held for 5 minutes.

4.3.2.2 Calibration standards

Prior to beginning the analysis of the sample extracts, quantitative conversion of GC area counts data to concentration of petroleum hydrocarbons (μg/ml or ppm) was performed by preparing a series of standard solutions. These standards are prepared by weighing the required amount of diesel fuel and diluting by volume of the hexane. A calibration curve for analysis of diesel fuel was prepared according to the results shown in Table 4-6 and plotted in Figure 4-2

Table 4-6 Diesel Fuel Concentration and Corresponding Peak Area Value

Diesel concentration (μg/ml)	Peak Area Values (graph units)
4200	951.8
3360	760.6
2520	534.5
1680	349.0
840	176.6
0.00	0.000

Based on the above data, a correlation between the peak area values and the corresponding concentrations was established:

concentration (μg/ml) = 4.3822 (μg/ml) x peak area value + 75.485(μg/ml) (4-2)

This formula was used for diesel contaminated samples to convert the peak area values into concentrations.

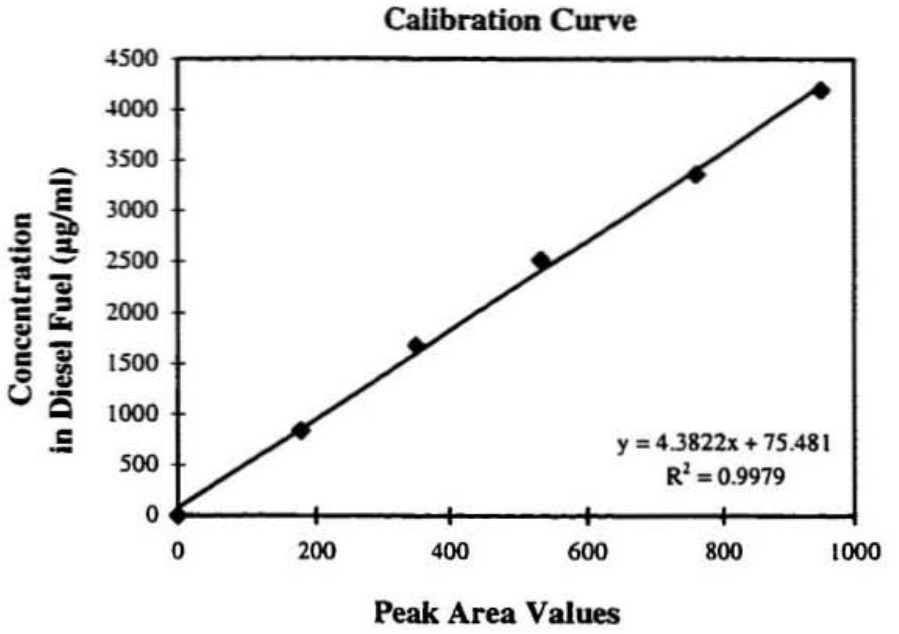


Figure 4-2 GC Calibration Curve

4.3.2.3 Calculation of TPH concentration in soil

The concentration of diesel fuel TPH ($\mu g/g$ or ppm) in a soil sample was calculated as follow:

$$TPH, ppm(\mu g/g) = \frac{C\ (\mu g/ml)}{W\ (g)} x\ V\ (ml) \qquad (4-3)$$

Where C= concentration in TPH for the prepared standard read from the calibration curve, Figure 4-2; W=weight of dry soil ; V=volume of hexane (solvent).

4.4 Bioremediation Tests on Soil Contaminated with Petroleum Hydrocarbons

In this section, the preparation of soil and chemicals in a close system reactor is presented and bacteria injection procedures are discussed.

4.4.1 Set up of the close system reactors and test procedures

Closed system reactors were built in 500 ml total volume glass jars with septa caps. The contaminated soil was weighed and transferred to the sterilized glass jars. Nutrient solutions, colony solution and surfactants were then mixed and introduced in the jars according to a well defined testing program.

Bioremediation tests were designed to investigate the effects of temperature of incubation, pH value, nutrients, surfactants, and amount of microorganisms on the rate of diesel fuel degradation. Temperatures of incubation were set at 5, 15 and 25°C, and pH values were changed within a range from 6 to 8. The pH of the contaminated soil as provided was from 6.8 to 7.0. Hydrochloric acid was used to adjust the pH value to 6, and sodium hydroxide was used to increase the pH to 8.

The influence of nutrients was assessed by using distilled water instead of the nutrient solution. The 20 ml mixed colony solution was centrifuged to get a pellet, resuspended in 20 ml distilled water and poured into the glass jar.

The importance of the amount of microorganisms was studied by comparing biodegradation using bacteria injection and biodegradation without bacteria injection. Enriched colony solution was seeded into the glass jar to increase the population of bacteria

in order to enhance the rate of bioremediation.

The composition of each glass jar is given in Table 4-7. Glass jars were put in incubators at different temperatures. Soil was mixed every week in order to supply oxygen to microorganisms. Samples were taken at scheduled times to analyze the concentration of TPH and to count bacteria.

4.4.2 Bacteria seeding

As indicated above for some tests, enriched colony was injected into the corresponding glass jar to increase the population of the bacteria. Each isolated colony was grown in a flask for two weeks as mentioned in section 4.2.3. The cells were collected by centrifuging at 12,000 rpm for 10 minutes. The pellets were resuspended in a flask with fresh nutrient solution and diesel fuel for another week, and the centrifugation procedure was repeated. The pellets of four kinds of enriched colonies were resuspended together in a 1000 ml flask with a fresh nutrient solution and diesel fuel for three days to form a solution of mixed colonies. This mixed colony solution was later used as a seed in glass jars so designated .

Table 4-7 Composition of Soil Added into Glass Jar

Glass Jar	Content	Incubation T°
BI-pH6-T25	300g contaminated wet soil, 20ml MS solution with enriched cultures, pH=6	25°C
BI-pH7-T25	300g contaminated wet soil, 20ml MS solution with enriched cultures, pH=7	25°C
BI-pH8-T25	300g contaminated wet soil, 20ml MS solution with enriched cultures, pH=8	25°C
BI-pH7-T25	300g contaminated wet soil, 20ml MS solution with enriched cultures, pH=7	25°C
BI-pH7-T15	300g contaminated wet soil, 20ml MS solution with enriched cultures, pH=7	15°C
BI-pH7-T5	300g contaminated wet soil, 20ml MS solution with enriched cultures, pH=7	5°C
BI-pH7-T25-Tri-0.5	300g contaminated soil, 20ml MS solution with enriched cultures, 1.5g Triton X-100 (0.5%w/w), pH=7	25°C
BI-pH7-T25-Twn-0.2	300g contaminated wet soil, 20ml MS solution with enriched cultures, pH=7, 0.6g Tween 60 (0.2%w/w)	25°C
NBI-pH7-T25	300g contaminated soil, 20ml MS solution, pH=7, No culture injection	25°C
BI-NN-pH7-T25	300g contaminated soil, 20ml distilled water with enriched cultures, pH=7	25°C
NBI-pH7-T25-Control	300g autoclaved soil, 1.5g diesel, 36ml distilled water, pH=7	25°C

Note: MS stands for Mineral Salt.

4.4.3 Preparation of the control jar

Autoclaved soil was used to prepare a control reactor. Soil was put into a capped glass jar. The glass jar was left in the autoclave for half an hour at 121^{0}C and 15 pounds of pressure per square inch (1056 g/cm^2). After that, the glass jar was left in the laboratory overnight. The same autoclave procedure was repeated the next day. Sterilized soil was mixed with diesel fuel to make a contaminated soil with a concentration of 5000 mg of diesel per kg of soil.

4.5 Surfactant Treatment of Soil Contaminated with Petroleum Hydrocarbons

Surfactants were utilized to remediate petroleum hydrocarbon polluted soils. These experiments were conducted in fixed wall permeameters and consisted of washing the contaminated soil contained in the column. The contaminant removal was analyzed in time. Procedures are presented hereafter.

4.5.1 Soil preparation

Soil was air dried at room temperature and screened on a Sieve #8 U.S. (2.36 mm). Diesel fuel was spread on the soil to prepare a contaminated soil with a concentration of 7000 mg diesel per kg of soil prior to column experiment. The mixing was carried out long enough to ensure that the contaminant distribution was homogeneous.

4.5.2. Preparation of the surfactant solution

Surfactant solutions of Triton X-100 and Tween 60 were used in these tests. They were prepared by dissolving the surfactants using distilled water in a magnetic stirring heating plate. The concentrations are reported as percent weight of surfactant in water. Surfactant solutions were used as an influent through the column to wash diesel fuel out of contaminated soil .

4.5.3 Experimental column set-up

As mentioned above, column tests were conducted in a permeameter consisting of a acrylic cylinder clamped between acrylic end plates. One porous disk with a filter paper was inserted at each end of the specimen, and two O-rings were used to seal the cylinder with the end plates. Polyethylene tubings were used for inflow and outflow. The cylinder was 5.08 cm (2.0 in.) in diameter and 10.16 cm (4.0 in.) in length.

The contaminated soil was compacted to a dry density of 1.88 to 1.99 g/cm^3 as shown in Table 4-8. Distilled water, 0.5% (w/w) Tween 60 solutions and 0.5% (w/w) Triton X-100 solutions were used as influents. Soils were initially saturated with water overnight before starting the washing test. The effluents were collected at different time intervals corresponding to increasing amount of percolating pore volumes.

Table 4-8 Information on compacted soil and influent

Sample I. D.	Dry Density (g/cm^3) as compacted	Void Ratio	Influent
D-water	1.99	0.20	Distilled water
Tween-0.5	1.88	0.25	0.5% Surfactant Tween-60
Triton-0.5	1.96	0.21	0.5% Surfactant Triton

4.5.4 Extraction and analysis of contaminant

The diesel fuel content of the effluent was determined using a liquid-liquid extraction procedure based on Standard Separatory Funnel Method 3501 (Test Methods for Evaluating soil waste, SW-864, 1982). The column effluent was collected in a glass jar. A 100 ml specimen was transferred to a cylinder and poured into a 250 ml separatory funnel. 20 ml hexane was used to wash the cylinder and was then transferred to the separatory funnel too. The separatory funnel was then capped and shaken for 5 minutes to partition the contaminants to the solvent phase. After a 2 minutes settling period, liquid in the funnel was separated into two phases: solvent phase and water phase. Water was drained out and a 10 ml portion of the solvent solution was transferred to a clean 20 ml glass vial for GC analysis.

4.5.5 Determination of hydraulic conductivity

Analysis of water flow in saturated soil are usually derived from Darcy's law which is based on the experimental observation of a linear relationship between the rate of flow and the hydraulic gradient. After the soil has been compacted and saturated with water, the head of water or surfactant solution was adjusted to give the desired hydraulic gradient. The percolation rates of water or aqueous surfactant solutions were determined during the testing period. The effluent was collected in a glass jar . Both effluent volumes and periods were recorded. The hydraulic conductivity in term of k (cm/s) was calculated by the following equation:

$$k = \frac{V_i}{A \cdot \Delta t_i} \frac{L}{h_i} \qquad (4-4)$$

where V_i (cm^3) = the effluent volume collected during the time interval Δt_i;

L = length of the sample (cm);

A = cross-sectional area of the specimen (cm^2);

Δt_i = individual time interval (s);

h_i (cm) = average water head difference between inflow and outflow during Δt_i.

Chapter 5

Results and Discussion

5.1 Biodegradation of Contaminated Soil in Closed System Reactors

5.1.1 Effect of temperature

The effect of temperature on the rate of biodegradation of petroleum hydrocarbons was evaluated in glass jars incubated at 5, 15, 25 °C for a period of 140 days (Section 4.4.1) . The incubation temperatures were selected based on the range of Newfoundland seasonal temperatures. Petroleum hydrocarbon degradation in the reactors was monitored by measuring the TPH concentration in the soil. The reduction of TPH concentration in closed system reactors versus time for each incubation temperature is plotted in Figure 5-1. The maximum rate of the degradation occurred at 25 °C. The concentration of TPH decreased from 6044 mg/kg of dry soil to 3004 mg/kg of dry soil. The hydrocarbons degraded to 50% of the initial value. Medium degradation rate happened at 15°C. The lowest degradation rate was obtained at 5°C, and only 17% of the hydrocarbons was reduced after 20 weeks of bioremediation treatment.

As the results show, temperature have a marked effect on the rate of the petroleum hydrocarbon degradation. At low temperatures, the biodegradation of TPH is limited or reduced. It is suggested to carry out remediation of TPH in the range of 15 to 25°C. Therefore, in situ remediation will not be efficient in the Newfoundland winter season due to cold weather.

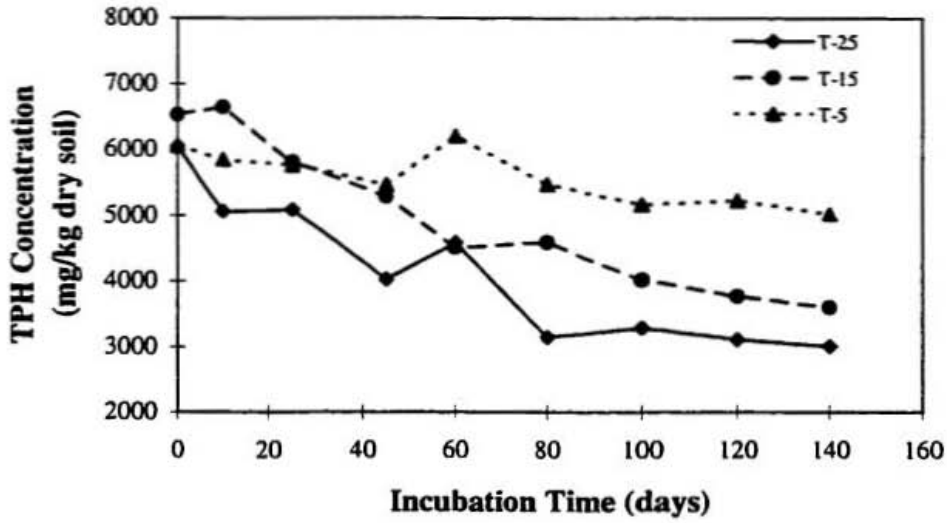


Figure 5-1 Effect of Temperature on Biodegradation of TPH
(pH at 7, with nutrients and bacteria injection)

5.1.2 Effect of pH

Results showing the effect of pH on the rate of degradation of hydrocarbons are presented in Figure 5-2. The pH values were selected as 6.0, 7.0, and 8.0 (Section 4.4.1). When the degradation was carried out at pH 6.0, the TPH were degraded to 38.4% of their original value during the 140 days period. At pH 7.0, the maximum hydrocarbon reduction was achieved with 50.3% of the initial TPH. At pH 8.0 the rate of the degradation was found

to be the lowest and the hydrocarbons were only 30.6% degraded.

Effective biodegradation process happened in a slight acid or neutral condition according to the experimental results. It looked like cultures had more active ability in the slight acid and neutral conditions than under alkali conditions. The native soil had a pH value of 6.2 in natural condition (Table 4-1). Cultures which were isolated from this contaminated soil have lived in a slightly acid condition for a long time and have adapted to this environment. Therefore when biodegradation tests were conducted under an acid or neutral conditions, significant hydrocarbon removal was observed. When biodegradation is carried out under alkali conditions, there is a gap between the native soil living conditions and the alkali environment, which prevents optimal growth of bacteria.

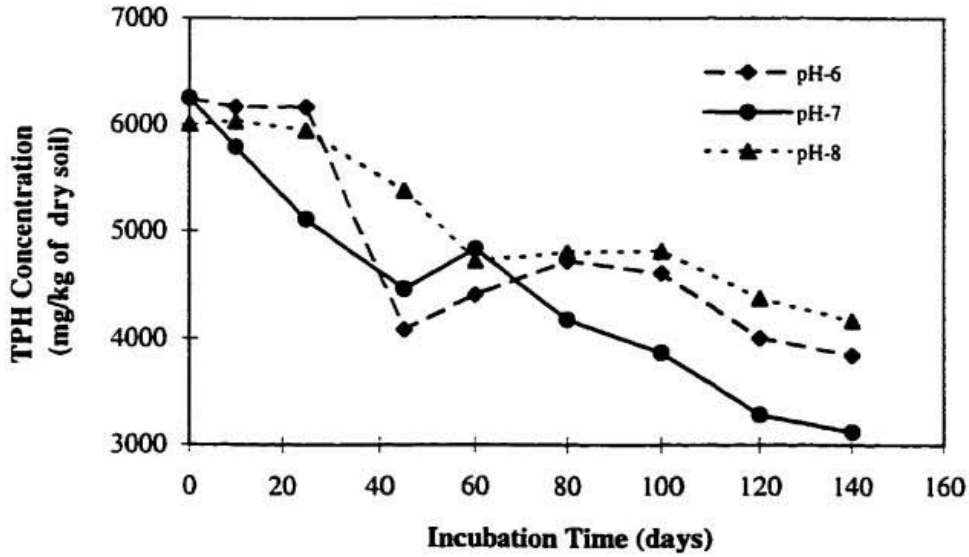


Figure 5-2 Effect of pH on Biodegradation of TPH
(Temperature at 25°C, with nutrients and bacteria injection)

5.1.3 Effect of Surfactants

To evaluate the effect of surfactants on petroleum hydrocarbon biodegradation in the diesel contaminated soil, three different treatments were performed:

(1) soil without surfactant;

(2) soil amended with 0.2% (w/w) of Tween 60 solution;

(3) soil added with 0.5% (w/w) Triton X-100 solution.

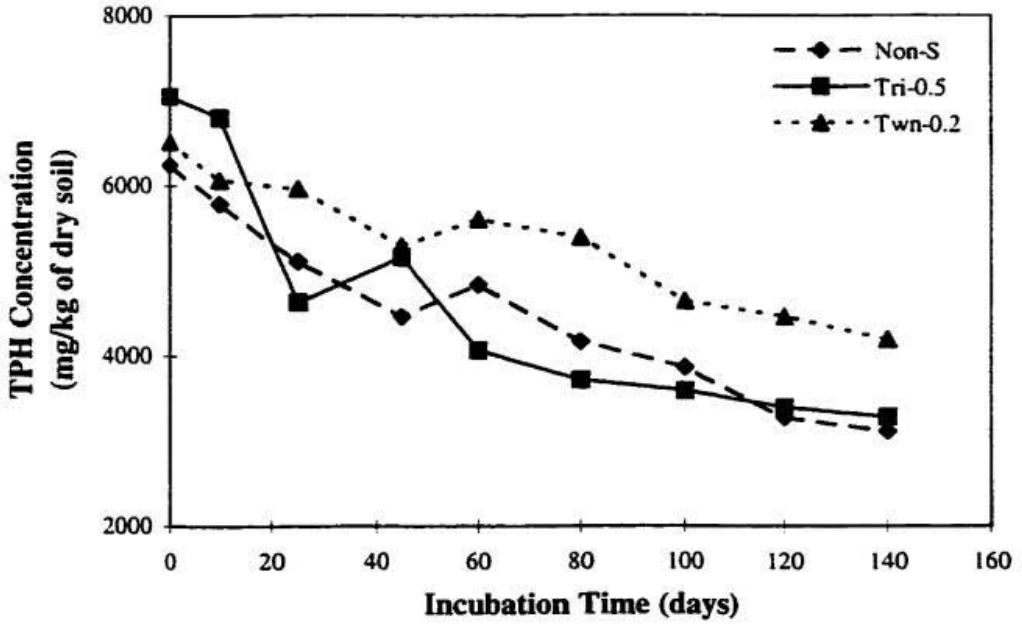


Figure 5-3 Effect of Surfactant on the Biodegradation of TPH
(Temperature at 25°C, pH at 7, with nutrients and bacteria injection)

The results are presented in Figure 5-3. The reactor containing Triton X-100 indicated a final reduction of TPH (After 20 weeks) of 53.4%, close to the non surfactant reduction. However the rate of degradation was found to be higher in the initial 10 weeks. It may be explained by the fact that surfactant is a kind of carbon source. With time going, it can be consumed by bacteria. If surfactant was added in the reactor after a certain time such as after 10 weeks, better degradation may be achieved.

In terms of percentage of the initial TPH, a 3% increase in efficiency was obtained by the addition of 0.5% of surfactant X-100 compared to non surfactant process. It is therefore found that surfactant X-100 may be useful in biotreatment of the petroleum

contaminated soil.

Unlike the effect of surfactant Triton X-100, an addition of 0.2% (w/w) surfactant Tween 60 did not enhance of the degradation rate of the TPH. It even appeared to have a negative effect on the rate of the TPH biodegradation when compared to treatment without surfactant addition. Only 35.5% of TPH removal was obtained in the biodegradation process. This result may imply that the surfactant Tween 60 inhibited the microbial activity thus decreasing the rate of biodegradation.

5.1.4 Effect of additional bacteria injection

5.1.4.1 Bacteria counting

Microorganism activity was monitored by bacteria counting. The populations of bacteria in the control autoclave soil, non bacteria injected soil, and bacteria injected soil were counted at day zero of the biodegradation process, and subsequently counted at 45, 100, and 140 days to evaluate the changes in bacteria population. The colony form unit per gram of soil were enumerated from appropriate dilutions of the above mentioned three soil samples after 48 additional hours of incubation at 25°C on TSA plates and calculated by the equation 4-1. The results are listed in Table 5-1.

Table 5-1 Bacterial Plate Counting (colony-forming units/g soil)

Sample incubation time	Control soil (cfu/g soil)	Non-Bacteria injected soil (NBI) (cfu/g soil)	Bacteria injected soil (BI) (cgu/g soil)
day 0	$50x10^1$	$22x10^5$	$42x10^5$
day 45	$24x10^3$	$39x10^5$	$13x10^7$
day 100	$43x10^4$	$33x10^6$	$40x10^7$
day 140	$75x10^4$	$21x10^7$	$29x10^9$

When bacteria counting was carried out on zero day, no bacterial activity was found in the autoclave control sample during the 48 hour initial incubation time, however bacteria were detected after 72 hours. Nevertheless, the colony that was observed was different from the colonies isolated in the contaminated soil. The soil may have been contaminated during operation, but due to its much lesser population, it still can be used as a control sample. As shown in Table 5-1, the population of the control soil reached $75x10^4$ at day 140. The populations of the non bacteria injected soil increased to $21x10^7$, and the bacteria seeded soil increased by a factor 10^4 to reache $29x10^9$ during the 20 weeks biodegradation period. These results also demonstrate that the microorganisms present in the soil with the TPH concentration up to 6000 mg/kg are capable of both surviving and increasing their numbers in the presence of the diesel fuel.

5.1.4.2 TPH reduction due to microbial activity

To evaluate the effect of the number of microorganisms on the degradation of TPH, the results obtained from non bacteria injected soil was compared to the result obtained form bacteria injected soil. Bacteria injected soil had a higher efficiency for the degradation of TPH than non bacteria injected soil. The results were presented in Figure 5-4. The TPH removal percentage after 20 weeks using bacteria seed is about 15% higher than the non seeded soil.

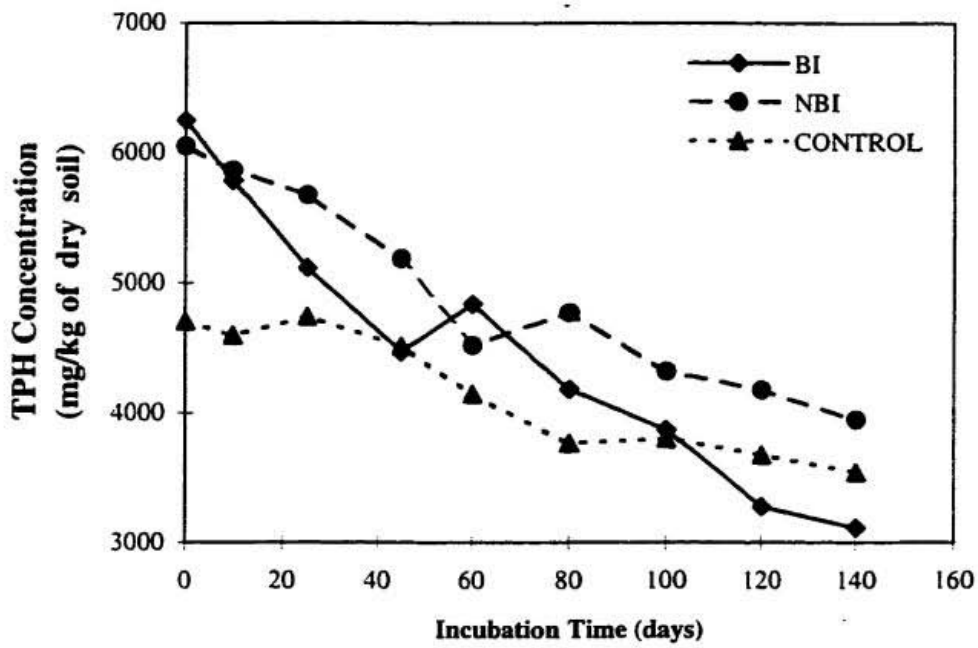


Figure 5-4 Effect of Bacteria Injection on the Biodegradation of TPH (Temperature at 25°C, pH at 7, with nutrients injection)

5.1.5 Effect of Nutrients

Microorganisms need nutrients to grow. Hence, biodegradation of hydrocarbons in the natural environment is limited by poor growth rate of microorganisms caused by nutrient deficiencies, especially in nitrogen and phosphorus. (Leahy and Colwell, 1990). Thus, when bioremediation is conducted, these nutrients are usually applied to the contaminated environment to simulate biodegradation (Prince, 1993). In this study, results of bioremediation with nutrients or without nutrients were compared as shown in Figure 5-5. The addition of nutrients is clearly effective in increasing the rate of biodegradation of the TPH.

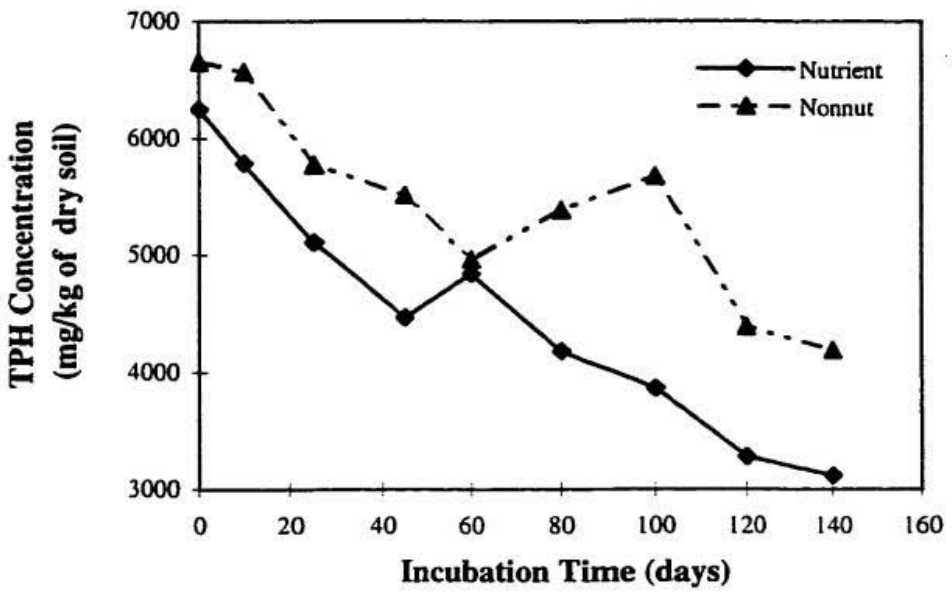


Figure 5-5 Effect of Nutrients on the Biodegradation of TPH
(Temperature at 25°C, pH at 7, with bacteria injection)

5.1.6 Summary of the laboratory biodegradation experiments

The growth potential of the bacteria in the diesel contaminated soil was determined by providing nutrients, and observing the ability of the microorganisms to grow with time using the fuel present in the soil. The samples were incubated under aerobic conditions with various temperatures, pH values and addition of surfactants. Later analysis of the soil indicated that part of the TPH was in fact consumed as carbon and energy sources for microorganisms. A summary of TPH removals is given in Figure 5-6. The efficiency of the biodegradation which was obtained at the temperature 25°C and pH 7 is 25% higher than the corresponding control sample. The GC profiles before and after incubation are given in Figure 5-7 and 5-8. An addition of 0.5% surfactant Triton X-100 solution, mineral salts and bacteria seeding assisted in degradating the petroleum hydrocarbon contaminated soils.

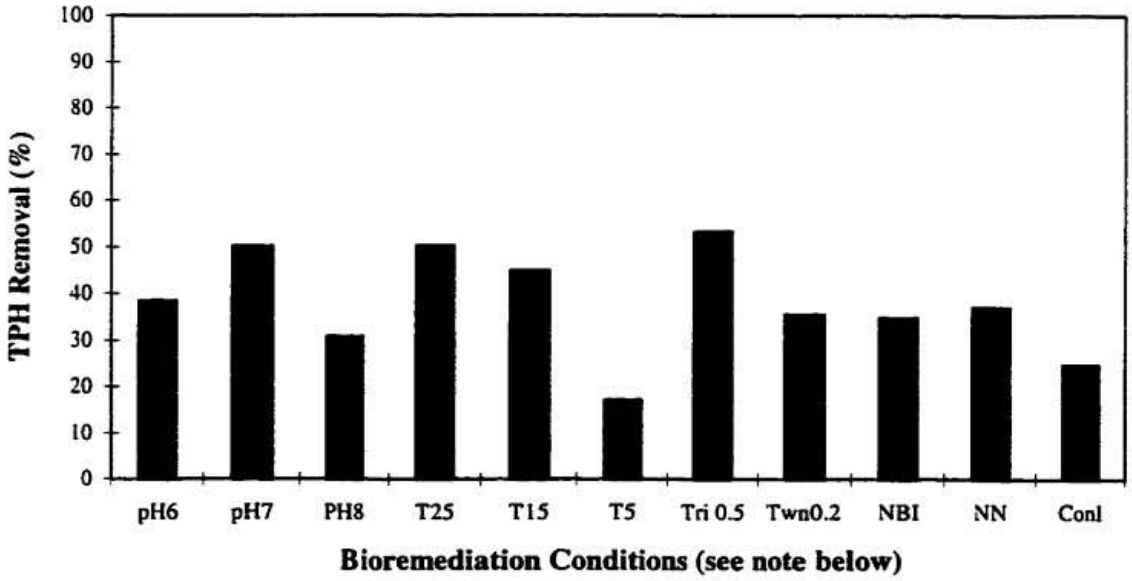


Figure 5-6 TPH Removal in Different Conditions of Bioremediation

Note: pH 6, 7, and 8: samples incubated at pH 6, 7, and 8 and temperature at 25°C with nutrients and bacteria injection

T 25, 15, and 5 : samples incubated at temperatures at 25, 15, and 5 °C and pH at 7 with nutrients and bacteria injection

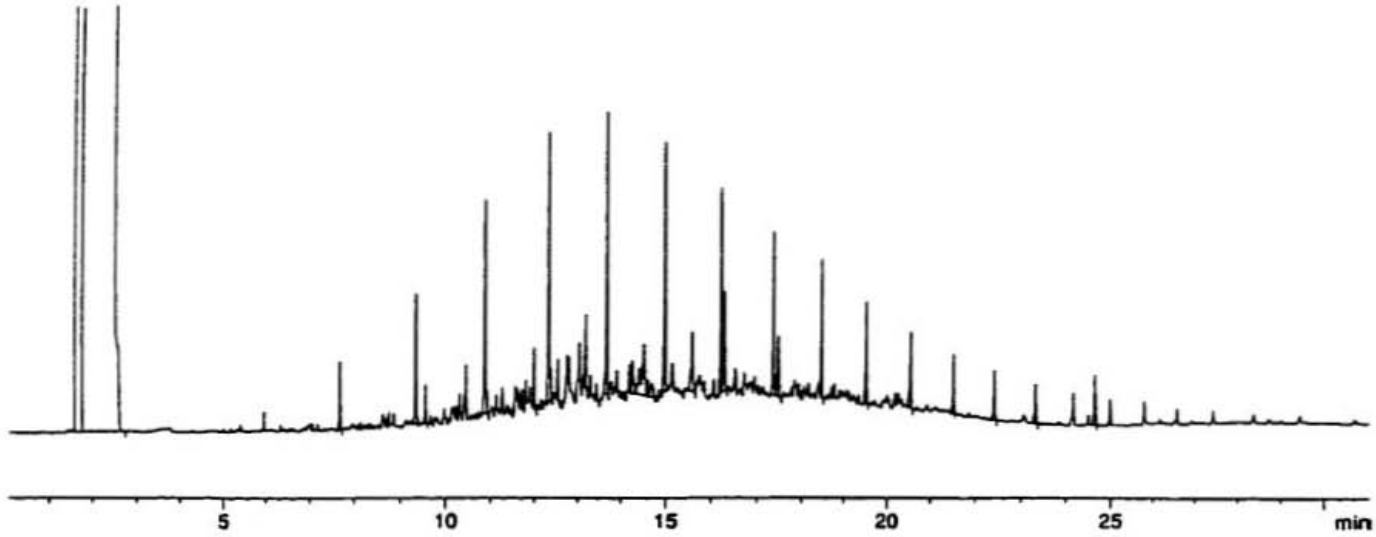
Tri 0.5 : sample with 0.5% surfactant Triton X-100 solution at 25°C, pH at 7 with nutrients and bacteria injected

Twn 0.2 : sample with 0.2% surfactant Tween 60 solution at 25°C and pH at 7 with nutrients and bacteria injected.

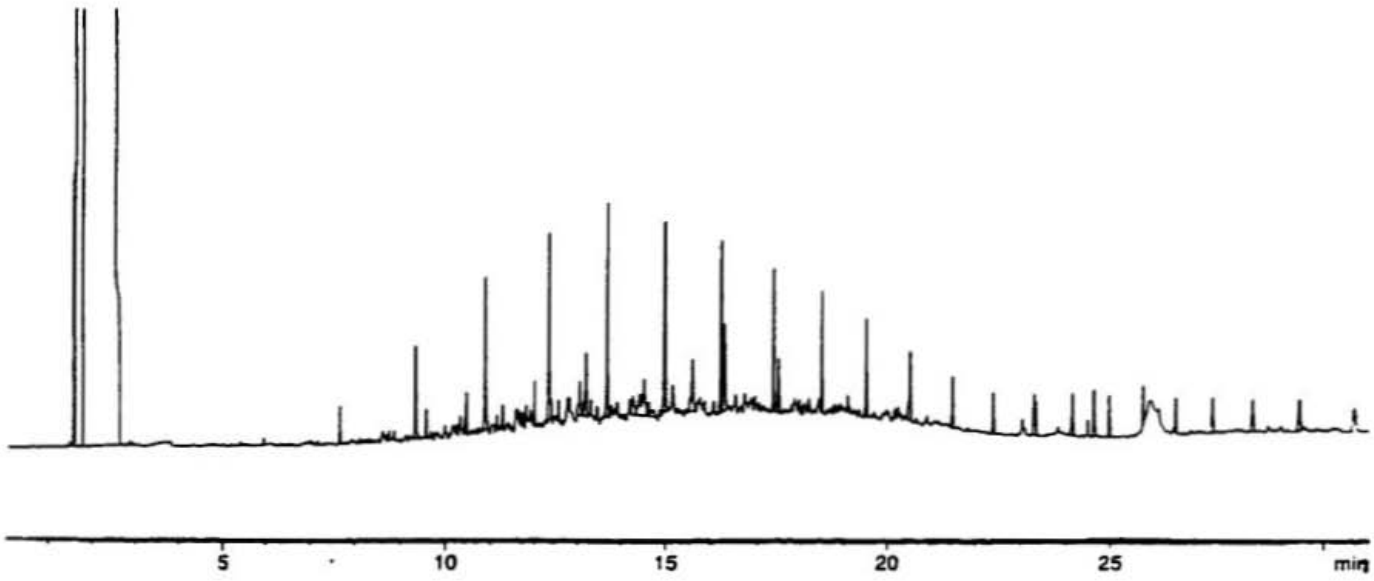
NBI: no bacteria injection incubated at 25°C, pH at 7 and nutrient injected

NN: no nutrient injection incubated at 25°C, pH at 7 and bacteria injected

Conl: control sample incubated at 25°C, pH at 7, no bacteria and nutrients injected



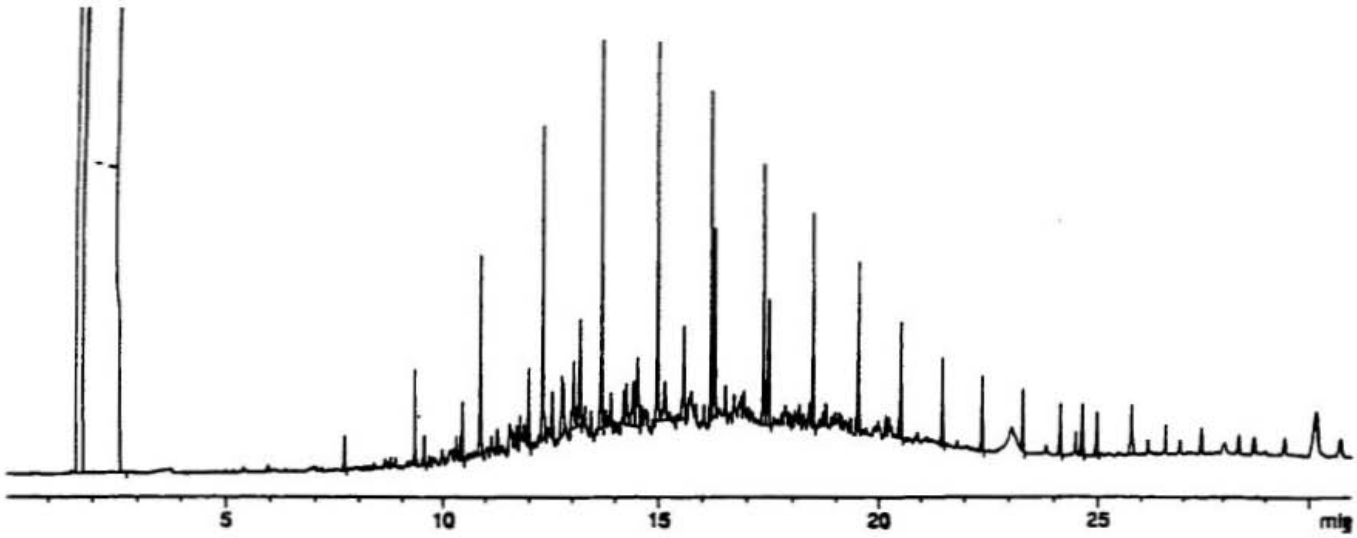
a. Before incubation



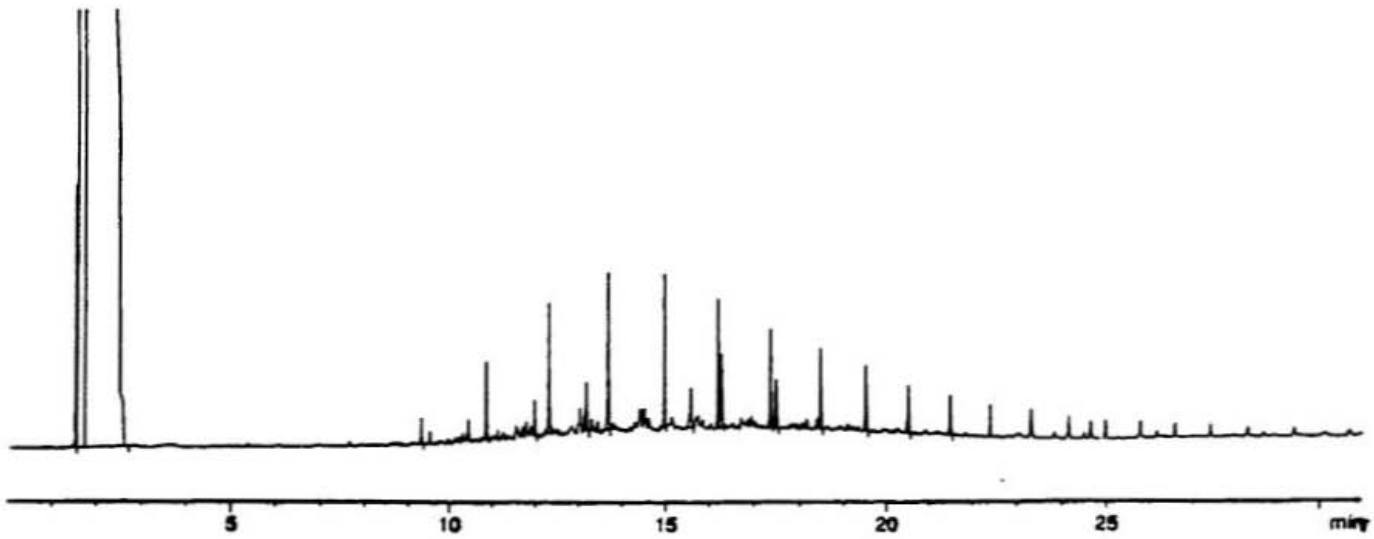
b. After incubation

Units for GC profile are equipment specific and are not indicated in this figure

Figure 5-7 GC Profiles of Control Soil Before and After Incubation



a. Before incubation



b. After incubation

Units for GC profile are equipment specific and are not indicated in this figure

Figure 5-8 GC Profiles of Treated Soil Before and After Incubation

5.2 Effect of Surfactants on Removal of TPH in Column Tests

5.2.1 Surfactant as an agent for removal of diesel fuel

5.2.1.1 Solubilization

One general mechanism by which surfactants can enhance the removal of contaminants is solubilization. Many of petroleum hydrocarbons, described as hydrophobic organic compounds, are relatively insoluble in water. Petroleum hydrocarbons may be adsorbed onto the soil or present in the subsurface aquifer as a discrete organic phase mixture of none aqueous phase liquid (NAPLs).

Surfactants can enhance the solubility of a hydrophobic compound in water due to the hydrophobic pseudophase of a micelle. Micelles are formed at the critical micelle concentration which is the aqueous surfactant concentration at which surfactant monomers form colloidal aggregates. Surfactant chemistry, temperature, ionic strength and the presence and type of organic additives determine the CMC. At the CMC, abrupt changes in solution properties such as surface tension occur (Canadia and Harwell, 1992).

Surfactants may partition between or adsorb to the interfaces of an oil-water-soils system. When surfactant is added to the aqueous phase, the polar head group interacts strongly with the water phase. The nonpolar hydrocarbon chain portion interacts very weakly with water molecules but partitions into hydrophobic organic compounds or petroleum hydrocarbons. The hydrophobic organic contaminants are thus desorbed from the soil and solubilized, allowing the implementation of remediation. Surfactant addition enhances the solubility of these contaminants in the aqueous phase. Such solubilized hydrocarbons are more available for conventional pump and treat strategies or bioremediation (Peter et

1992).

5.2.1.2 Mobilization

Mobilization of the contaminant by a surfactant is another way to remediate contaminated soil. It depends on the surface tension reduction. This has been used as one of a criteria for judging the effectiveness of a surfactant in-situ washing. Low surface tension increases wetting of the soil and provides for better contact between surfactant and contaminant. (Texas Research Institute, 1982b).

5.2.2 Experimental results

Removal of TPH from the soil was conducted in columns (see Section 4.5). Three columns were set up, and distilled water, 0.5 % (w/w) of surfactant solution of Triton X-100 and 0.5% (w/w) surfactant solution of Tween 60 were used as influent to wash the contaminated soil through a column individually. The removal of TPH (diesel) was calculated using the following equation:

$$W(mg) = \frac{C\left(\frac{\mu g}{ml}\right) \times V_{in}(\mu l) \times 10^{-6}}{V_{eff}(ml)} \times V_{sol}(ml) \qquad (5\text{-}1)$$

Where: W=Amount of removed diesel

C= Concentration of the diesel (obtained from the calibration curve, Figure 4-2)

V_{in}=Volume injected in the GC

V_{sol}= Volume of solvent (Hexane)

V_{eff}=Volume of effluent

$$Removal\ of\ diesel\ (\%) = \frac{Amount\ of\ removed\ diesel\ (mg)}{Original\ amount\ \ of\ diesel\ (mg)} \qquad (5-2)$$

In column 1, the distilled water washing resulted in the 5.3% of removal of TPH after using 130 pore volumes of influent. Figure 5-9 shows that Petroleum Hydrocarbons are removed very slowly with increasing percolation. In column 2, 155 pore volumes washing by the 0.5% aqueous solution of Tween 60 yielded a total 21.7% removal of TPH from soil as shown in Figure 5-10. In column 3, a total of 67.8% diesel removal from soil was obtained when washed by 170 pore volume of the 0.5% surfactant solution Triton X-100. Figure 5-11 indicates that removal was slow in the early 40 pore volumes. The removal efficiency increased between 50 and 110 pore volumes, then stabilized.

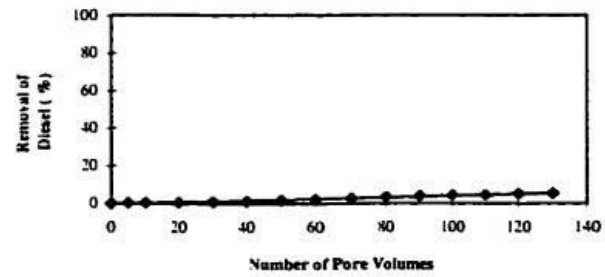


Figure 5-9 Removal of Diesel by Distilled Water

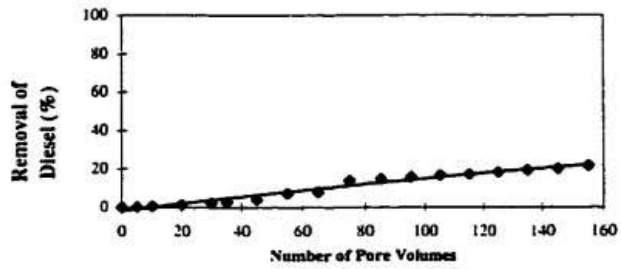


Figure 5-10 Removal of Diesel by 0.5% Surfactant Tween 60

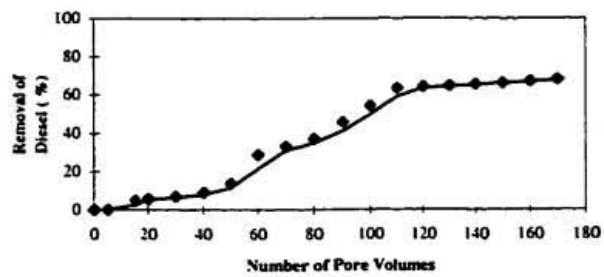


Figure 5-11 Removal of Diesel by 0.5% Surfactant Triton X-100

5.2.3 Discussion of results

An improvement in the mobility of petroleum hydrocarbons in a soil matrix has been demonstrated using surfactant aqueous solution. Lab-scale experiments using soil columns are helpful to characterize the process of diesel mobilization by surfactant solutions. The results of three column experiments using distilled water and different surfactants lead to the following comments. Distilled water did not mobilize trapped diesel fuel in the soil matrix because most petroleum hydrocarbons like diesel are hydrophobic compounds, and the aqueous solubility of the diesel only is 0.2 mg/l. The 0.5% of Tween 60 solution moderately mobilized the diesel fuel, and its overall performance was better than that of the water washing. This is due to solubility enhancement of the diesel fuel. The 0.5% of surfactant Triton X-100 solution at concentration of up to 37 times its critical micelle concentration (CMC) resulted in a high diesel removal efficiency.

5.2.4 Hydraulic conductivity

Hydraulic conductivities were calculated by the equation 4-4 and the results are shown in Table 5-2.

Table 5-2 Hydraulic Conductivities Obtained in Column Tests

Column characteristics	Hydraulic conductivity (cm/s)	Dry Density (g/cm^3)
Distilled water washing	1.95×10^{-5}	1.99
Tween 60 solution washing	2.06×10^{-5}	1.88
Triton X-100 solution washing	2.41×10^{-5}	1.96

Chapter 6

Conclusions

6.1 Summary and Conclusions

A study has been carried out in the Argentia site (Newfoundland) to identify typical soil conditions involving contaminant treatment and simulating conditions in a controlled testing facility. The potential for bioremediation using local soil and indigenous bacteria for the degradation of petroleum hydrocarbons (diesel fuel) has been evaluated. The incubation conditions used for this treatability study in closed system reactors included temperature, pH value, surfactant and nutrient addition, and bacteria seeding. Diesel fuel leaching by surfactants has also been conducted in column tests. Based on the results obtained from these experiments, the following conclusions can be drawn.

1. Degradation of total petroleum hydrocarbon using indigenous microorganisms is possible.
2. Petroleum removal efficiencies in terms of TPH removal can reach 50% over a period of 140 days in a closed system reactor within the range of experimental conditions investigated in this study.
3. TPH removal decreases with decreasing temperature in the closed system reactors. A maximal degradation rate was achieved at temperature 25°C.
4. Optimal rate of degradation of TPH is obtained at a neutral or slightly acid pH condition.

5. Addition of surfactant Triton X-100 may be useful to degradate the TPH. Surfactant Tween 60 does not enhance the biodegradation of the TPH.

6. Microbiological growth with diesel fuel as sole carbon source was clearly observed by bacteria counting results.

7. Nutrient requirements have been demonstrated by the results of enhancement of TPH bioremoval.

8. Surfactants are useful in increasing the solubility of petroleum hydrocarbons in pore water, thus increasing the potential for bioremediation.

6.2 Recommendations

1. The laboratory experimental work should be confirmed by additional testing such as duplication, finer range of parameter variation, etc.

2. Bacteria growth in-situ should be studied to validate the laboratory findings.

3 The influence of the type of electron acceptor (oxygen , hydrogen peroxide, etc.) should be evaluated.

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