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The Effect of Temperature on the Rate and Extent of Crude Oil Biodegradation in a Soil Slurry

by

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ABSTRACT

The proper design and management of hydrocarbon-contaminated soil biological treatment systems requires an understanding of the rates at which the hydrocarbon contamination is degraded. The effect of temperature on the rate and extent of crude oil biodegradation was investigated in a soil slurry over 121 days. Biodegradation of Total Extractable Hydrocarbons (TEH) at 5°C and 20°C was measured gravimetrically using Soxhlet extraction with dichloromethane, microbial community numbers were estimated using a 96-well plate most-probable-number technique, and carbon fractions were measured using GC-FID. It was found that the rate of treatment TEH degradation at 5°C from 0-121 days was the same as the rate of treatment TEH degradation at 20°C from approximately 42-121 days. In addition, a two-stage first order kinetics model can approximate the rate of degradation of TEH at 20°C, and a single-stage first-order kinetics model can approximate the rate of degradation of TEH at 5°C.

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1.0 INTRODUCTION

Hydrocarbon contamination issues are widespread in freshwater, marine and terrestrial environments. There are many techniques available to remediate soil contamination at a site including *in-situ* methods, such as soil vapour extraction and *ex-situ* methods such as soil removal and disposal, incineration, and chemical treatment. One *in-situ* technique for the remediation of hydrocarbon-contaminated soils is bioremediation, which is the application of biological treatment for the clean-up of hazardous materials (Cookson 1995). Bioremediation is the use of microorganisms to transform contaminants into less harmful compounds. These biotransformations include mineralization, which is the conversion of contaminants to carbon dioxide and water, as well as transformations to biomass, humic material or more slowly converted metabolites (Huesemann 1994). Bioremediation technologies optimize conditions for microbial degradation by aeration, pH control and nutrient addition, and have been shown to be effective in reducing hydrocarbon concentrations. Examples of *in-situ* bioremediation technologies include bioventing and biosparging; *ex-situ* bioremediation technologies include landfarming, biopiles and slurry bioreactors. Compared to a technique such as excavation and disposal, bioremediation can be less costly. However, in many cases, it is more scientifically complex and requires a longer time period to lower contaminant concentrations to acceptable remediation criteria.

In cold climates, such as the sub-arctic and arctic regions, alpine habitats and colder seasons in temperate zones, ecological communities and human populations are potentially more exposed to chemical contamination from industry because the contaminants can accumulate and persist longer than in warmer environments. During much of the year in cold-climate regions, temperatures are below freezing and growing periods, during which organisms, such as plants, must mature and reproduce, are short. Cold-climate ecosystems have low productivity and low diversity, making them less resilient to perturbations (Bourdeau *et al.* 1989). The added stress of hydrocarbon contamination may have a profound influence on the ability of the ecosystem to survive,

and natural recovery of the ecosystem may be extremely slow (Bourdeau *et al.* 1989).

Because of their remoteness, use of technologies such as excavation and disposal for treating many cold-climate contaminated sites can be costly. In addition, a lack of transportation infrastructure makes the use of large machinery destructive. In these cases, *in situ* bioremediation may be a non-invasive, less costly, preferred option if it can reduce the hydrocarbon concentration to levels accepted by regulators.

Recently, bioremediation in cold climates has been more widely considered as a remedial option (Wardell 1995). Biodegradation of many components of hydrocarbons has been reported at low temperatures in a wide variety of cold-climate sites such as alpine soils (Margesin and Schinner 1997a, 1997b, 1997c, 1997d 1998), Alaskan aquifer sediments (Bradley and Chapelle 1995), seawater (Horowitz and Atlas 1977, Siron *et al.* 1995) and arctic soils (Westlake *et al.* 1978, Ramert *et al.* 1993).

Conventional understanding, with respect to bioremediation in cold climates, is that a decrease in temperature inhibits the growth and development of microbial communities and thus reduces the rate of biodegradation (Colwell and Walker 1977, Travis 1990, Margesin and Schinner 1999a). It is well known that temperature affects biological systems (Atlas and Bartha 1993, Campbell 1993). For every 10°C decrease in temperature a reaction rate decreases by about one half; this is known as the Q_{10} relationship (Radel and Navidi 1994). This is generally true for a specific enzyme; when the temperature is reduced by 10°C below its optimum temperature, the enzyme's activity is reduced by 50%. The temperature effect on biodegradation is often expressed as the Q_{10} value, which gives the factor by which the rate increases when temperature is raised by 10°C. The Q_{10} value has been used to predict that the rate of biodegradation in cold climates should be much slower than in warmer climates. This prediction, however, does not take into account the fact that microbial communities are capable of adapting to cold temperatures and can attain a growth rate higher than predicted (Margesin and Schinner 1999a).

Laboratory studies can be misinterpreted to suggest that rates of biodegradation in cold climates are reduced compared to warm climates. Using organisms from warm environments to characterize degradation rates at low temperatures may not reflect rates in cold environments because of the different microbial communities that would naturally exist in cold environments. In addition, many of the laboratory studies of oil degradation were carried out at temperatures higher than those found at natural sites. Therefore, the organisms adapted to growing at the higher temperatures were artificially selected and enriched resulting in degradation results not comparable to those actually occurring in a colder climate.

Cold-adapted microorganisms play a large role in the biodegradation of contaminants and organic matter in permanently cold areas and in habitats subject to large seasonal variation of temperature. Knowledge of the biodegradation rates of cold-adapted organisms is important to evaluate the persistence of organic pollutants and for the design and evaluation of bioremediation as a site-remediation option.

The physical state of crude oil in soil is affected by temperature. Changes to degradation rates can be affected by the physical changes in hydrocarbon compounds due to temperature. The complexity of the soil environment, dynamics of the microbial community, and the physical state of the oil make kinetic modeling of contaminant degradation in soil and the characterization of the effects of temperature difficult.

Current literature does not characterize how the rate of biodegradation over time changes with temperature. The objective of this study was to investigate the effects of cold temperatures on the rate and extent of biodegradation of crude oil in soil. Specifically, the objective was to compare the degradation rate and/or extent of biodegradation of crude oil in soil between 5°C and 20°C in a soil slurry. Changes over time in the rate of biodegradation for the two temperatures was examined. The degradation curves of crude oil at 5°C and 20°C were fitted to a first-order kinetics model. Data including microbial enumeration and gas chromatography was investigated in an attempt to suggest possible explanations for differences between degradation at 5°C and

20°C. The results of this research can be used as a stepping stone for future research to construct experiments that will determine the mechanism for the effect of cold temperature on crude oil biodegradation. Once the mechanism for the effect of cold temperatures on biodegradation is determined, techniques may be developed to improve bioremediation in cold-climates.

2.0 LITERATURE REVIEW

2.1 FACTORS AFFECTING BIOREMEDIATION

2.1.1 Abiotic Factors

There are many abiotic factors that affect the rate and extent of biodegradation of petroleum hydrocarbons by bacteria in soil. These factors, some of which are: oxygen availability, temperature, nutrients, pH and moisture, influence both the growth of microbial populations and the physical characteristics of hydrocarbons within the soil. Interactions among these factors and the relative impact of each individual factor depends on site characteristics, indigenous microbial community composition, and hydrocarbon characteristics.

2.1.1.1 Oxygen

During bioremediation, some heterotrophic bacteria use organic compounds as a carbon source to generate energy in the form of ATP (adenosine 5'-triphosphate). Enzymes catalyze the metabolism of the organic compounds resulting in the repeated oxidation (i.e. loss of electrons) of the hydrocarbon molecules (Cookson 1995). During this process, molecular oxygen acts as the electron acceptor. If oxygen is not present, aerobic biodegradation will not occur (Cookson 1995). Nitrate, sulfate, and iron can also serve as electron acceptors. However, in general, biodegradation of hydrocarbons does not occur in anaerobic conditions as rapidly as it does in aerobic conditions (Atlas 1981).

2.1.1.2 Temperature

Microorganisms are particularly susceptible to temperature change because they are unicellular and poikilothermic (Atlas and Bartha 1993). Therefore, a microbial cell's temperature is the same as surrounding temperature. For a microbial species there is a minimum temperature below which growth does not occur, an optimal temperature at which growth is most rapid, and a maximum temperature above which growth is not possible (Prescott, Harley and Klein 1995). These three temperatures are known as the cardinal temperatures and are depicted in Figure 2-1 (Prescott, Harley and Klein 1995). Mesophilic organisms have an optimal growth range of 20-45°C and thermophiles have an optimal growth range of 55-65°C (Prescott, Harley and Klein 1995). Psychrotrophic and psychrophilic organisms have a lower optimal range and will be discussed in more detail in section 2.2.1. It should be noted that these definitions are how the existing literature categorizes bacteria and the boundaries between the groups are neither distinct nor well defined.

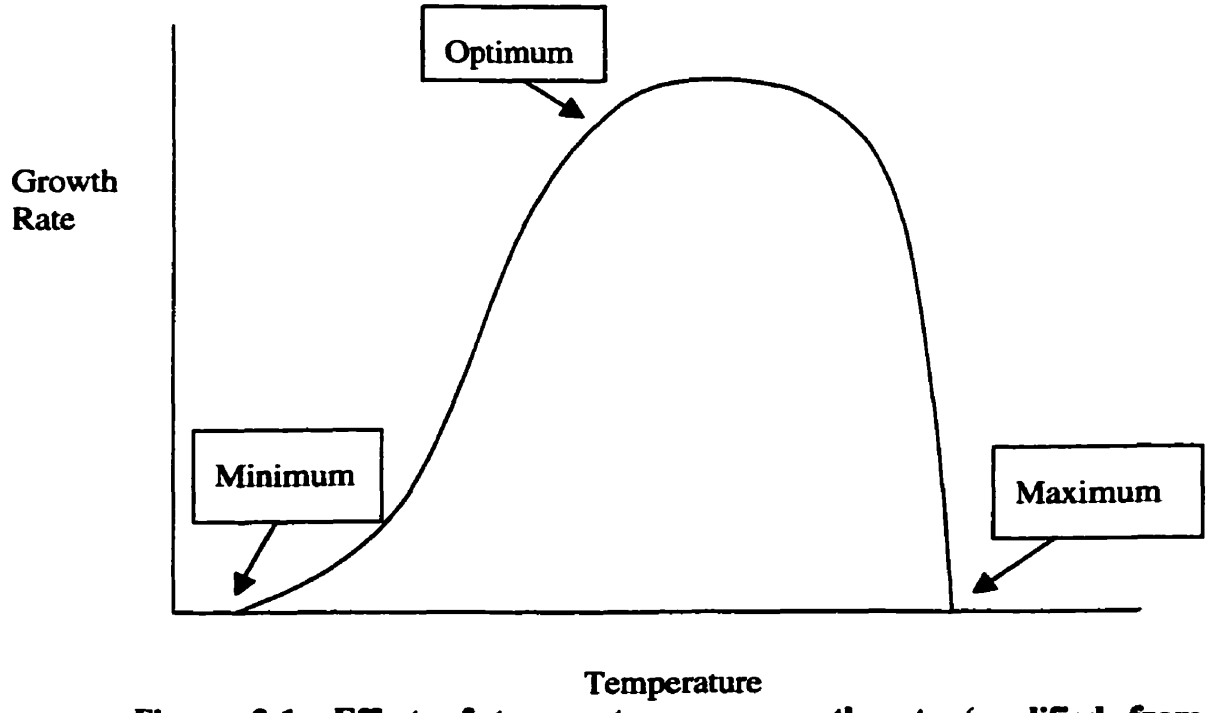

Figure 2-1: Effect of temperature on growth rate (modified from Prescott, Harley and Klein 1995)

Cardinal temperatures for a microbial species are not fixed, but depend on other environmental factors such as nutrients and other growth factors (Chablain *et al* 1997). Growth factors are organic compounds that are required because they are essential cell components and which cannot be synthesized by the organism (Prescott, Harley and Klein 1995). For example, *Crithidia fasciculata*, a flagellated protozoan, will grow in a simple medium at 22-27°C, but will not grow at 33-34°C without the addition of metals, amino acids, vitamins, and lipids (Prescott, Harley and Klein 1995). At these higher temperatures the metals, amino acids, vitamins, and lipids are growth factors. The effect of temperature on enzyme-catalyzed reactions is important. At low temperatures, an increase in temperature increases the growth rate because the enzyme-catalyzed reaction rates increase (Figure 2-1). At temperatures beyond the maximum, microorganisms quickly cease to function because enzymes, transport carriers and other proteins denature from the heat (Prescott, Harley and Klein 1995). Because biodegradation reactions are enzyme-catalyzed reactions, temperature affects on the rate of biodegradation of petroleum hydrocarbons. The rates of metabolic biochemical reactions, used in bioremediation, are governed by the Arrhenius equation (Cookson 1995). As temperature increases so does the rate of the biochemical reactions responsible for biodegradation. Further discussion of the Arrhenius equation can be found in section 2.2.3

In the natural environment, temperature varies vertically within the soil horizons, daily, and seasonally. This variation in temperature results in responsive variation by microbial communities (Atlas 1981). Communities can become adapted to temperatures that are below what may be considered optimum for mesophiles. This results in more rapid or more extensive biodegradation in colder climates by adapted communities than anticipated when considering biodegradation by mesophilic communities in cold climates. The proportion of mesophiles, psychrophiles, and psychrotrophs may determine the rate and extent of biodegradation in cold climates. Currently, research is underway to determine differences between communities at 5°C and communities at 20°C

(Rowsell *et al.* personal communications). The effect of temperature on biodegradation is the focus of this project and therefore will be examined in greater detail throughout the thesis.

2.1.1.3 Nutrients

Microorganisms use hydrocarbon compounds to produce more microbial biomass during bioremediation. The nutrient requirements for this process can be approximated from the nutrient composition within a microbial cell (Cookson 1995). The composition of biomass is approximately $C_5H_7O_2NP_{0.03}$ along with other trace elements. Carbon is supplied from the organic source (petroleum hydrocarbons); hydrogen and oxygen are supplied from water; nitrogen, phosphorus, and sulfur are supplied from inorganic sources. The remaining elements such as potassium, manganese, calcium, iron, cobalt, and zinc are provided from inorganic salts, usually present in most soils in adequate concentrations (Cookson 1995). All of the components making up microbial biomass must be available in appropriate concentrations for biodegradation of hydrocarbons to occur.

Natural nutrient replenishment of nitrogen and phosphorus in the soil are often too low to sustain bioremediation. As a result, biodegradation can be nutrient limited causing the rate of degradation to be extremely slow. To prevent this limitation, nitrogen and phosphorus fertilizers can be added to the soil in a process known as biostimulation. During oily-sludge bioremediation, Dibble and Bartha (1979) found that adding nutrients in small frequent applications rather than larger less frequent application resulted in better biodegradation. In addition, they found that a carbon to nitrogen (C:N) ratio of 60:1 and a carbon to phosphorus (C:P) ratio of 800:1 worked best. During experimentation by Margesin and Schinner (1997a, 1997b, 1997c) a C:N ratio of 10:1 worked well for the degradation of diesel fuel in soil at 5°C. The exact quantity of nitrogen and phosphorus depends on site conditions and hydrocarbon concentrations present. Nitrogen and phosphorus are typically measured in experiments seeking to optimize biodegradation.

2.1.1.4 pH

Soil pH can be highly variable and must be considered when optimizing bioremediation. The pH of the environment in which the microorganisms live affects processes such as cell membrane transport and equilibrium of catalyzed reactions (Cookson 1995). Most heterotrophic bacteria prefer to grow at neutral to slightly alkaline pH (Atlas 1981, Cookson 1995). Dibble and Bartha (1979) found the optimal pH for the biodegradation of oily sludge to be approximately 7.8.

2.1.1.5 Moisture

Water availability directly affects movement and growth of microorganisms. Oil decomposition occurs at the oil-water interface of free oil (McGill *et al.* 1981) and in the dissolved phase. Soil bacteria normally occupy less than 1% of the total soil-pore space. Therefore, movement of organisms or substrate is essential to decomposition (McGill *et al.* 1981). All cell requirements are transported from the aqueous environment into the cell. Moisture content of the soil affects many physical processes, some of which are the availability of contaminants and nutrients and the transfer of gases (Cookson 1995).

2.1.2 Biotic Factors

2.1.2.1 Microbial Community

Hydrocarbon compounds occur naturally and microorganisms have evolved the ability to utilize them to produce the energy required for growth. Microorganisms capable of degrading petroleum hydrocarbons include bacteria, fungi, yeast, as well as some algae (Cookson 1995). Zobell (1964) has identified more than 100 species representing 30 microbial genera capable of utilizing hydrocarbons. These hydrocarbon-utilizing microorganisms are widely distributed in freshwater, marine and terrestrial ecosystems (Atlas 1981). The proportion of the total heterotrophic community represented by hydrocarbon-utilizing bacteria is variable depending on site characteristics (Leahy and Colwell 1990). Individual microbial species can metabolize only a limited range of hydrocarbon substrates therefore, a consortium of organisms is required to metabolize a complex mixture of hydrocarbon compounds. Often, the mixed consortium of microorganisms can accomplish more than the individual sum of their parts (Cookson 1995). For example, one organism may synthesize a needed component for another organism to degrade a particular hydrocarbon. Some of the more important degrading bacteria listed in a review done by Leahy and Colwell (1990) include *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Flavobacterium*, *Nocardia*, and *Pseudomonas spp.* The abiotic factors, including the ones listed above and others specific to local environmental conditions for each site, have a selective influence on the degrading community (Atlas 1981). This influence is discussed in the following section.

2.1.2.2 Adaptation and Previous Exposure

Prior exposure to hydrocarbons from either natural sources or human contamination plays a role in how rapidly degradation occurs (Leahy and Colwell 1990). Previous contamination results in an adapted or acclimatized community of microorganisms. Leahy and Colwell (1990) gave three mechanisms of the adaptation to hydrocarbon contamination described below: induced enzyme production, genetic exchange and microbial community change.

One mechanism is induction and/or depression of enzymes. The presence of a hydrocarbon substrate can induce the production of enzymes necessary to degrade that specific hydrocarbon. The organism then has an increased number of enzymes for degradation and bioremediation occurs more rapidly. A second mechanism is a genetic exchange resulting in new metabolic capabilities of the microorganisms to degrade the contaminant of concern. This primarily occurs from amplification by means of selective enrichment, gene transfer or mutation of genes involved in metabolism of the hydrocarbon substrate. Transfer of highly mobile plasmid DNA via transformation or conjugation results in the recipient organism achieving hydrocarbon-utilizing ability. A third mechanism is selective enrichment of the community by organisms able to transform the compounds of interest. Studies have shown an increase in the proportion of hydrocarbon-utilizing organisms within the microbial community after exposure to hydrocarbon contamination. Pinholt *et al.* (1979) examined microbial changes during oil decomposition and found the number of aerobic bacteria was 3 times higher in oily soil than in the control. During decomposition, there was an increase in oil degrading bacteria from 3.5% to 50% of the total population after a fuel-oil spill (Pinholt *et al.* 1979).

2.1.2.3 Bioaugmentation

Indigenous organisms are preferred for use in bioremediation because they are adapted to specific site conditions and will most likely perform best under those

conditions. However, indigenous organisms may not have the capability to degrade certain types of contamination (Errampalli *et al.* 1997). Bioaugmentation, also called seeding, involves the introduction of allochthonous microorganisms into the contaminated environment to increase the rate or extent of biodegradation (Leahy and Colwell 1990). Studies have shown mixed results with respect to bioaugmentation and this topic will be discussed in greater detail with respect to cold climates in section 2.4.

Seeding allows the use of new molecular techniques to enhance bioremediation. Two such techniques described by Errampalli *et al.* (1997) are genetically engineered microorganisms (GEMs) and encapsulation. GEMs contain a suite of enzymes capable of degrading the target contaminants. GEMs must possess characteristics that will make their use beneficial to bioremediation, some of which are competitiveness in a chemically polluted environment, not pathogenic to plants and animals, no production of toxic byproducts, no antibiotic resistance and do not participate in high-frequency gene transfer of undesirable traits. Encapsulation involves using a biodegradable nontoxic algal biopolymer to encapsulate microorganisms. Encapsulation allows microorganisms to increase their survival rate and maintain metabolic abilities in extreme environments such as wet-dry cycles, freeze-thaw cycles and exposure to protozoan predators. By maintaining a small tolerable microenvironment within the capsule, microorganisms can then survive more heterogeneous conditions of the soil environment. This technique has increased the rate of degradation and made seeding bacteria easier to store, transport, and apply. The disadvantage is that the bacteria have reduced mobility in soil (Errampalli *et al.* 1997). These techniques have not been widely studied therefore their use in the field is limited.

2.1.3 Bioavailability

Under environmental conditions that are not limiting (i.e. oxygen, nutrients, and moisture are sufficiently supplied; correct pH and microbial community) the rate and extent of hydrocarbon degradation can be greatly affected by limitations in bioavailability of the hydrocarbon compounds. The bioavailable fraction of hydrocarbons is the fraction

that is accessible to microorganisms. Bioavailability is affected by physical barriers, such as tight adsorption within soil pores, and micropores, rates of mass transfer such as diffusion and fluid flow, and moisture.

The fate of organic chemicals in the soil depends on processes such as adsorption, desorption, volatilization, photolysis (light degradation), hydrolysis and biodegradation (Winegardner 1996). When considering bioavailability the most important process is adsorption and desorption that is defined by Greenland and Hayes (1981) as a process by which a chemical species passes from one bulk phase to the surface of another where the chemical accumulates without penetrating the structure of the second bulk phase. Desorption is the reverse process. Adsorption/desorption should not be confused with absorption which involves the transfer of a molecule from one phase to another via their interface and this transfer alters the composition of the second bulk phase (Greenland and Hayes 1981). There is both chemical adsorption and physical adsorption, however the more important process in the case of bioavailability is physical. Physical adsorption is a rapid, non-activated process which occurs at all interfaces. Transport processes like diffusion or fluid flow to an interface are rate determining and the chemical nature of the adsorptive species is preserved (Greenland and Hayes 1981). Factors such as the chemical structure of the contaminant and soil characteristics (particle size distribution and organic matter content) influence adsorption of petroleum contaminants in soil (Winegardner 1996) and as a result they influence bioavailability.

The effect of bioavailability can be shown by the decrease in biodegradation with increased aging of the contaminated soil. Aging, as defined by Alexander (1995), is the change in availability of a compound with time. This change involves volatilization of lighter compounds and sequestration of compounds within the soil matrix. It is the process by which organic compounds become increasingly desorption-resistant in soil and is associated with the continuous diffusion of the contaminant into more remote sites within the soil (Alexander 1995). Aging studies are of interest because soil that has been exposed to hydrocarbons for a long period of time may respond differently than fresh

contamination to bioremediation. Chung and Alexander (1995) showed that mineralization by bacteria decreased as aging increased. Hatzinger and Alexander (1995) showed that the extent and rate of mineralization decreased with increased aging time.

The influence of organic matter on sequestration of hydrocarbons and bioavailability has received some attention. Sequestration of hydrocarbons in soil may occur by the partitioning of the hydrocarbons into solid organic matter within the soil making the hydrocarbons inaccessible to microorganisms (Alexander 1995). If this is the case, it may be expected that a higher portion of organic matter in the soil will effect the rate and extent of biodegradation. Studies do not show conclusive evidence for the effect of organic matter on bioavailability. In studies by Salanitro *et al.* (1997), hydrocarbon degradation differences between soils with high (4.7%) and low (0.3%) organic matter were attributed more to oil type than organic matter. More detailed investigations are required to determine the affects of organic matter on bioavailability of petroleum hydrocarbons in soil. The effects of hydrocarbon characteristics and soil properties illustrate how contamination in soil is highly variable. Site specific analysis is required to determine the relative importance of hydrocarbon characteristics and soil properties such as soil organic matter to bioremediation at a site.

2.1.4 Biodegradability

The size of the biodegradable portion of hydrocarbon can affect the rate and extent of degradation. The biodegradable fraction of petroleum hydrocarbons is the fraction of hydrocarbons that can be degraded by microorganisms. This fraction contains hydrocarbon compounds that are the correct size and shape to be metabolically compatible with the microorganism. Material that is not biodegradable or degradable at an extremely slow rate is considered recalcitrant. The structural framework of the recalcitrant material may be too bulky to pass through the bacterial membrane or the structure can not properly align with the active site of the enzymes responsible for their breakdown, an effect known as steric hindrance (Huesemann 1997).

Most bioremediation projects require between 3 months to 5 years. For the recalcitrant portions of the hydrocarbon contamination to be degraded, the bacterial population would have to adapt enzymes or synthesize new enzymes capable of attacking the structurally complicated molecules (Huesemann 1997). The natural process of evolution is much slower than 5 years and has an unpredictable outcome. For example, the bacteria may develop resistance against the contaminant instead of developing the ability to degrade it. Genetic engineering may be able to speed up and control the process. However, in most cases these options are not considered during site remediation.

Hydrocarbon characteristics play a large role in biodegradability. Petroleum contamination consists of a complex mixture of compounds. These compounds can be fractionated using silica gel chromatography into saturates (aliphatic fraction), aromatics, asphaltic (polar fraction) and resins (Atlas 1981, Leahy and Colwell 1990). Saturates include n-alkanes, branched alkanes and cycloalkanes. Aromatics include structures with at least one benzene ring. In general, the degradability of the hydrocarbon compounds decreases from n-alkanes (most degradable) to branched alkanes to low molecular weight aromatics to cyclic alkanes to asphaltic to resins (least degradable) (Leahy and Colwell 1990).

Studies have indicated that aromatics with up to six rings can be degraded (Atlas 1981), however, Huesemann (1995) observed that four- and five-ring saturates with less than 44 carbon atoms were not degraded. The discrepancies in the data are most likely the result of different treatments and different duration of degradation. The low degradation rates of complex compounds can not be explained solely by insolubility because normal alkanes are insoluble and are readily degradable (Huesemenn 1995). Studies have shown the extent of oil and total petroleum hydrocarbon biodegradation is correlated with initial molecular composition (Huesemann 1995). These experiments were performed on soils that were freshly spiked, therefore sequestration could only play a minor role in incomplete degradation.

Biodegradation of relatively recalcitrant material has been ascribed to cometabolism (Leahy and Colwell 1990). Cometabolism is the degradation of one compound only in the presence of another organic material that serves as a primary electron source (Cookson 1995). When an organism grows on a primary substrate, the enzymes generated can also transform a secondary substrate. The transformation of the secondary substrate is not associated with the organism's energy production, carbon assimilation or other growth processes. As a result the secondary substrate is usually only partially degraded (Cookson 1995). Cometabolism allows for the partial degradation of complex branched alkanes and aromatic hydrocarbons (Atlas 1981).

It is hypothesized that during microbial degradation, microbes produce hydrocarbons of different molecular weight and structure than the original mixture of hydrocarbons (Atlas 1981). Analytically, many of these compounds can not be resolved and identified. During GC analysis an "unresolved hump" is formed by the compounds that can not be resolved into individual peaks (Huesemann 1997).

Because natural systems, including soil development and microbial communities, are extremely complex, it is most likely that the explanation for the rate and extent of hydrocarbon biodegradation in soil will involve all of the abiotic and biotic factors listed above.

2.2 MICROORGANISMS IN COLD CLIMATES

2.2.1 Definitions

Since the proposal of the term "psychrophile" by Schmidt-Nielson in 1902 (cited in Gounot 1991 and Russell 1990), the definitions of organisms capable of growing and reproducing at cold temperatures has been disputed. The most widely accepted definitions, and the definitions used throughout this project, were put forth by Morita (1975). Morita defined psychrophiles as organisms having an optimum temperature for growth at 15°C or lower and a maximum temperature for growth at 20°C or below. Psychrotrophic organisms have an optimal temperature for growth at 15°C or higher and a maximum temperature for growth above 20°C and as high as 40°C. Figure 2-2 shows a typical growth curve for a marine psychrophile. The optimal growth temperature is 4°C and the lower limit is −1.8°C, the freezing point of seawater (Morita 1975). The organisms will not grow above 10°C and generally show a restricted range of growth. In an Antarctic pond kept from freezing by high concentrations of $CaCl_2$, microorganisms have been observed as low as −24°C (Atlas and Bartha 1993). The practical limit to low temperature growth is the freezing temperature of the cell contents and the surrounding water.

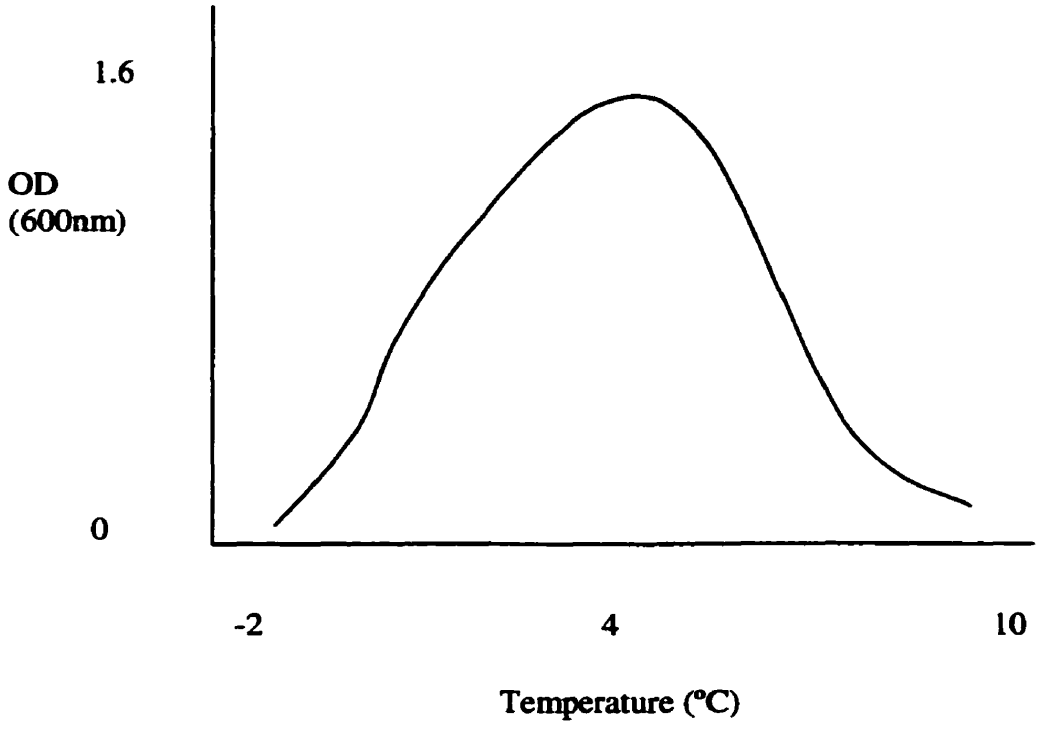

Figure 2-2: Growth of an Antarctic marine psychrophile during 80-hour incubation measured by optical density (OD) (source: Morita 1975).

Psychrophiles, cold-loving organisms, are restricted to permanently cold habitats with stable thermal regimes (Russell 1990). Psychrotrophs, cold-tolerant organisms, are widespread in cold habitats that undergo diurnal or seasonal thermal fluctuations and are considered more adaptable because they can grow over a wider temperature range (Russell 1990). Genera of Gram-negative and Gram-positive bacteria, cyanobacteria, fungi and eukaryotic algae contain psychrophilic and psychrotrophic organisms (Gounot 1991); however, the emphasis in this project will be on the bacteria.

2.2.2 *Physiological Adaptations*

In order to optimize biodegradation by psychrophiles and psychrotrophs their physiology, ecology, genetics and biochemical catabolic pathways must be understood. The adaptations that allow psychrophilic microorganisms to grow in temperature ranges that are prohibitive to most mesophilic organisms are worth consideration. Most mesophilic organisms have control mechanisms that shut off protein synthesis if the temperature falls to approximately 5°C (Inniss and Ingraham 1978). Psychrophilic organisms have a method to override these controls. The ability of psychrophiles and psychrotrophs to grow at low temperatures depends on the adaptive changes to the organism. There are two types of adaptive changes, 1) genotypic changes, those that occur over an evolutionary time-scale and eventually produce new species or subspecies and 2) phenotypic changes, those that occur within the organism's lifetime. Phenotypic changes can have a time-scale from minutes to seasons or longer. Hochachka and Somero (1984) acclimatization as phenotypic adaptation to multiple variables in the natural environment. The target of adaptation is to maintain the function of enzymes and structural molecules, therefore the qualitative and quantitative changes in proteins and lipids enable psychrophiles and psychrotrophs to grow at low temperatures (Hochachka & Somero 1984). The following section contains a brief overview of some of the physiological adaptations that help psychrophilic and psychrotrophic bacteria to grow and reproduce in cold temperatures.

2.2.2.1 Lipid Composition and Membrane Fluidity

Phenotypic adaptation occurs in the lipid composition of the bacterial cell membrane according to the growth temperature. In general, the proportion of unsaturated fatty acids and/or short chain lipids increases as the growth temperature decreases to maintain the optimal degree of membrane fluidity (Atlas and Bartha 1993). The usual lipid content of most bacteria is between 2% and 5%, most of which is in the cell membrane (Jay 1996). Jay (1996) cites sources showing as much as a 50% increase in the content of unsaturated bonds of fatty acids from mesophilic and psychrotrophic

Candida spp. in cells grown at 10°C compared to cells grown at 25°C. This phenomenon is not related specifically to psychrophiles; it is also seen in mesophiles (Russell 1990). The difference between mesophiles and psychrophiles is the time scale of the adaptive changes after a sudden decrease in temperature (Russell 1990). Psychrotrophs are quicker at adapting to a down-shift in temperature whereas mesophiles are quicker at adapting to an up-shift in temperature. The consequences of adapting the lipid composition of the cell membrane is to increase the efficiency of electron transport, ion pumping, and solute uptake at low temperatures (Russell 1990 and Jay 1996).

2.2.2.2 Cold Adaptations in Proteins

Proteins of cold-adapted microorganisms are cold stable (Margesin & Schinner 1998). Non-covalent interactions within the polypeptide backbone and between side-chains of amino acids stabilize proteins (Russell 1990). These interactions include hydrogen bonds, van der Waals interactions and hydrophobic interactions. Cold-active enzymes have high flexibility in tertiary structure at low temperatures. Proteins within cold-adapted species are not prone to cold denaturation and the enzymes have higher catalytic efficiencies because they have a looser formation, allowing conformational changes to occur with less energy input (Margesin and Schinner 1994). Activation energies are lower in cold-adapted homologues of an enzyme than in the warm-adapted homologue of an enzyme (Hochachka and Somero 1984). The sacrifice of cold-adapted protein modifications is a greater heat-sensitivity and a shift of the optimum activity toward lower temperatures (Morita 1975). Finally, formations of more enzymes at low temperatures may compensate for the slower rate of enzymatic activity (Margesin and Schinner 1994).

Cold Shock Proteins (csps) have been found to be important for bacterial survival at lower temperatures (Jones *et al.* 1987, Gumley and Inniss 1996). Although the csps functions are still undetermined, several csps have been identified and are thought to function mostly in transcription and translation as well as ensuring that heat-shock proteins are repressed (Russell 1990, Jones and Inouye 1994). Initial investigative

studies found that shifting *Escherichia coli* from 37°C to 10°C resulted in a 4-hour lag followed by a new rate of growth (Jones *et al.* 1987). During the lag phase, the number of proteins produced was greatly reduced and 13 proteins were made at 3 to 300 times the rate at 37°C. The protein with the highest rate of synthesis at 10°C was not produced at 37°C (Jones *et al.* 1987).

It has been found that ribosomes from psychrophiles and psychrotrophs are better able to synthesize proteins at lower temperatures than ribosomes from mesophiles. Russell (1990) described laboratory experiments showing that ribosomes from psychrophilic organisms produced proteins with a very low miscoding rate compared to those from mesophiles at low incubation temperatures. Bertoli and Inniss (1978) found that ribosomes from some psychrotrophs have different factors associated with them as compared with those from mesophiles, which could affect the translation process.

2.2.2.3 Other Adaptations

Other noted adaptations of psychrotrophic and psychrophilic organisms are the increased production of pigments, differential substrate utilization, and larger cells (Jay 1996). In cold climates, extra pigmentation may help adsorb solar radiation and make better use of a temperature increase as well as protect from the strong ultraviolet radiation at high altitudes (Margesin and Schinner 1994). Psychrotrophs formed acid and gas when fermenting glucose and other sugars at 20°C and lower but formed only acid when fermenting glucose and other sugars at higher temperatures (Jay 1996). The larger cell size may be due to the increased protein and RNA production (Jay 1996) or from the higher content of lipids within the cell membrane that, in part, could be a mechanism of increasing the efficiency of membrane transport by increasing membrane surface area (Margesin and Schinner 1994).

An easy way to compare the molecular determinants of psychrophily would be to isolate mutants of one thermal group (mesophiles) that are able to grow at temperatures characteristic of another thermal group (psychrophiles) (Russell 1990). However, all

structural and metabolic proteins of psychrophiles have to be functional at low temperatures and consequently the number of mutations in genes encoding a variety of functions required to obtain a psychrophile mutant from a mesophilic bacteria is highly unlikely (Gounot 1991).

It becomes apparent that there is no single component that can be identified as the molecular determinant of psychrophiles and psychrotrophs and it is an overall cellular phenomenon allowing the organisms to be fully functional and grow at low temperatures (Russell 1990). This is exemplified by the results of the experiments by Gumley and Inniss (1996) who inserted the plasmid containing the toluene degrading gene from a mesophile into the psychrotroph *P. putida* Q5. They found that the transconjugant produced more csps than the parent that are not necessarily required for growth at low temperatures (Gumley and Inniss 1996). This suggests that the insertion has created a metabolic stress in the transconjugant that was reflected in the increased csps production and shows the complexity of cold adaptations in psychrophiles and psychrotrophs.

2.2.3 Growth Rate

Prior to describing the effects of temperature on growth, the growth of bacteria in the presence of an adequate nutrient supply at a constant temperature will be reviewed. This information is summarized from Prescott, Harley and Klein (1995).

Consider microorganisms grown in a batch culture, that is, within a closed system with a single batch of medium. The growth of the organisms reproducing by binary fission can be plotted as the logarithm of cell number versus incubation time and results in four distinct phases (Figure 2-3).

The first phase is called the lag phase where there is no immediate increase in cell number. This phase may vary in time depending on the microorganisms and the growth conditions. It usually results because the microorganisms present are synthesizing new cell components. For example, if the medium or substrate is different from what the organisms have been growing in, new enzymes may be needed to be produced in order to

use the substrate.

The second phase is the exponential phase or the log phase. This phase occurs when the organisms are growing and dividing at the maximum rate possible given their genetic potential, nature of the medium and growing conditions. The rate of growth during this phase is constant.

The third phase is the stationary phase where population growth ceases and the total number of viable organisms remains constant. This phase occurs if there is a balance between cell division and cell death or if the population ceases to divide but remains metabolically active. The cause of the stationary phase could be the limitation of nutrients or the accumulation of toxic waste products or a combination of both. The fourth and final phase is the death phase during which there is a decline in the number of viable cells.

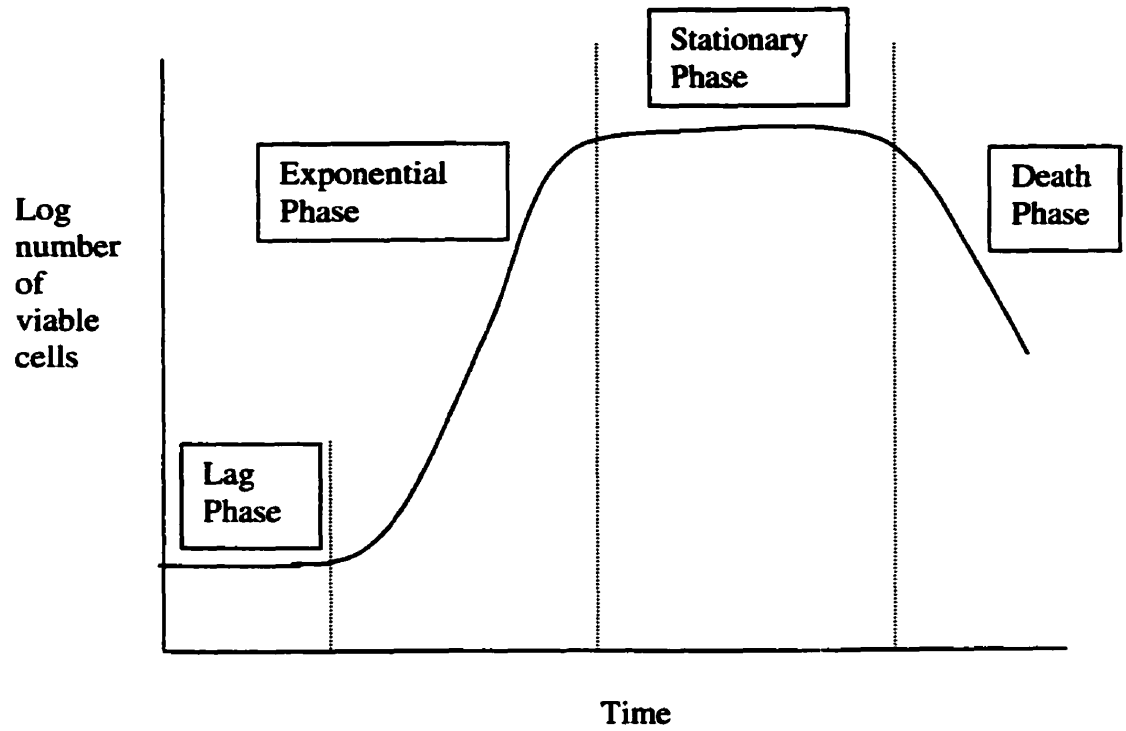

Figure 2-3: Microbial growth curve in a closed system

Now consider a bacterial species, growing on a water soluble, non-toxic organic compound used as a carbon and energy source. The system is well aerated, and inorganic nutrients and growth factors are present in excess. In conditions where this organic substrate, essential for growth, is limited, the substrate would be depleted and growth would cease. The effect of a limited substrate can be defined using the Monod equation described below.

$$u = \frac{u_{max} S}{K_s + S}$$

Where u is the specific growth rate, u_{max} is the maximum specific growth rate, S is the substrate concentration and Ks is the substrate concentration at one half u_{max} that is a constant. This effect of substrate concentration on specific growth rate is shown in Figure 2-4.

In conditions where the substrate concentration is much greater than Ks, growth is not affected by the substrate concentration, and the decay of the substrate follows zero-order kinetics. In conditions where the substrate concentration is much smaller than Ks, growth rate is exponential and the decay of the substrate follows first-order kinetics.

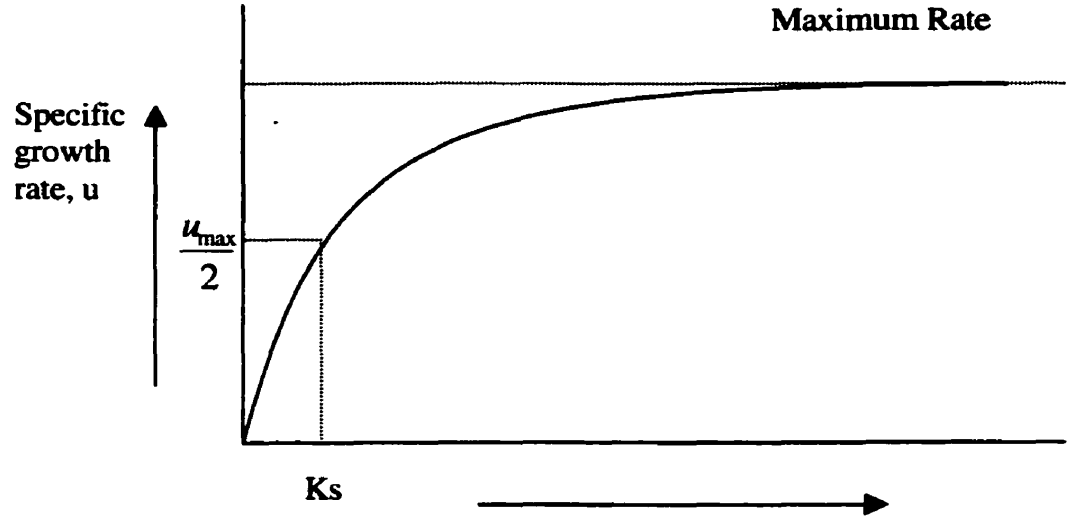

Figure 2-4: Plot showing effects of limiting nutrients on the specific growth rate

A Swedish chemist named Arrhenius, in the late 1800s, developed the following equation to describe how temperature affects the rate of a homogeneous chemical reaction (Radel and Navidi 1994). He found that the rate constant of the reaction increases exponentially with temperature according to the following equation.

$$k = Ae^{-Ea/RT}$$

Where k is the rate constant, A is a constant called the collision or frequency factor, E_a is the activation energy of the reaction, R is the universal gas constant and T is the absolute temperature. The logarithmic form, described below, predicts a linear relationship between the log of the rate of the reaction and the reciprocal of absolute temperature.

$$\ln k = \left(\frac{-E_a}{R}\right)\left(\frac{1}{T}\right) + \ln A$$

This type of plot, an Arrhenius plot, has been used by microbiologists to describe the effects of temperature on cell growth. Temperature affects the conformation of cellular macromolecules and other constituents that determines the rate of enzyme reactions governing biochemical metabolic reactions (Russell 1990, Cookson 1995). E_a in the reaction above is referred as the temperature characteristic (u) and the bacterial growth rate is substituted for the reaction rate (Ingraham 1958, Harder and Veldkamp 1967, Morita 1975). If a plot of ln k versus 1/T is made then the slope of the linear portion of the curve equals –u/R and the temperature characteristic can be calculated (Figure 2-5). At temperatures above and below the linear portion of the curve, there is a deviation from linearity and growth eventually ceases.

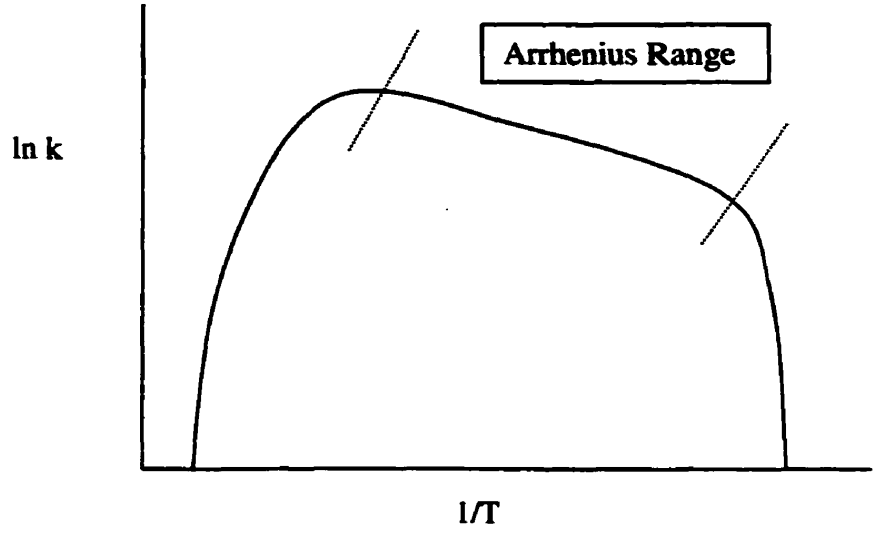

Figure 2-5: Arrhenius plot

An early hypothesis for growth at low temperatures was suggested by Ingraham (1958) who used a comparison between the Arrhenius curves of a psychrophilic *Pseudomonas* and mesophilic *Escherichia coli.* The temperature characteristic of the psychrophilic bacterium was lower than the mesophilic bacterium and therefore it was suggested that the temperature characteristic could be used to distinguish psychrophiles from mesophiles (Ingraham 1958). This concept was found to be erroneous because the same results were not found for three species of *Vibrio* and for yeast (Shaw 1967, Haight and Morita 1966). Harder and Veldkamp (1967) compared four psychrophilic bacteria and found that there was a difference in the temperature range at which the Arrhenius plots are linear not a difference in the temperature characteristic. Low u values may not correspond to psychrophilic properties. Morita (1975) states that making an Arrhenius plot for microorganisms growth response is excessive when growth curves give the same result.

An alternative model was proposed by Ratkowsky *et al.* (1992, 1993) who showed that the Arrhenius plots are curves rather than straight lines. They proposed a linear square root relationship

$$\sqrt{k} = b(T - T_{min})$$

where k is the specific growth rate, b is the slope of the regression, T is the absolute temperature and T_{min} is the temperature where the regression line cuts the temperature axis. The activation energy, E_a, is related to temperature by the expression

$$E_a = \frac{2RT^2}{T - T_{min}}$$

This relationship was found to apply to data for 43 strains of bacteria for temperatures ranging from the minimum temperature to just below the optimal temperature (Ratkowsky *et al.* 1992, 1993).

2.3 BIODEGRADATION RATES

First order kinetics and half-life periods are most commonly used for describing biodegradation. Kinetic models are based on substrate concentration and biomass (Suthersan 1997). In first order kinetics, the rate of biodegradation is directly proportional to concentration of substrate and can be summarized by the equation

$$\text{Rate} = -dC/dt = k$$

where k is a rate constant and C is the concentration of substrate. In this case, a plot of the logarithm of the chemical concentration remaining against time would give a straight line.

Natural environments are highly complex, both physically and chemically, and microbial communities are heterogeneous. It is therefore questionable to apply models, such as the first order kinetic model, to biodegradation in natural ecosystems (Alexander 1999). The concentration of the substrate is important in biodegradation models. Therefore, any process that lowers the concentration of substrate will affect biodegradation rates. In addition, the response of microbial communities to organic compounds does not depend on the total concentration but mainly on the contaminant water-soluble concentration (Suthersan 1997). Diffusion and sorption kinetics contribute greatly to bioavailability and thus the biodegradation rate (reviewed previously in section 2.1.3).

Hydrocarbon contaminants, such as crude oil, are a mixture of compounds that often degrade at a different rate. The typical pattern of degradation of hydrocarbons within soil seen during bioremediation has been labeled as the "hockey stick" curve (Huesemann 1997, Alexander 1995). It is characterized by an initial phase of rapid degradation followed by a second phase of slow degradation, during which the concentration of hydrocarbons remains relatively constant (Figure 2-6). Various hydrocarbon mixtures will vary in the initial and final degradation rates as well as the concentrations of remaining compounds.

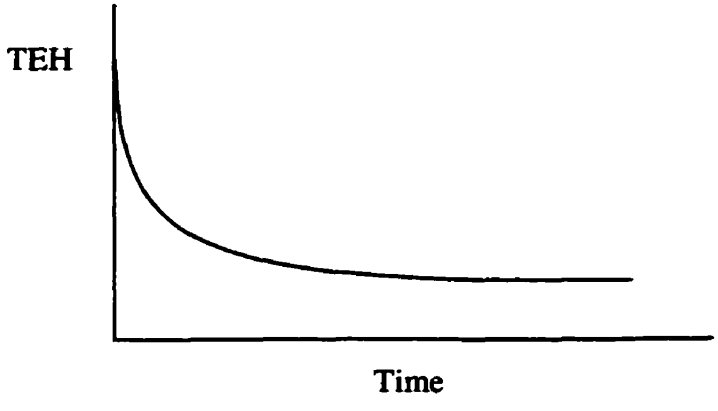

Figure 2-6: Degradation pattern of hydrocarbon contamination

Other factors such as acclimatization of the microbial consortium, weathering, complexation, biodegradability, pH, and moisture affect degradation rates (Suthersan 1997). Finally, temperature plays are role in determining degradation rates and the following discussion will focus on comparing degradation rates at different temperatures.

All reaction rates increase with increasing temperature. A general and very approximate rule of thumb is that reaction rates approximately double for every 10°C rise in temperature (Radel and Navidi 1994). This can be explained in terms of the kinetic theory. At higher temperatures a larger fraction of molecules have sufficient energy to react; that is, a higher fraction of molecules have the appropriate activation energy.

The above rule of thumb has been used to describe the response of bacterial processes such as biodegradation to temperature by defining what is called the temperature coefficient (Q_{10}). Q_{10}, described by the equation below, denotes the factor by which the rate of biodegradation increases for each 10°C rise in temperature.

$$Q_{10} = (R_{i+t}/R_i)^{(10/t)}$$

Where R_{i+t} is the reaction rate at temperature i+t, R_i is the reaction rate at temperature i and t is the change in temperature. Q_{10} is calculated when degradation rates are determined for only two temperatures and have been cited in numerous sources (Gibbs *et al.* 1975, Gibbs and Davis 1976, Yeung *et al* 1997, Dalyan *et al.* 1990). It has been shown that for various substrates including glucose, acetate, and formate, Q_{10} degradation values for psychrotrophs are lower than those for mesophiles (Ingraham and Bailey 1959). However, when cell free extracts were employed the Q_{10} values were the same for psychrotrophs and mesophiles (Ingraham and Bailey 1959). Ingraham and Bailey (1959) suggest that this is because the difference in temperature response of psychrophiles and mesophiles depends on the structural integrity of the cell.

It should be realized that the influence of temperature is more complex than the Q_{10} description. Cold adaptations of the microorganisms potentially affect Q_{10} values. Solubility of petroleum hydrocarbons, influencing toxicity levels, could also affect rates of degradation. In addition, the effect of temperature on the number of collisions with energy greater than or equal to the reaction activation energy depends on the magnitude of Ea (Radal and Navidi 1994). Therefore, the Q_{10} rule is not always obeyed. For example, the Ea must be 50kJ for the reaction rate to double when the temperature increases from 15 to 25°C (Masterton *et al.* 1985). If Ea were appreciably greater than 50kJ the reaction rate would less than a double for a 10°C rise; if Ea were smaller than 50kJ the reaction rate would be more than a factor of two.

2.4 BIOAUGMENTATION IN COLD-CLIMATES

It has generally been shown that bioaugmentation with psychrotrophic organisms has not been significantly beneficial to biodegradation in cold climates. However some literature suggests, in some cases, bioaugmentation still is appropriate (Whyte *et al.* 1998, Alexander 1999). It is difficult to differentiate between the role of indigenous microorganisms from the role of added microbes during bioaugmentation studies. The following discussion summarizes some of the findings with respect to cold-climate bioaugmentation.

Margesin and Schinner (1997a) studied the efficiency of indigenous soil microorganisms as well as the efficiency of bioaugmentation with psychrotrophic inoculum, from an oil contaminated alpine soil, on diesel oil degradation in soil at 10°C and 20°C. They found that with fertilization at a C:N ratio of 50:1 the psychrotrophic inoculation increased the degradation by only 2-7% at 10°C. With fertilization at a C:N ratio of 10:1 there was no difference between the degradation with the inoculum and without the inoculum. This study showed the degradation at 10°C of diesel oil was highest by indigenous microorganisms in the presence of fertilization. Margesin and Schinner (1997a) point out that nutrients may intensify competition between indigenous and non-indigenous microorganisms.

During an additional study by Margesin and Schinner (1997b), they isolated a psychrotrophic yeast from a diesel oil contaminated alpine soil to study the effect of temperature on diesel oil degradation as well as the effect of inoculation on indigenous microbes. They isolated the effects of the inocula on degradation by subtracting degradation of the non-inoculated samples from the inoculated samples. They found that the inoculum degradation was greatest after 5 days at 10-15°C and greatest after 10 days at 4°C. However as degradation time increased the activity of the inoculum decreased. These data show that the inoculum activity was only high during the period when indigenous activity was low suggesting again, that there is competition between indigenous and inoculated microorganisms. Margesin and Schinner (1997b) suggest that

it is possible that the indigenous organisms are using the inoculated population as a nutrient source, known as cryptic growth. These studies suggest that bioaugmentation is not that helpful and indigenous organisms are better than introduced psychrotrophic organisms at metabolizing diesel oil in soil.

During a treatability study on soil contaminated with 1000-5000 mg/kg diesel fuel at a site with an ambient temperature of 5-8°C, bioaugmentation with cold-adapted hydrocarbon degraders was studied (Wilson 1999). The organism used was a strain of *Rhodococcus* sp. Q15 and classified as a psychrotrophic bacterium. This strain was isolated from the Canadian Arctic and Great Lakes and is capable of degradation of n-alkanes from C_{15} to C_{32} at 5°C. The results of the treatability test showed that indigenous microbes, with fertilization, could degrade 66 to 84% of the diesel fuel within 40 days and bioaugmentation was not significantly helpful (Wilson 1999). Biostimulation seems to be more important than bioaugmentation.

Whyte *et al.* (1998) characterized *Rhodococcus* sp. Strain Q15 ability to mineralize alkanes and diesel oil at low temperatures. They found that bioaugmentation with this strain decreased the lag time and increased the rate and extent of mineralization at 5°C. However, this occurred when the inoculum was supplemented with yeast extract that contains growth factors and vitamins that may be important for the success of bioaugmentation.

The studies by Wilson (1999) and Margesin and Schinner (1997a, 1997b) showed that bioaugmentation with pure cultures of psychrotrophic organisms did not enhance bioremediation rates or extents beyond biostimulation alone. Whyte *et al.* (1999) used a consortium of enriched indigenous degradative microbes that showed slightly different results. To quickly increase the indigenous diesel-degrading population from contaminated site samples, Whyte *et al.* (1999) prepared soil slurries supplemented with 0.1% diesel (v/v) and 25ppm yeast extract (w/v) and incubated the samples for up to 6 weeks at 5°C, which is close to the site temperature. This enriched population should then be acclimated to on-site conditions such as low temperature, soil characteristics and

nature of contaminants. The results of augmenting the fertilized soil with the indigenous consortium, was a decrease in the lag phase of C_{16} mineralization at 5°C when compared to mineralization with fertilization alone. Whyte *et al.* (1999) found that after 21 days of incubation the total petroleum hydrocarbon (TPH) reduction was the highest in the bioaugmented and fertilized samples compared to the fertilized alone samples. However, after 45 days of incubation, TPH removal was higher in the fertilized alone samples than the bioaugmentation and fertilization samples.

It is commonly thought that the lag phase during biodegradation in cold-climates may be due to the time required for the indigenous microorganisms to acclimatize to the conditions (Atlas 1981, Leahy and Colwell 1990, and Suthersan 1997). However, a second explanation for the lag phase may be a decrease in bioavailability, due to decreased solubility and decreased desorption of the hydrocarbons lower temperatures. Whyte *et al.* (1998) found that as temperature decreased the mineralization of longer-chain alkanes (C_{28} to C_{32}) was reduced to a greater extent than the mineralization of shorter chains (C_{12} to C_{16}). Octacosane (C_{28}) and dotriacontane (C_{32}) form relatively large crystals at 0 and 5°C thus decreasing the bioavailability of the compounds making them more recalcitrant. Whyte *et al.* (1998) suggest that successful bioremediation in cold-climates may depend on application of cold-active solubilizing agents to increase the bioavailability of the long-chain alkanes.

The physical state of the hydrocarbons at lower temperatures may result in the presence of more toxic compounds, which may limit biodegradation and result in a lag phase in cold-climates. Examples of toxic compounds include benzene and toluene, as well as short chain alkanes, that, if present in high concentration, become toxic to bacterial cells by dissolving the cell membrane. As temperature decreases evaporation of toxic compounds decreases allowing microbes to be exposed to a higher toxicity level from the soluble toxic compounds. This toxicity decreases the biodegradation rate.

Atlas (1975) studied the biodegradation of 7 different crude oils at 10 and 20°C with microbial communities from Pacific and Arctic seawater samples. They found that

the greatest evaporation occurred with the lighter oils. The lightest oil, had the greatest delay of degradation at 10°C as compared with degradation at 20°C. It was suggested that the decrease in evaporation at lower temperatures results in the retention of more toxic compounds decreasing the rate of degradation and producing a lag phase. Atlas (1975) found that oxygen consumption by *Pseudomonas* sp. during oil degradation, was inhibited by fresh light oils, however, heavier oils did not show inhibition. Preweathering of the lighter oils removed the inhibition. The lack of inhibitory fraction suggests that heavier oils are less affected by a temperature drop because the lack of evaporation will produce less of an inhibitory effect.

The above studies show that the lag phase during low temperature biodegradation may be due to a decrease in bioavailability of higher carbon compounds and a decrease in volatilization. The viscosity of the oil increases at low temperatures and the volatilization of toxic short-chain compounds is reduced possibly delaying biodegradation (Atlas 1991). This would suggest that bioaugmentation may not be helpful in reducing the lag phase if the cause of the lag phase is increased in toxicity of compounds that have not volatilized. It is very likely that at contaminated cold-climate sites the bacterial community is already adapted to their environment. If however, the initial bacterial count is extremely low adding an enhanced local consortia along with nutrients and growth factors may initially speed up the process of degradation.

The above literature review describes the complexity of the effect of temperature on a natural soil system contaminated with crude oil. Temperature can influence microbial community composition, microbial community growth, and the physical state of the hydrocarbon contamination. This study does not separate the effects of temperature on community composition, microbial growth or the physical state of soil in soil. It characterizes crude oil biodegradation in soil at 5°C and 20°C which is one of the first steps towards understanding the effect of temperature on the rate and extent of biodegradation in cold climates.

3.0 METHODS AND MATERIALS

3.1 SOIL ACQUISITION, STORAGE, AND PRETREATMENT

The source soil was obtained in March 1997 from a flare pit site outside Edmonton, Alberta and was transported to Olds College, Olds, Alberta (Varga 1997a, 1997b). The soil was then thawed and partially dried indoors by spreading, and for a week turning daily with a backhoe. Two passes with a windrow turner (Double T Equipment Manufacturing Ltd.) homogenized the soil. Soil not used in the biopile experimentation was transported to Houston for characterization by Colorado Analytical Laboratory. In July 1998, the soil was returned from storage in Houston and homogenized by using a modification of the method suggested in the sample preparation section of ASTM D 5369 (ASTM 1993). A brief summary of the method includes emptying out the soil on a tarp and flattening the soil with a suitable straightedge until it is uniformly spread. Then, remixing the soil by lifting a corner of the tarp and drawing it across to the opposite corner so the soil rolls over and over and does not slide and repeating the flattening and rolling procedure 10 times. The soil was inadvertently exposed to –20°C from April 1999 to September 1999 because of problems with the cold room. At all other times the soil was stored in 20L plastic pails at 5°C. The soil was sieved using a No. 10 sieve (2mm) one week prior to the start of experimentation.

3.2 SOURCE SOIL CHARACTERIZATION

The homogenized and sieved soil was analyzed for soil pH, moisture content, particle size distribution, organic matter content and dichloromethane (DCM) total extractable hydrocarbons (TEH) measured gravimetrically. It should be noted that the definition of TEH used within this project does not include silica gel clean up or measurement by gas chromatography (GC) equipped with a flame ionization detector (FID). The definition is not the same as the one used by Alberta Environmental Protection (AEP 1996). The definition of TEH used in this project is the same as what AEP defines as "oil and grease". The oil within the soil was characterized by measuring pentane insolubles.

3.2.1 Initial Soil pH

The pH of the soil was measured potentiometrically in a supernatant liquid that is in equilibrium with a soil suspension of 1:2 soil-to-liquid mixture. A digital pH/mV meter with a combination electrode and automatic temperature compensator was used. The method used was modified from Thomas (1996)

3.2.1.1 pH in water

Approximately 20g of air-dry soil was added to a 50mL beaker followed by 40mL of distilled deionized water. The mixture was thoroughly stirred for 10 seconds with a glass stir stick and then further stirred four to five times over a 30-minute period. The mixture was then allowed to settle for 30 minutes. The electrodes were inserted into the supernatant and the pH was recorded once the reading had stabilized after approximately 1 minute. The pH was measured for three replicates, and was recorded as pH_W.

3.2.1.2 pH in 0.01*M* $CaCl_2$

A 0.01 Molar $CaCl_2$ solution was made by dissolving 1.47g of $CaCl_2.2H_2O$ in 1L of distilled deionized water. The pH of this solution was between 5.0 and 6.5. The same procedure as described above for pH_W was followed, substituting 40mL of $CaCl_2$ solution for 40mL of distilled deionized water. The pH was measured for three replicates, and was recorded as pH_{CaCl2}.

3.2.2 Moisture Content

The moisture content of the soil was determined using a modification of the methods described in ASTM method D2216 (ASTM 1992). The following is a summary of the procedure used. The mass of a clean dry aluminum weigh dish was recorded. Approximately 10g of wet soil was added to the dish and the mass of the container and the soil was recorded. The sample was placed in a drying oven at 110°C, dried for 24 hours, removed from the oven, and allowed to cool in a desiccator at room temperature. The mass of the container and the oven-dried soil was recorded and the gravimetric soil moisture content (g/g) was calculated using the following equation.

$$\text{Gravimetric Soil Moisture}\left(\frac{g}{g}\right) = \frac{[\text{wet soil mass}\,(g)] - [\text{oven dry mass}\,(g)]}{[\text{oven dry mass}(g)]}$$

3.2.3 Particle-Size Distribution

The Hydrometer Test, described by Day (1965), Gee and Bauder (1986), and ASTM method D 1140 (ASTM 1997), was used to determine the particle-size distribution of material <0.075mm within the soil. A summary of the method is presented below.

A Calgon solution, which consists of sodium hexametaphosphate, which acts as a dispersing agent, was prepared by dissolving 50g of Calgon in 1L of distilled water. The hydrometer was then calibrated by adding 100mL of Calgon solution to the sedimentation cylinder and filling the cylinder to the 1L mark with distilled water. The suspension was mixed thoroughly with a plunger and allowed to reach room temperature. The temperature was recorded and the hydrometer was carefully lowered into the solution. The R_L value, which is a measure of grams of soil colloids per litre, was recorded as the upper edge of the meniscus surrounding the stem.

Once the hydrometer was calibrated, approximately 40g of soil was placed in a 600mL beaker and an equal amount of soil was used for determination of oven-dry weight (method described in section 4.2.2). Calgon solution (100mL) and approximately 400mL of distilled water was added to the beaker and the sample was left to soak overnight. The sample was then transferred to the sedimentation cylinder and the level of the liquid in the cylinder was brought to 1000mL with distilled water. Once the temperature of the suspension became constant, the temperature was recorded. A plunger was used to thoroughly mix the contents and immediately after mixing the time was recorded. The hydrometer was carefully lowered into the suspension and after 30 seconds a reading was taken. Without removing the hydrometer, a second reading was taken after one minute. The hydrometer was removed, rinsed and dried. Without remixing the suspension between measurements, the hydrometer was carefully lowered into the suspension about 10 seconds before each hydrometer reading was taken. Readings (R) were taken at 3, 10, 90, 270, 720 and 1080 minutes.

The concentration of the suspension (c), in grams of soil colloids per litre, for each time interval was calculated using $c=R-R_L$. The summation percentage (P) was calculated using $P=100(c/c_o)$ where c_o is the oven dry weight of the sample. The corresponding particle size was calculated using the equation

$$X(\mu m) = y/\sqrt{t}$$

where t is the sedimentation time in minutes and y is the sedimentation parameter obtained from Day (1965). The values of y obtained from Day (1965) are for a temperature of 30°C, therefore they must be corrected for a temperature of 19.5°C. The value of y is corrected by multiplying y by

$$\sqrt{n/n_{30}}$$

where n is the viscosity of water at the temperature of measurement and n_{30} is the viscosity at 30°C. The summation percentage was plotted against particle size on a semilogarithmic scale.

To segregate particles coarser than 0.075mm a modified sieving technique was used (Gee and Bauder 1986). Sieving has numerous limitations, therefore these results should be used with caution, that is, only as a rough estimate. An example of a limitation is particle shape. A particle, whose shape permits passage through a sieve only in one orientation, has a limited chance of getting through, therefore requires prolonged shaking (Gee and Bauder 1986). In addition, organic matter was not removed prior to particle-size analysis which affects the results. The following summarizes the sieving method used.

A sample was dried to a constant mass at 110°C and the mass was recorded. Calgon solution (100mL) was added to a 600mL beaker and 300mL of distilled water was added. The mixture was left to soak overnight. Once soaking was complete, the mixture was vigorously stirred and poured onto the top sieve of a stack of four sieves, numbers 30, 60, 120, and 200. The container with the mixture was thoroughly rinsed onto the top sieve and the specimen was washed with a gentle stream of water no warmer than 32°C and being careful not to allow the middle and bottom sieves to overflow. Each

sieve was washed until the water that ran through the sieve was clear. The weight of labeled Watman 541 filter paper (particle retention > 2.7μm, Fisher Scientific) was recorded and the contents of each sieve was transferred onto the corresponding filter paper. The water was allowed to filter through and the paper with the sieve contents was placed in a drying oven at 110°C. After 24 hours of drying, the papers were removed and placed in the desiccator to cool at room temperature, after which the weight of paper and sample was recorded. The percent mass smaller than 0.6, 0.25, 0.125, and 0.075mm was calculated. The mass values were then adjusted to account for the loss of sample during manual sieving.

3.2.4 Organic Matter Content

The organic matter content of the soil was determined using the Loss On Ignition (LOI) method described in Nelson and Summers (1996). A summary of the method involves heating crucibles in a muffle furnace at 400°C for 2 hours and then allowing them to cool in a desiccator at room temperature to determine their tared weight to ±0.1mg. One to three grams of air-dried soil ground to <0.4mm was added to the crucibles and tared. The samples were then heated at 105°C for 24 hours and allowed to cool in a desiccator. The samples were weighed to 0.1mg. The oven dry weight of the samples were obtained by subtraction and the samples were placed in a muffle furnace at 400°C for 16 hours. The samples were again cooled in a desiccator and the weight obtained to 0.1mg. The organic matter content was determined for the soil prior to extraction with DCM (five replicates) and following extraction with DCM (five replicates). The following calculation was used to calculate organic matter content in percent and then converted to mg/kg.

$$\mathrm{LOI}(\%) = \frac{[\text{soil weight heated to } 105^\circ C\,(g)] - [\text{soil weight heated to } 400^\circ C\,(g)]}{[\text{soil weight at } 105^\circ C\,(g)]}$$

3.2.5 DCM Extractable TEH

The initial hydrocarbon content of the soil was determined gravimetrically by Soxhlet extraction using dichloromethane (HPLC grade from Fisher Scientific). DCM was chosen because it was found to extract topped crude oil efficiently from both mineral and organic soils (McGill and Rowell 1980). Although the soil used in this experiment does not contain topped oil that was added to the soil, the conditions of the field soil are similar because normal volatile losses in the field of the light ends occurs when the soil is sampled and prior to extraction. Modifications to the methods described by McGill and Rowell (1980) and in the ASTM method D 5369 (1993) were used and are summarized below.

Prior to the extraction process, all the extraction glassware was washed with FL-70 detergent (Fisher Scientific), rinsed with tap water, rinsed with distilled water, rinsed with acetone and rinsed with DCM. Approximately 20g of wet soil was weighed to ±0.001g in a 80mL beaker. An equal weight of granular anhydrous sodium sulfate (Certified ACS Fisher Scientific) was added to the beaker and stirred. The mixture was allowed to stand for 20 minutes. The mixture was then transferred to a glass mortar to be ground. Additional sodium sulfate was used as a dry rinse to be sure all the soil was transferred from the beaker to the mortar. The ground sample was placed in a cellulose thimble, using sodium sulfate to dry rinse the mortar, and a cotton plug was used to prevent soil material from floating out of the thimble. The thimble was placed in the Soxhlet apparatus so that the thimble extended just above the siphon arm of the Soxhlet apparatus. Approximately 350mL of DCM was added to a 500mL flat-bottom flask containing 3-4 teflon boiling chips and the flask was attached to the extractor. The flasks were heated on hot plates to approximately 40°C and the mixture was extracted for 48 hours.

Once the extraction was complete, the apparatus was allowed to cool and was disassembled. The thimbles were drained of DCM and removed from the extractor. The remaining DCM in the extractor was added to the flask. The flask containing the 350mL

solvent reservoir was attached to a distillation apparatus and the DCM was distilled off until approximately 15mL of DCM remained in the flask. Condensation from the outside of the Soxhlet apparatus caused water to be introduced to the system so 3-4mL of ethanol was added to the remaining DCM and extract to aid in the evaporation of water from the extract. The remaining DCM and extract was transferred to an aluminum-weighing dish tared to ± 0.001g. Two or three washings of 3-4mL of DCM with glass Pasteur pipettes were used to rinse out the flask. The remaining DCM was allowed to evaporate in a fumehood at room temperature for 48 hours. The dish was reweighed and the amount of oil determined on an oven-dry soil basis using the following equations.

$$\begin{array}{c}\text{Oven Dry}\\ \text{Mass of Soil}\\ \text{Extracted}\end{array} = \begin{array}{c}\text{Mass of}\\ \text{Wet Soil}\\ \text{Extracted}\\ \text{(g)}\end{array} - \left(\begin{array}{c}\text{Mass of}\\ \text{Wet Soil}\\ \text{Extracted}\\ \text{(g)}\end{array} * \begin{array}{c}\text{Soil}\\ \text{Moisture}\\ \text{Content}\\ \text{(\%)}\end{array} \right)$$

$$\text{Oil Content} = \frac{[\text{mass of oil } (mg)]}{[\text{mass of oven dry soil extracted } (g)] - [\text{mass of oil } (g)]} * 1000 \; {}^{g}/_{kg}$$

All DCM extractable TEH results are reported on an oven-dry soil mass basis.

3.2.6 Pentane Insolubles

The pentane insoluble portion of the extracted oil from the soil was determined using a method similar to that described in ASTM D 4055 (1992). Following Soxhlet extraction of the soil and distillation of the DCM, the remaining DCM was evaporated in 80mL beakers instead of aluminum trays. Approximately 2mL of toluene (Fisher Scientific) was added to the beaker followed by approximately 50mL of pentane per gram of hydrocarbon. The pentane was used to transfer the oil from the beaker into a flask. A stir bar was added to the flask and the mixture was heated slightly on a hot plate while being continuously stirred, allowing the oil to fully contact the pentane. The contents of the flask were then transferred to a glass centrifuge tube and were centrifuged at 175 x *g* (*g* is the standard acceleration of gravity) for approximately 5 minutes. The supernatant was transferred back to the original beaker and the pentane was allowed to evaporate in the fumehood. The pentane insolubles remaining in the centrifuge tube were transferred using DCM to a tared aluminum weight tray. This tray was placed in the fumehood to allow the DCM to evaporate at room temperature. Three replicates were performed. The percent of TEH that is pentane insoluble material and the percent recovery were calculated using the following formulas.

$$\%\ \text{Pentane Insolubles} = \frac{[\text{mass of pentane insolubles } (g)]}{[\text{mass of TEH } (g)]} * 100$$

$$\%\ \text{Recovery} = \frac{[\text{mass of pentane insolubles}(g)] + [\text{mass of pentane solubles}(g)]}{[\text{mass of TEH}(g)]} * 100$$

3.3 EXPERIMENTAL DESIGN AND APPARATUS

3.3.1 Preparation of Control and Treatment Slurries

Soil slurries are a bioremediation technique designed to optimize abiotic conditions for degradation. They consist of a mixture of soil in water in various ratios and greatly enhance biodegradation rates over solid treatment systems by maximizing the contact between microorganisms, the hydrocarbons, nutrients, and oxygen (Cookson 1995). Increased soil moisture results in a larger amount of solubilized contaminant, therefore increasing bioavailability.

There are numerous designs available for slurry biodegradation of contaminated soil. Some examples include a vertical tank equipped with an impeller or air sparger for mixing or a rotating drum equipped with lifters to provide internal mixing (Gray *et al.* 1994, Cookson 1995). The vertical tank with impeller has problems mixing a heterogeneous mixture. The solids content must be low and finely sieved for proper mixing to occur. Both the designs are mechanically intense and costly. It is for the reasons listed above that a sacrificial flask treatability laboratory design was chosen for this experiment.

There are two types of slurries within the experiment, control slurries and treatment slurries. The soil slurries were made up to contain a soil to water ratio of 1:3 (dry-wt/wt) based on the gravimetric moisture content described in section 4.2.2. Each slurry contained approximately 20g of soil and 60mL of chlorine free tap water combined in a 250mL Erlenmeyer flask. Beakers containing water were left open to the atmosphere overnight to allow the chlorine from tap water to volatilize. Foam stoppers were used to prevent dust and associated bacteria from entering the system while allowing for complete aeration. Aeration of the slurries was achieved by rotating the flasks at 300 rpm on a New Brunswick orbital shaker table (model G.10) for the duration of the experiment. The combination of shaking the flasks and filling the flask to approximately $1/5^{th}$ volume allowed for aerobic growth conditions (Alef and Namipleri 1995).

Ammonium nitrate was added to attain a 400mg of nitrogen per kilogram of soil. Monobasic and dibasic potassium phosphate was added to maintain a concentration of 50mg/L of phosphate. Typically approximately 50% of added phosphorus remains available to microorganisms (Walworth and Reynolds 1995). To account for potential precipitation of phosphorus, the concentration of phosphorus added was increased to 100mg/L. The monobasic and dibasic potassium phosphate was used as a buffer solution. A C:N ratio was not used because this type of nutrient application is better suited for complete carbon mineralization (Alexander 1999). Addition of all nitrogen estimated as necessary for complete degradation in a single application may be inhibitory to maximum biodegradation rates (Walworth and Reynolds 1995). Therefore the concentrations listed above were chosen and better suited for slurry studies (Alexander 1999). A detailed description of the nutrient addition calculations and solution preparation can be found in Appendix A.

The control flasks account for the loss of TEH due to sources other than biodegradation such as photodegradation, adsorption, and volatilization. Abiotic control flasks contained soil that was autoclaved at 121°C for 1 hour. Sodium azide was added to the slurry to prevent reinoculation of airborne bacteria. The effective concentration of sodium azide is 1mmol/L (Pramer and Bartha 1972). To be sure microorganisms are completely inhibited from growth, 50 times this concentration was used, which is 0.20g in each flask. Sodium azide calculations can be found in Appendix A.

3.3.2 Design, Sampling, and Statistical Analysis

The experiment consisted of two sets of treatment flasks, one treatment at 5°C and one treatment at room temperature or approximately 20°C ± 5°C. Each treatment consisted of 128 flasks, 40 of which were controls. There were a total of 13 sampling time intervals for each treatment. For the time intervals of 3, 7, 14, 30, 60, 90 and 121 days, there were ten replicate treatment flasks sacrificed and 4 replicate control flasks sacrificed. For the time intervals 1, 5, 10, 21, 45 and 75 there were three replicate treatment flasks sacrificed and two replicate control flasks sacrificed. Because the air temperature within the room dropped approximately 5°C overnight the flasks were sacrificed at the same time on each sampling day.

Because the number of Soxhlet extraction apparatuses and shaker table space was limited, the start days for the flasks were staggered. For each time interval the replicate treatment and control flasks were split into two groups. One group was started two days following the start of the first group. This prevented the storage of samples after sacrifice. The delay between the start of the first flasks and the start of the last flasks was two months. The 5°C treatment was started one month prior to the starting the 20°C. To determine if this delay in start date affected the initial TEH content of the soil (effect of storage), 12 extractions of the source soil were carried out following the start of the last flasks. A two-sample t-test was used to determine if there was an effect of storage, that is, if the source soil TEH changed over time. The flasks were labeled as treatment or control, 5° or 20°C, and with the sample time interval, and the replicate number. Examples include T5-D1-R1 and C20-D121-R4, that are 5°C treatment flask, day one, replicate 1 and 20° control flask, day 121, replicate four respectively.

The extent of TEH disappearance (for the controls and treatments) was assessed as a percentage of loss of the initial slurry TEH concentration. Linear regression analysis and multiple regression analysis were performed on the TEH degradation data using Microsoft Excel software. Of the 256 TEH degradation data points, 4 were considered outliers and removed because their values were too high due to the presence of water in the extract. The statistical program, Systat, was used to fit the treatment degradation data and the GC fraction C_{16}-C_{24} data to the following two-phase first order theoretical model using nonlinear regression analysis.

$$TEH = Ae^{k1t} + Be^{k2t}$$

Where TEH is the total extractable hydrocarbons (mg/kg), A is the initial mass of rapidly degraded hydrocarbons (mg/kg), B is the initial mass of slowly degraded hydrocarbons, t is time (days), k_1 is the degradation rate constant for the readily degradable hydrocarbon fraction (day^{-1}), k_2 is the degradation rate constant for the slowly degradable hydrocarbon fraction (day^{-1}). The use of this model was based on the assumption that the degradation rate of hydrocarbons is positively correlated with the hydrocarbon pool size in the soil.

The mathematical basis for the above model is that the degradation curve can be fit to a first order degradation model that consist of an infinite number of stages described by the following equation.

$$TEH = Ae^{k1t} + Be^{k2t} + Ce^{k3t} + De^{k4t}....$$

Two stages were chosen because they were found to sufficiently describe the degradation curve therefore, the terms in addition to the ones described by the theoretical model are considered to be not significantly different from zero.

3.3.3 Analysis

At each time interval, all 14 flasks (days 3, 7, 14, 30, 60, 90) or 5 flasks (days 1, 5, 10, 21, 45, 75) were completely sacrificed and the entire 20g soil sample Soxhlet extracted with DCM. This allowed the degradation to be assessed by monitoring the changes in gravimetric DCM extractable TEH over time. The sample extract was then redissolved in CS_2 for GC analysis. CS_2 was chosen as a solvent for GC analysis instead of DCM because it does not result in large tailing solvent peak and solubilizes more hydrocarbons. Details of the procedure for sacrificing of slurries and GC analysis follow in sections 3.5 and 3.8 respectively.

Microbial community sizes were estimated using a 96-well most-probable-number (MPN) method. This method is described in detail in section 3.7. For each treatment temperature at every time interval, total heterotroph population size and oil-degrader population size was estimated for incubation growth at 5°C and 20°C. Incubation at 5°C gives an indication of the combined number of psychrotrophs and psychrophiles. Incubation at 20°C gives an indication of the number of mesophiles and psychrotrophs. Three replicates for each of the 13 time intervals listed above were determined to give a description of microbial growth. These replicates were averaged and plotted over time. One control flask from each time interval was monitored for microbial growth to be sure the controls remained abiotic.

Throughout the duration of the biodegradation run, each flask was compensated for water loss by evaporation by the addition of the nutrient solution (Appendix A). During the trial described in section 4.8, it was determined that 2mL of solution per week would maintain the slurry with a soil to water ratio of 1:3 (dry-wt/wt). The pH of the slurry was monitored, using a digital pH/mV meter described in section 4.2.1, at time intervals greater than those monitored during the trial run to ensure an optimal pH was maintained. Nutrients were monitored when possible at periodic intervals by ion chromatography described in section 4.6.

3.4 REPRODUCIBILITY TEST

The goal of this experiment was to detect a statistically significant difference in the rate and endpoint of hydrocarbon degradation between 5°C and 20°C. A statistically significant difference occurs when the signal caused by the effect of temperature is greater than the noise due to experimental error. The accuracy to which DCM extractable TEH can be determined is limited by the variation between random samples. Three sources of variation were identified in this experiment. 1) Variation due to soil sampling that is affected by soil homogenization following acquisition from the field. 2) Variation due to analytical procedures such as Soxhlet extraction that is affected by centrifugation, transferring of sample, grinding, adsorption to laboratory equipment, extraction time etc. and 3) Variation due to temperature treatment.

To determine the combined variation caused by soil sampling and analytical procedures, a sample reproducibility test was conducted. During this test, approximately 20g soil samples were added to the flasks with approximately 60mL of distilled water. The 5 replicate flasks were mixed for 4 hours and then centrifuged and ground and oven dried as described below in section 4.5. A two-sample t-test was performed to determine if there is a significant difference between these slurry samples and the initial DCM extractable TEH. This reproducibility test gives the percent recovery of TEH from the slurry assuming that no hydrocarbons were lost during the 4 hours of mixing.

Because the control soils were autoclaved at 121°C prior to being added to the slurry, a test was run to determine if autoclaving the soil would affect the DCM extractable TEH. Three samples of soil were taken and autoclaved for 1 hour at 121°C and then extracted as described above in section 4.2.5. These results were then compared with the initial DCM extractable TEH using a two-sample t-test.

3.5 SACRIFICING OF SLURRIES

At each time interval, once mixing was complete, the flasks were allowed to settle for approximately 10 minutes and a 1mL sample was taken from three treatment slurries and one control slurry for microbial enumeration. To perform Soxhlet extraction on the soil in the slurries, all of the water must be removed. This was accomplished by transferring the slurries into a 100mL stainless steel centrifuge tube using approximately 25mL of distilled water to rinse the flask. Prior to transfer the weight of the empty centrifuge tube was recorded. The samples were then centrifuged for 60 minutes at a relative centrifugal force of approximately 14,200 x *g*. The supernatant was gently poured from the centrifuge tube into a beaker for nutrient analysis. The soil sample remaining in the tube was oven dried for 24 hours at 60°C and the weight of the centrifuge tube and oven-dry sample was recorded. The oven-dried sample was then transferred into a mortar using a stainless steel spatula and the sample was ground and homogenized with anhydrous sodium sulfate. The remaining extraction process is as described in section 3.2.5.

3.6 NUTRIENT ANALYSIS

Cations, including sodium, ammonium, magnesium, calcium and potassium, and anions, including nitrate, chloride, phosphate, and sulphate, were analyzed using ion-exchange chromatrography (IC). A Dionex 2000i was used for cations with a Dionex CS15 250mm long by 4mm diameter column. The column temperature was 44°C and the sample loop was 25µL. The eluent was 20mMolar methanesulfonic acid (MSA) at a flow rate of 0.6mL/min. The regenerant was 50mMolar tetrabutylammoniumhydroxide (TBAOH) at a flow rate of 1.2mL/min.

For anions, a Dionex 4000I AS4A 250mm long by 4mm diameter column was used. The column temperature was ambient and the sample loop was 25µL. The eluent was 1.8mM sodium carbonate plus 1.7mM sodium bicarbonate at a flow rate of 2mL/min. The regenerant was 25mM sulfuric acid with a flow rate of 4mL. Both ICs were equipped with guard columns and operated in chemical suppression mode. As

mentioned above, approximately 25mL of distilled water was used during the transfer of the slurry from the flask to the centrifuge tube. This amount had to be varied depending on the consistency of the sample. As a result, the nutrient values obtained are not exact and should only be used as estimates.

During the course of the experimental run, nutrient concentrations had to be increased to maintain excess concentrations. The time at which the concentration of nitrate went below the acceptable level of 100mg/L differed for the two treatments. Two mL of ammonium nitrate solution at a concentration of approximately 5700mg/L was added to the 20°C treatment on day 18 and was added to the 5°C treatment on day 90. This increased the concentration of the solution by approximately 150mg/L. To be sure the phosphorus was available, 2mL of a 500mg/L phosphorus solution was added on days 12, 21, 33, 46, 66, and 105 to both the 5°C treatment and the 20°C treatment. This increased the phosphorus concentration at each addition by approximately 15mg/L.

3.7 MICROBIAL ENUMERATION - MPN

Throughout the degradation experiment the heterotrophic and oil-degrading microbial populations were measured using the 96-well plate most-probable-number procedure described by Haines *et al.* (1996). To enumerate the heterotrophs present in the slurry, 180µL of $1/10^{th}$ strength tryptic soy broth was added to 11 of the 12 rows in 96-well microtiter plates using an 8-channel pipette. The first row was left empty and 20μL of undiluted slurry sample was added to the second row. The method suggests adding 200µL of sample to the first row, however the thickness of the slurry samples made adding 20μL directly to the second row more efficient. The contents of the second row were then mixed using the pipette and 20μL was transferred from the second row to the third row and mixed. This process was continued for all rows except the last, which was used as a sterile control. A new set of pipette tips were used for each transfer and mixing involved sucking the entire contents of the wells into the tips and replacing it into the well 3 to 4 times. After the ten-fold dilutions were complete, the lid was placed on the plate and the plate was sealed along the edges with parafilm. The plates were allowed

to incubate for 7 days at room temperature or in a fridge at 5°C. Once incubation was complete, 50µl of 3g/L of filter sterilized iodonitrotetrazolium violet (INT) indicator solution was added to each well starting from row 12 and working backwards, down in dilution, using the same pipette tips. The indicator was allowed to sit for 24 hours after which time the wells were scored. Wells containing a red or pink precipitate were scored positive and the number of positive wells in each row was recorded.

The oil-degraders were enumerated as described above for the heterotrophs with the following differences. 180µL of Bushnell-Haas broth was added instead of tryptic soy broth. Following the dilution of the samples, 2µL of filter sterilized diesel fuel was added to each of the wells individually using a single-channel pipette. Diesel fuel was used as a carbon source even though crude oil is the carbon source in the experimental slurries because Haines *et al.*(1996) found that crude oil underestimated the hydrocarbon degraders present. The oil-degrading plates were incubated for 14 days instead of 7 days and then scored the same as described above.

Once the scores of the plates were determined, the data was input into a computer program described by Klee (1993)[1] to obtain estimates of MPN. The three replicate MPN values were averaged and plotted over time.

3.8 GC ANALYSIS

Samples from the 5°C and the 20°C treatments were analysis by a Varian 3800 GC using a Rtx-2887 column that was coated with 100% dimethyl polysiloxane. The column was 10 meters long with an inside diameter of 0.53mm and 2.65µm film thickness. The method used was a modification of ASTM method D 2887 (ASTM 1984). Hewlett Packard boiling point calibration standard was used. The boiling point calibration standard included the following components (% by weight): n-pentane (8.32),

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[1] Obtained from the US EPA

n-hexane (4.38), n-heptane (4.55), n-octane (4.67), n-nonane (4.77), n-decane (9.71), n-undecane (4.92), dodecane (19.91), n-tetradecane (10.14), n-pentadecane (5.11), n-hexadecane (10.28), n-heptadecane (5.17), n-octadecane (2.21), n-eicosane (1.30), n-tetracosane (0.90), n-octacosane (0.90), n-hexatriacontane (0.91), n-tetracontane (0.92), and n-dotriacontane (0.90). Sample injection size is 1.0μL. Helium was used as a carrier gas at a constant flow rate of 20.0mL/min. The injector temperature is held at 40°C for 0.10min then increased to 400°C at a rate of 200°C/min and held for 5.00min and then decreased to 350°C at a rate of 100°C/min. The oven temperature was at 40°C for 1.00min and increased to 360°C at a rate of 35°C/min. The FID temperature was 360°C.

The hydrocarbon sample was split into carbon fractions that included C_{10}-C_{16}, C_{16}-C_{24}, C_{24}-C_{36}, and C_{36}-C_{40} chosen on the basis of the new Canada-wide standards for petroleum in soil (CCME 2000). These fractions were identified in the GC method by boiling point temperatures that correspond to the carbon number. The cumulative area under the chromatogram for each fraction was calculated and the resulting percentage of total sample was recorded. Three replicate treatment samples for each time interval at 5°C and 20°C were analyzed and the resulting carbon fraction percentages were plotted versus time. Regression analysis was performed on each carbon fraction over time using Excel. This method allowed for a visual qualitative comparison of the GC fingerprints.

A modification of the above method was used to determine the percentage of the sample that was residual. The residual is the portion of the crude oil sample that remains on the column. Carbon compounds with greater than C_{40} atoms are considered residual because the highest standard used was C_{40}. Above C_{40} the peaks are not integrated and become less resolved. This fraction should be distinguished from the pentane insoluble fraction that does not include alkanes and is defined by the method of extraction. Approximately 0.1000g of oil extract was weighed to four decimal places and then added to a tared GC vial. Approximately 0.0100g of the internal standard (Hewlett-Packard 5080-8723) was weighed to 4 decimal places and added to the same GC vial. An additional vial containing only approximately 0.1000g of oil extract was added to a

second GC vial. The vials were then filled to the neck with carbon disulfide. Three replicates of an initial soil extract, day 121 at 5°C and day 121 at 20°C were analyzed.

3.9 TRIAL RUN

A two-week trial run was performed at 20°C to anticipate any problems, to determine the amount of water loss due to evaporation, to monitor pH, to determine if sodium azide alone would be sufficient to eliminate the bacteria, and to test liquid-liquid extractions. Four time intervals were chosen (1,3,7, and 14 days). At each interval, three replicate treatment flasks and one control flask were set up as described in section 4.3. The same methods as described for the experimental run were used in the trial run.

To determine the amount of water loss due to evaporation, the flasks, including soil, nutrient solution and foam stopper, were initially weighed. Prior to sacrifice at each time interval, the flasks were reweighed and the amount of water loss determined by subtraction. Liquid-liquid extractions were performed on the supernatant from the slurries after centrifugation. This method is described below in section 4.9. The pH was measured as described in section 4.3.3 at each time interval to be sure the degradation conditions remained optimal. Finally, microbes were enumerated at time interval 14 days using the spread plate method (described in section 4.10) to determine if the control was abiotic.

3.10 LIQUID-LIQUID EXTRACTION

During the trial run to determine if the liquid portion of the slurry contained significant amounts of hydrocarbons extractable by DCM, a liquid-liquid extraction was performed using a separatory funnel. Once the slurry sample from a flask was centrifuged, the supernatant was carefully poured into a separatory funnel. Approximately 30mL of HPLC grade DCM was added to the funnel. The mixture was shaken for approximately 2 minutes while venting the separatory funnel frequently. The mixture was then allowed to settle until a sharp separation between the water and the DCM has formed without bubbles. The DCM was then discharged through the stopcock

into a tared aluminum weigh dish and allowed to evaporate at room temperature in the fumehood. The amount of DCM extractable material in the aqueous phase was then determined gravimetrically. The weight of the TEH extractable material from the aqueous phase was added to the weight of the TEH extractable material from the soil to determine if the aqueous phase material was significant.

3.11 MICROBIAL ENUMERATION – SPREAD PLATE

Total heterotrophs were determined for the control flasks during the trial run using a modification the spread-plate method described in Zuberer (1994). One-tenth strength Tryptic Soy Agar media was prepared by autoclaving 1.5g of tryptic soy both and 7.5g agar in 1L of distilled deionized water. Aseptic technique was used to pour 15 plates for each sample. The plates were allowed to cool overnight and then labeled with sample identification and dilution factor.

Phosphate buffered saline solution was used as a diluent and was prepared by adding 8.5g of NaCl, 0.3g of KH_2PO_4, and 0.6g of Na_2HPO_4 to one litre of deionized distilled water. Nine mL of the diluent was added to 8, 10mL test tubes and the tubes were autoclaved for 25min at 121°C. A sterile pipet tip was used to transfer 1mL of slurry sample to the first of the 8 test tubes, tube 1, and vortexed for 10 seconds resulting in a 10^{-1} dilution. After tube 1 had been vortexed, a sterile 1mL pipet was used to transfer 1 mL of solution from tube 1 to tube 2 that was then vortexed resulting in a 10^{-2} dilution. This technique was continued for subsequent test tubes up to a 10^{-8} dilution.

Three replicate plates for 5 of 8 dilutions were plated by using a sterile pipet tip to transfer 0.1mL from the dilution test tube to the plate. The dilutions used were 10^{-4} to 10^{-8}. A flame-sterilized bent glass rod was used to spread the aliquot evenly across the surface of the agar. The plates were allowed to dry, sealed with parafilm to prevent moisture loss, then incubated at room temperature for 7 days. Following incubation, all colonies on plates containing 30 to 300 colonies were counted and recorded. The average colony forming units (CFU) per mL of slurry was determined by multiplying the average

plate count from the three replicate plates by the dilution factor.

4.0 RESULTS

4.1 SOURCE SOIL CHARACTERIZATION

4.1.1 Initial Soil pH

All three replicates for pH_w and pH_{CaCl2} were the same. The pH_w was 7.2 and the pH_{CaCl2} was 6.6.

4.1.2 Initial Moisture Content

The initial moisture content of the source soil at the beginning of the experimental set up was 0.0522g/g ± a standard error of 0.0003g/g. The initial moisture content of the soil at the end of the experimental set up was 0.0434g/g ± a standard error of 0.0004g/g. These data can be found in Table B-1 of Appendix B. This change in moisture content should not affect the TEH results from the slurries because the samples were oven-dried prior to extraction.

4.1.3 Particle Size Distribution

Figure 4.1 shows the particle size distribution of the contaminated source soil. The soil contains 3% clay, 25% silt and 72% sand and the United States Department of Agriculture (USDA) texture classification is a sandy loam, which is considered a coarse textured soil. These data can be found in Table B-2 and B-3 in Appendix B.

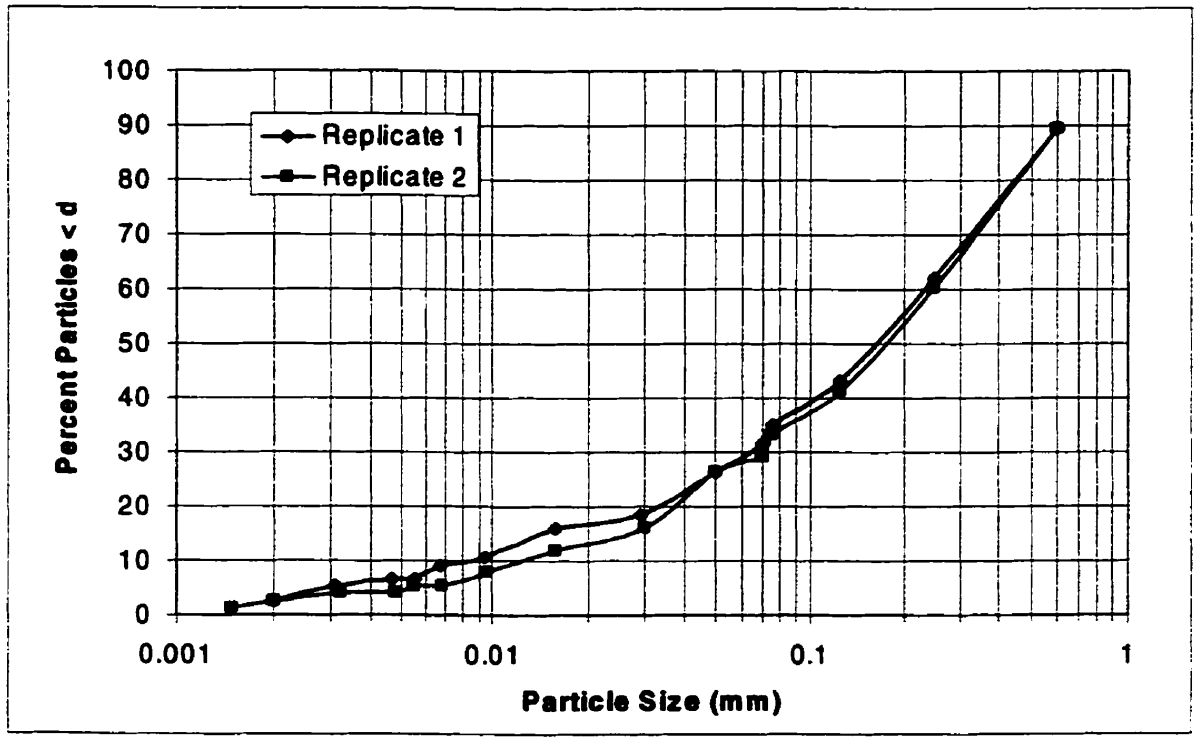


Figure 4-1: Particle size distribution of source soil

4.1.4 Organic Matter Content

The organic matter content of the oil-contaminated soil was 60500mg/kg ± 300mg/kg. The organic matter content of the extracted soil was 30800mg/kg ± 300mg/kg. These data can be found in Table B-4 of Appendix B. The natural organic matter content of the extracted soil is more representative than the oil-contaminated soil because at 400°C hydrocarbon compounds will be lost resulting in a higher organic matter content.

4.1.5 DCM Extractable TEH

The initial hydrocarbon content of the soil at the beginning of the experimental set up was 43440mg/kg ± 70mg/kg. The initial hydrocarbon content of the soil at the end of the experimental set up was 42500mg/kg ± a standard error of 100mg/kg. There was a significant difference between the mean of the initial hydrocarbon content of the soil at the beginning of the experimental setup and the mean of initial hydrocarbon content at the end of the experimental setup (two sample t-test, $p<0.05$, $df = 22$). The difference in TEH between the beginning of the set up and the end of the set up is 980mg/kg ± 320mg/kg. It is most likely that aeration from mixing of the soil prior to set up and during set up allowed for a small amount of biodegradation. This difference will not affect the results because the TEH in the soil is much larger than the difference. These data can be found in Table B-5 of Appendix B.

4.1.6 Pentane Insolubles

The pentane insoluble material was found to be 6.5% ± a standard error of 0.4% of the TEH. The percent recovery was 98.8%. These data can be found in Table B-6 of Appendix B. A summary of the initial soil characteristics can be found in Table 4.1

Table 4-1: Summary of Source Soil Characterization

pH_w		7.2
pH_{CaCl2}		6.6
Moisture Content		0.05g/g
Particle Size Distribution	Clay	3%
	Silt	25%
	Sand	72%
Organic Matter Content		30800mg/kg
Initial TEH		43440mg/kg
Pentane Insolubles		6.5% of TEH

4.2 REPRODUCIBILITY TESTS

The average DCM extractable TEH from the slurry reproducibility test was 40600mg/kg ± 200mg/kg standard error. There is a significant difference between the initial TEH of the source soil and the initial slurry TEH (two-sample t-test, $p<0.05$, df=15). The difference between the initial TEH of the soil and the initial TEH of the slurry is 2800mg/kg ± 400mg/kg. This difference will affect all the values of TEH of the treatment and control slurries by a constant amount and therefore will not change the results of the analysis. These data can be found in Table B-7 of Appendix B.

The average DCM extractable TEH from the autoclaved initial source soil was 44100mg/kg ± 300mg/kg standard error. When all three values of TEH are included in the analysis, there is a significant difference between the initial TEH of the source soil and the autoclaved initial TEH of the source soil (two-sample t-test, $p<0.05$, df=13). However, when the third value is removed, because it contained water, there is no significant difference between the initial TEH of the source soil and the autoclaved initial TEH of the source soil (two-sample t-test, $p>0.05$, df=12). These data can be found in Table B-8 of Appendix B.

4.3 TRIAL RUN

The average water loss from the soil slurries per week was 2mL. These data can be found in Table B-9 of Appendix B. Sample TT20-D14-R1 (TT is Trial Treatment) shows excessive water loss because dissolved oxygen uptake rate, using an oxygen probe, was attempted and failed on the sample causing loss of soil and water. This value was not used during the average calculation for that time interval. A standard 2mL per week was determined to be an appropriate compensation for water loss to maintain the slurry concentration. If all hydrocarbons present in the slurry were to degrade, a maximum of approximately 0.8g of hydrocarbon weight could be lost throughout the duration of the treatment. It is unlikely this amount will be lost in one week and the weight difference due to degradation is not considered to significantly alter the soil to water ratio in the slurry.

The average mass of extractable hydrocarbons in the aqueous phase of the slurry is 2.5mg. These data can be found in Table B-10 of Appendix B. The aqueous phase TEH added to the solid phase TEH does not significantly alter the total DCM extractable TEH, therefore liquid-liquid extractions were not done for the experimental run. The average pH values at time intervals 1, 3, 7, and 14 days are 7.6, 7.0, 7.3, and 7.2 respectively. These values show that the pH is within an acceptable range during degradation. At 14 days the control flask contained 2.4 x 10^6 CFU per mL of slurry. This concentration is high enough to affect the rate of degradation within the control flasks. Therefore, the soil in the control flasks for the experimental runs was autoclaved prior to being added to the slurry.

4.4 TREATMENT DEGRADATION

During the biodegradation treatments at both temperatures, the pH values remained between 6.9 and 7.6. Nitrate levels stayed within the acceptable range of 100 to 300mg/L. The nutrient data can be found in Table B-11 of Appendix B.

Figure 4-2 and 4-3 show the remaining TEH over time in the treatment and control flasks at 5°C and 20°C respectively. These results show that there was a significantly greater decrease in the treatment flasks than there was in the control flasks. Based on the initial slurry TEH value of 40600mg/kg described in section 4.2, there was an abiotic loss of 4.0% in the 5°C control flasks in 121 days (final value of 39000 mg/kg). There was an abiotic loss of 4.7% in the 20°C control flasks in 121 days (final value of 38700 mg/kg). There was no significant difference between the rate of abiotic loss at 5°C and the rate of abiotic loss at 20°C (Multiple Regression Analysis, $p>0.05$, $df=76$). The rate of abiotic loss at 5°C and 20°C determined from regression analysis was 17.2 mg/kg/day. Based on the initial slurry TEH value of 40600mg/kg, the 5°C treatment flasks showed a 19.0% decrease in 121 days (final value of 32900 mg/kg). The 20°C treatment flasks showed a 44.3% decrease in 121 days (final value of 22600 mg/kg). The control and treatment data can be found in Table B-12 and B-13 of Appendix B.

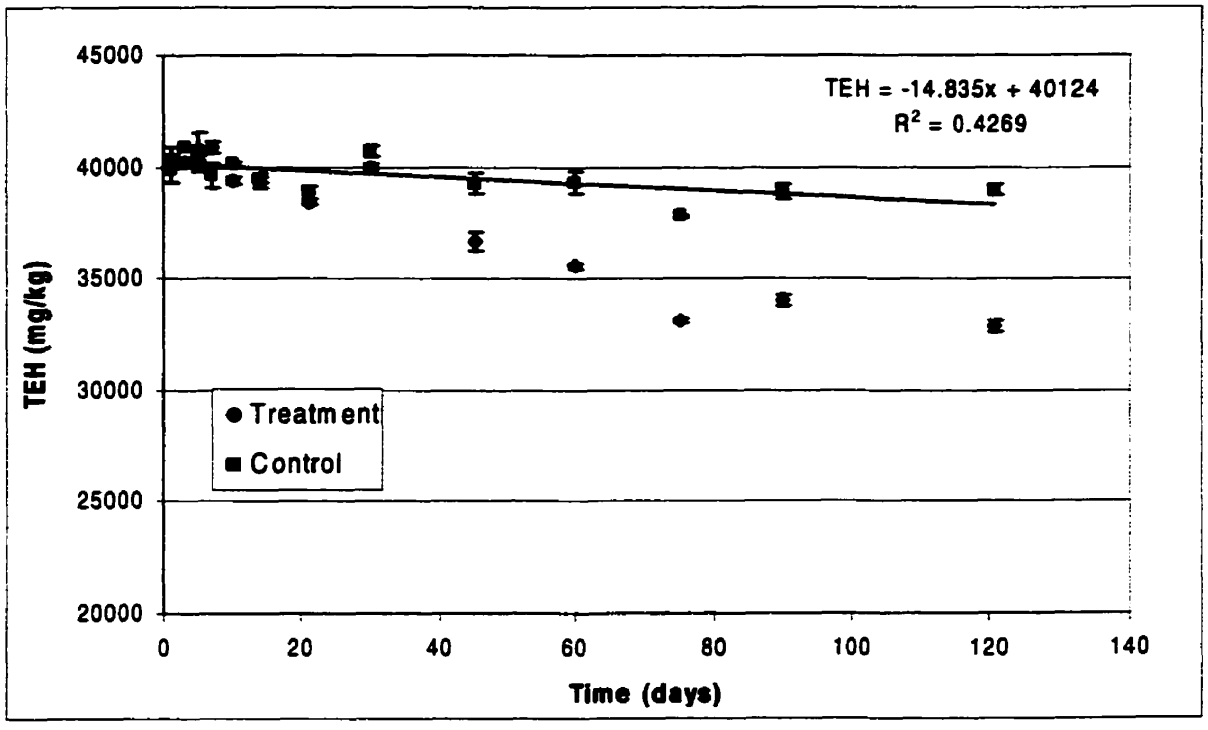


Figure 4-2: TEH remaining over time in control and treatment at 5°C including standard error bars and linear regression equation for the abiotic control

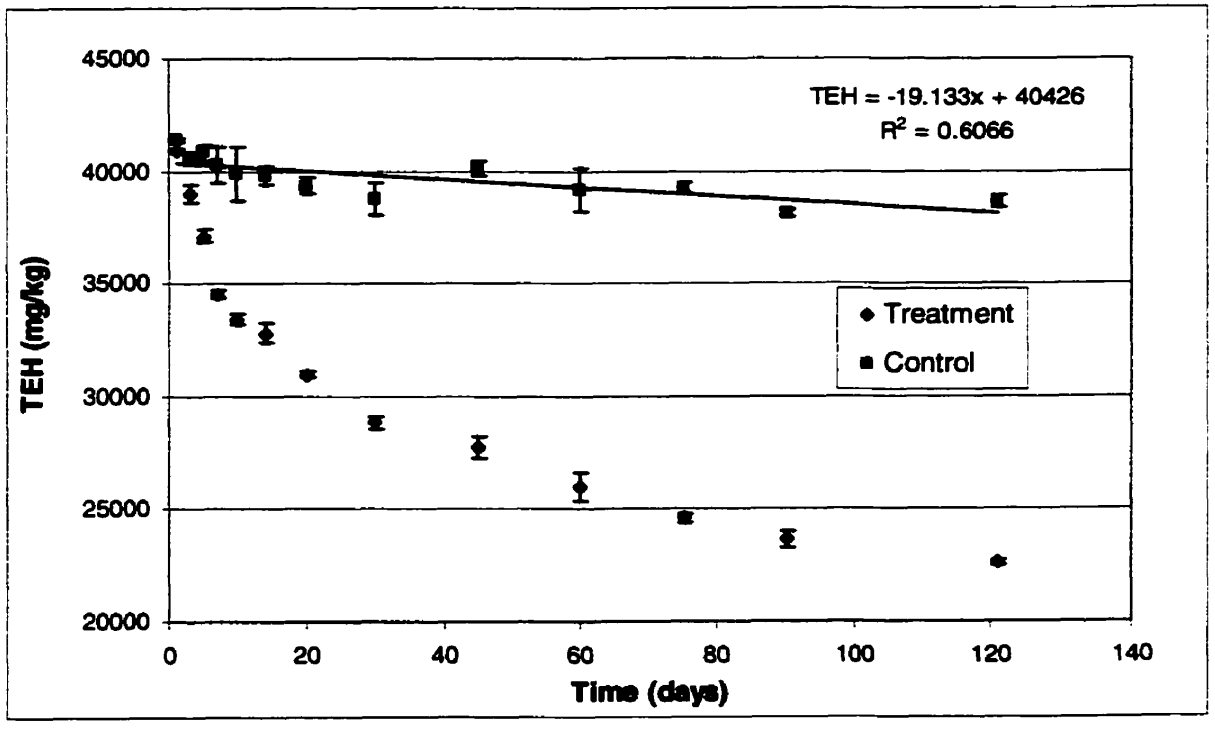


Figure 4-3: TEH remaining over time in control and treatment at 20°C including standard error bars and linear regression equation for the abiotic control

Figure 4-4 shows the TEH remaining over time in the 5°C and the 20°C treatments. There is no significant difference between the rate of biodegradation at 5°C and the rate of biodegradation at 20°C after 21 days (Multiple Regression Analysis, $p>0.05$, $df=132$). Day 21 was chosen as the start of the 20°C regression analysis because it was the time interval that produced the best-fit regression at 20°C. Day 21 is somewhat arbitrary and therefore it may not be the exact day during which the rates become the same. The rate of TEH loss in the 5°C treatment flasks throughout the experimental duration, and determined from linear regression analysis, was 69.2 mg/kg/day. The rate of TEH loss in the 20°C treatment flasks after 21 days is the same (69.2 mg/kg/day). The rate of abiotic loss is subtracted from the rate of TEH loss in the treatments to determine a rate of biodegradation that is 52.0 mg/kg/day. In summary, the rate of biodegradation at 5°C and the rate of biodegradation at 20°C after 21 days is 52.0 mg/kg/day. After the initial phase of biodegradation, the rate of biodegradation is independent of temperature.

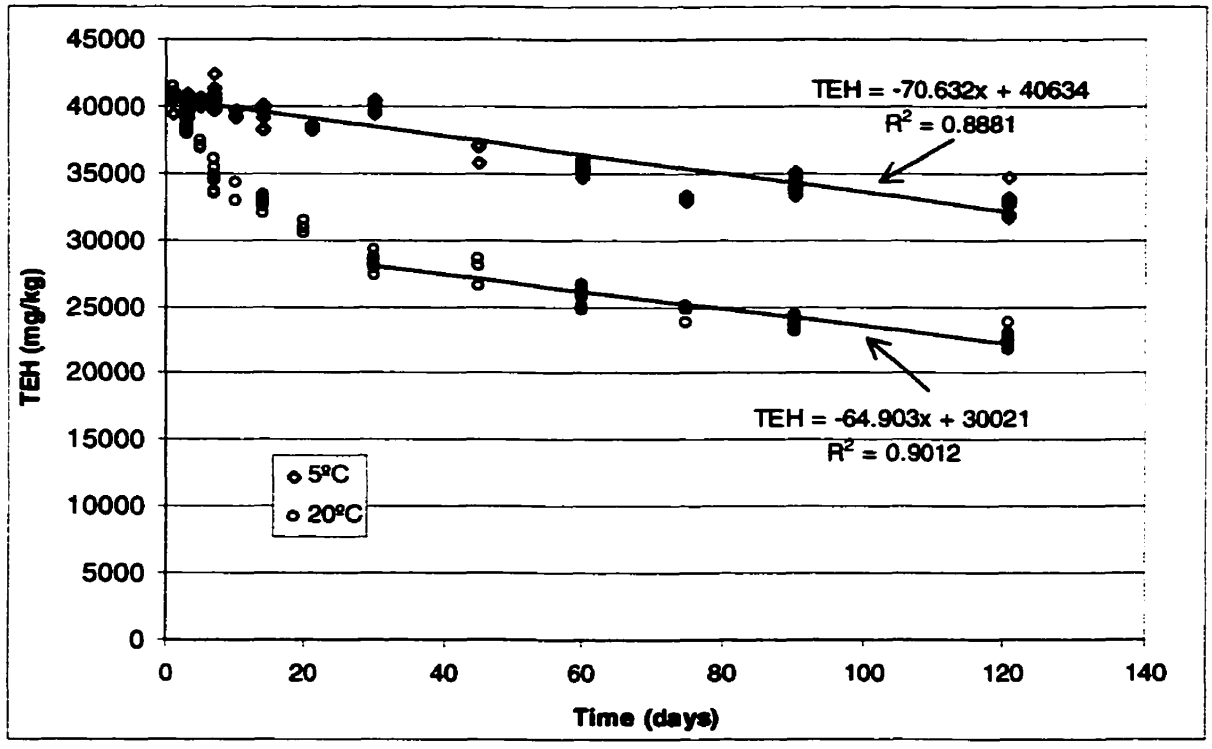


Figure 4-4: TEH remaining over time at 5°C and 20°C including linear regression equations

The treatment degradation data along with the model fit is presented in figure 4-5. The results from fitting the 5°C treatment degradation data to the model described in section 3.3.2 are presented in Table 4-2. A single-stage first-order kinetics model describes the decrease in treatment TEH at 5°C. At 5°C the degradation can not be separated into two stages. The rate constant for treatment degradation at 5°C is between –0.0021/d and –0.0018/d. The results from fitting the 20°C treatment degradation data to the model described in section 3.3.2 are presented in Table 4-3.

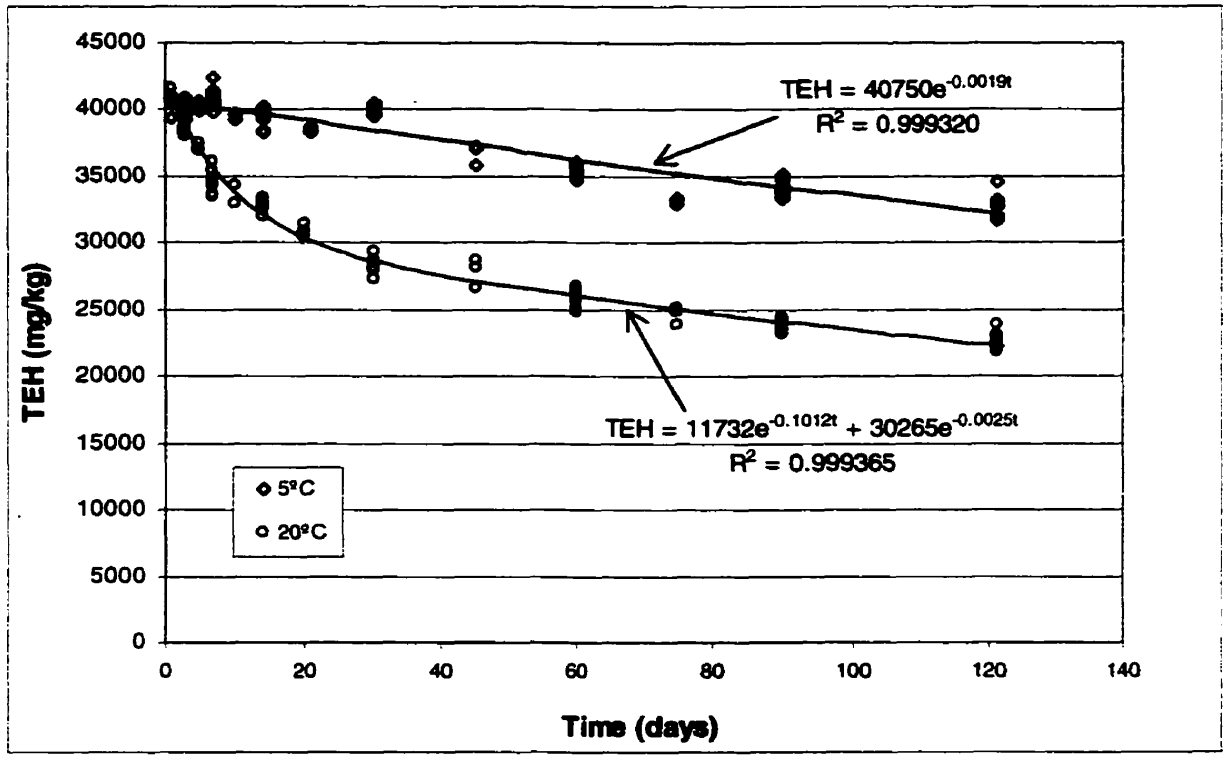


Figure 4-5: TEH remaining over time for the 5°C and 20°C treatments including nonlinear regression models

Table 4-2: Nonlinear Regression Results for 5°C Treatment Degradation Data

Parameter	Estimate	Asymptotic Standard Error (ASE)	Parameter/ ASE	Lower 95% Confidence Interval	Upper 95% Confidence Interval
B	40750mg/kg	159mg/kg	255	40433mg/kg	41067mg/kg
k_2	-0.0019/d	0.000075/d	-25	-0.0021/d	-0.0018/d

Table 4-3: Nonlinear Regression Results for 20°C Treatment Degradation Data

Parameter	Estimate	Asymptotic Standard Error (ASE)	Parameter/ ASE	Lower 95% Confidence Interval	Upper 95% Confidence Interval
A	11731mg/kg	484mg/kg	24	10768mg/kg	12695mg/kg
k_1	-0.101/d	0.0097/d	-10	-0.1205/d	-0.0818/d
B	30265mg/kg	469mg/kg	64	29331mg/kg	31199mg/kg
k_2	-0.0025/d	0.00017/d	-13	-0.0029/d	-0.0022/d

A two-stage first-order kinetics model describes the decrease in treatment TEH at 20°C. There is between 10768 and 12695mg/kg of rapidly degraded hydrocarbons and between 29331 and 31199mg/kg of slowly degraded hydrocarbons at 20°C. The rate constant for degradation of the rapidly degraded hydrocarbons in the treatment slurries at 20°C is between –0.1205/d and 0.0818/d. The rate constant for degradation of the slowly degraded hydrocarbons at 20°C is between –0.0029/d and –0.0022/d.

Because the rate constants for the 5°C and 20°C are not the same rate constants they cannot be compared to each other. However, to show how the effect of temperature on the rate of degradation changes with time, Figure 4.6 shows the first derivative of the degradation model curves at 5°C and 20°C. This figure shows that at approximately 42 days the rate of change of TEH degradation at 5°C and 20°C is the same.

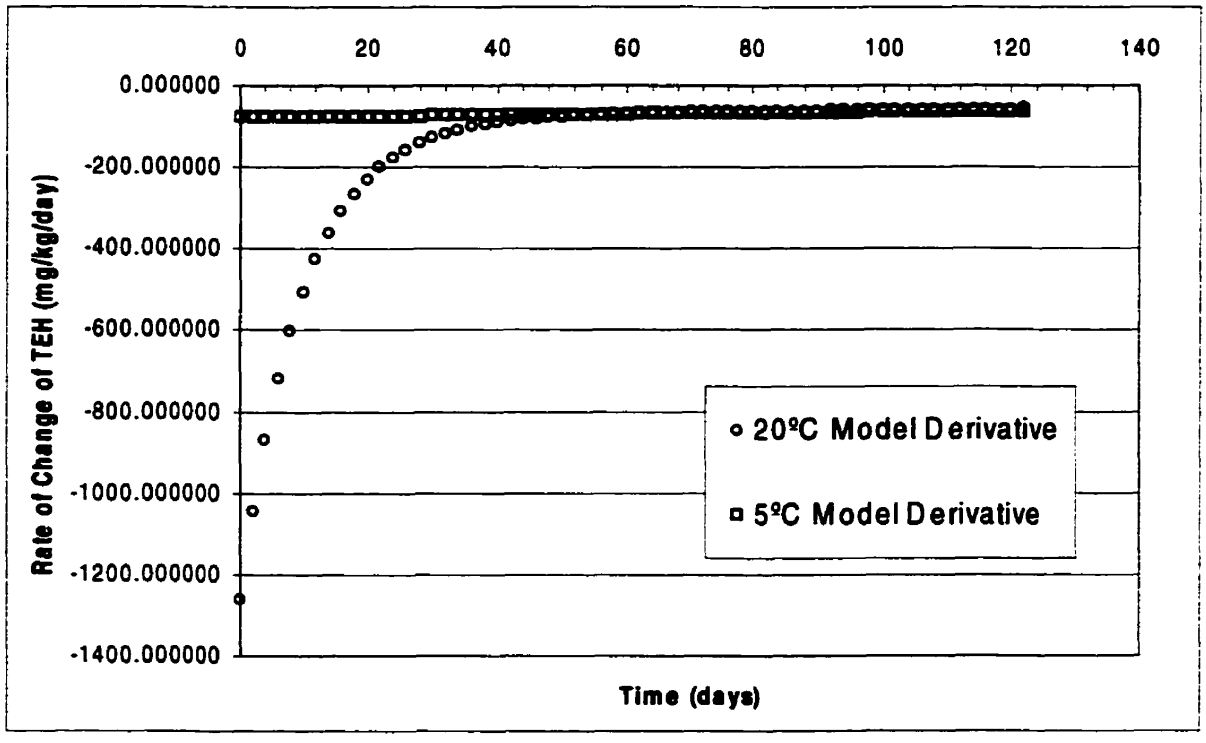
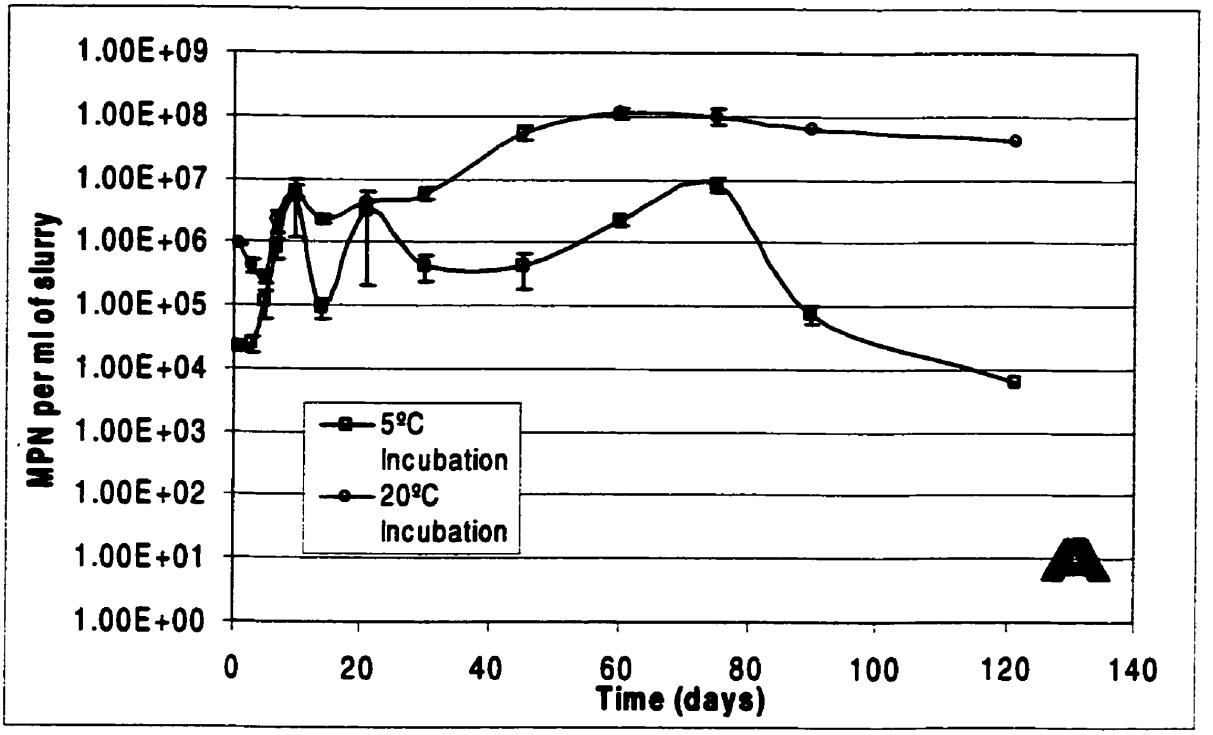


Figure 4-6: Rate of change of TEH over time for 5°C and 20°C treatment

4.5 MICROBIAL ENUMERATION

Within the wells of the 96-well plates of the MPN determination for the control flasks there was no formation of pink precipitate in both the 5°C and the 20°C treatments. This indicates that there are no measurable numbers of bacteria in the control flasks of the treatments.

Figures 4-7 and 4-8 show the MPN of heterotrophs and oil-degraders incubated at 5°C and 20°C for the 5°C treatment and the 20°C treatment. In general, the microbial enumeration data shows that nutrients were not limiting biodegradation throughout the duration of the experiment because there was no spiking of microbial numbers corresponding to the time when nutrients were added. The microbial data shows that there was little microbial population growth occurring throughout the duration of the experiment because there is no noticeable initial increase in MPN showing exponential growth. Figure 4-8 (B) shows that the 5°C and 20°C incubated oil-degraders in the 20°C treatment decrease in MPN starting at day 21. The MPN data can be found in Tables B-14 to B-17 of Appendix B.



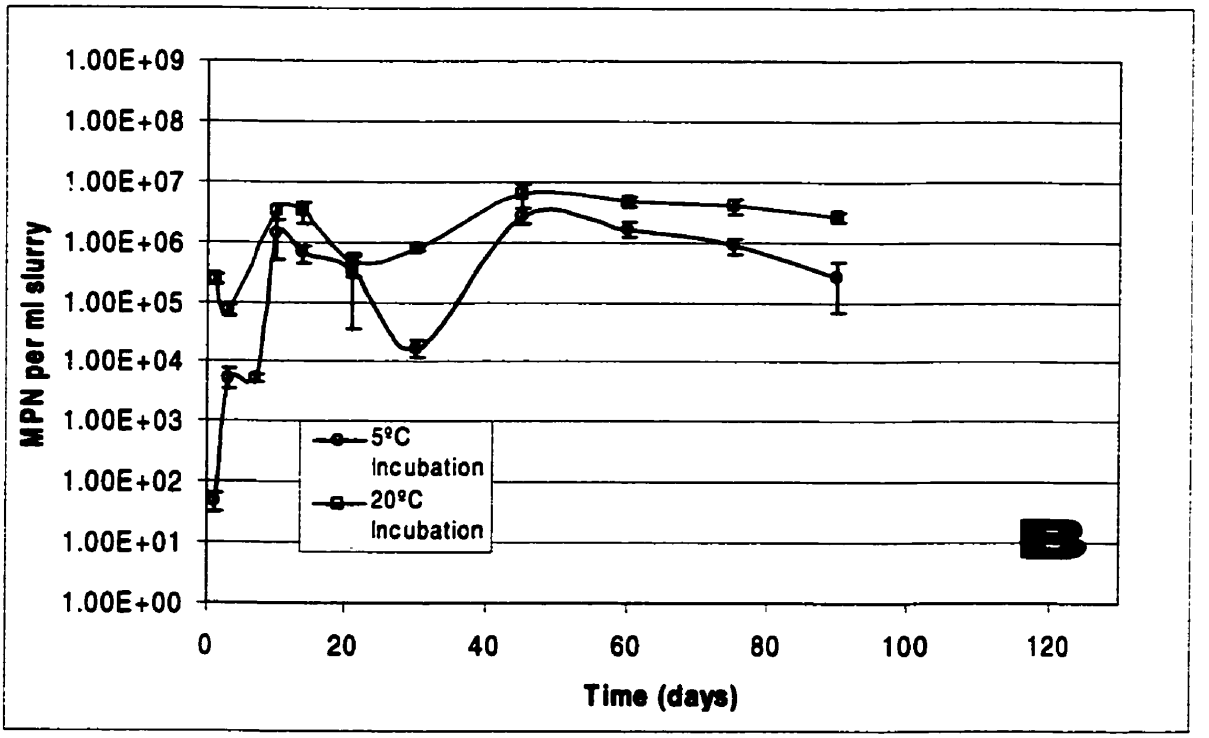


Figure 4-7: A) Average heterotroph MPN for 5°C treatment including standard error (n=3) B) Average oil degraders MPN for 5°C treatment including standard error (n=3)

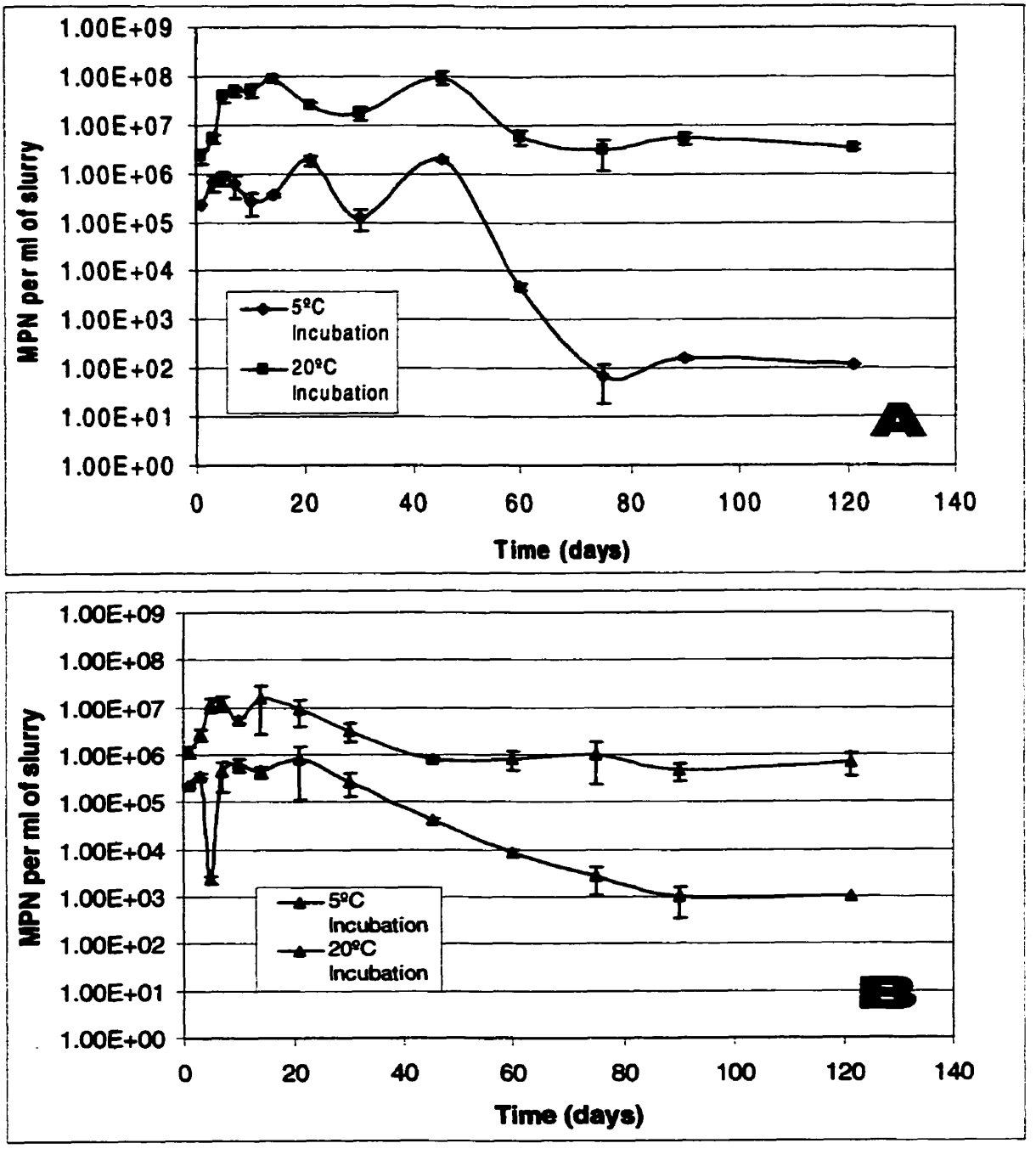


Figure 4-8: A) Average heterotroph MPN for 20°C treatment including standard error (n=3) B) Average oil degraders MPN for 20°C treatment including standard error (n=3)

4.6 GC ANALYSIS

Figures 4-9 show the change in percentage of carbon fractions in the 5°C and 20°C treatment flasks. It should be noted that the data presented are not absolute values but relative values. The GC fraction data show that the fraction most acted upon at both 5°C and 20°C is C_{16}-C_{24}. There was no significant change over time in the C_{24}-C_{36} fraction and the C_{36}-C_{40} fraction (Linear Regression, $p>0.05$, df=75). There was a significant change in C_{10}-C_{16} fraction (Linear Regression, $p<0.05$, df=75). However a very small portion of this fraction remains in the extract and this fraction is greatly influenced by volatilization and will not be considered with respect to the rate of biodegradation. The results of fitting the C_{16}-C_{24} fraction data at 5°C and 20°C to the model described in section 3.2.2 are presented in table 4.4 and 4.5 respectively. Table 4-6 presents a summary of the nonlinear models for the treatment degradation data at 5°C and 20°C and the GC fraction C_{16}-C_{24} at 5°C and 20°C.

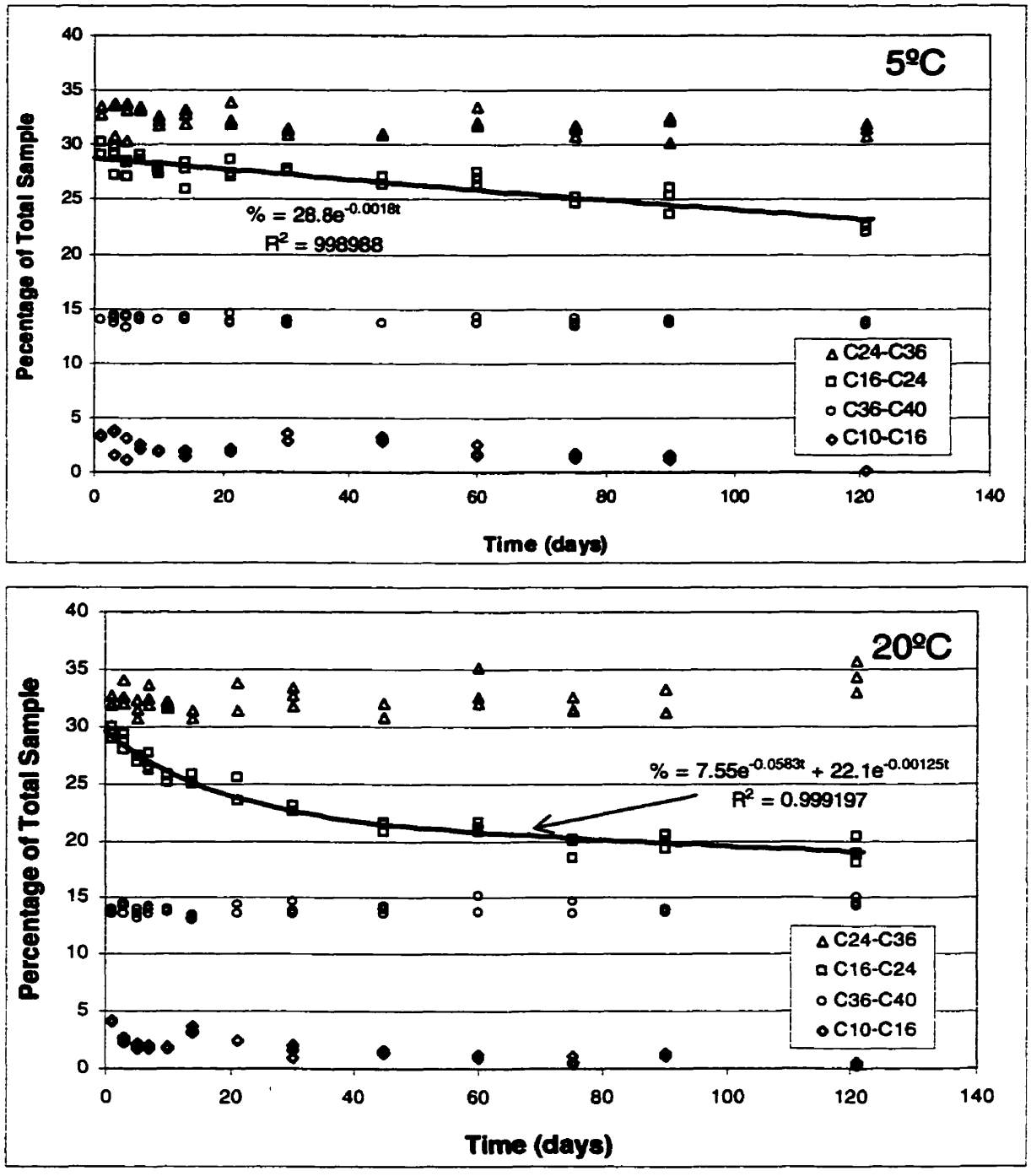


Figure 4-9: Percentage of total sample of GC fractions over time (n=3) for 5°C treatment and 20°C treatment

Table 4-4: Nonlinear Regression Results for GC Carbon Fraction C_{16}-C_{24} at 5°C

Parameter	Estimate	Asymptotic Standard Error (ASE)	Parameter/ ASE	Lower 95% Confidence Interval	Upper 95% Confidence Interval
B	28.77%	0.211%	135	28.34%	29.20%
k_2	-0.0018/d	0.000153/d	-11	-0.0021/d	-0.0015/d

The decrease in percentage of the total sample of carbon fraction C_{16}-C_{24} at 5°C is described using a single-stage first-order kinetics model where the rate constant is between –0.0021/d and –0.0015/d.

Table 4-5: Nonlinear Regression Results for GC Carbon Fraction C_{16}-C_{24} at 20°C

Parameter	Estimate	Asymptotic Standard Error (ASE)	Parameter/ ASE	Lower 95% Confidence Interval	Upper 95% Confidence Interval
A	7.548160%	0.994542%	7.589580	5.527006%	9.569313%
k_1	-0.058329/d	0.014014/d	-4.162079	-0.086809/d	-0.029848/d
B	22.072846%	0.000525%	20.315708	19.864830%	24.280863%
k_2	-0.001251/d	0.000525/d	-2.382528	-0.002318/d	-0.002318/d

A two-stage first-order kinetics model describes the decrease in percentage of total sample of the carbon fraction C_{16}-C_{24} at 20°C. The rate constant for the rapidly degraded portion is between –0.086/d and –0.029/d. The rate constant for the slowly degraded portion is –0.0023/d.

Table 4-6: Summary of Degradation Models

Data	Temperature	Model
Treatment Degradation	5°C	$TEH = 40750e^{-0.0019t}$
	20°C	$TEH = 11732e^{-0.1012t} + 30265e^{-0.0025t}$
GC Carbon Fraction C_{16}-C_{24}	5°C	$\% = 28.8e^{-0.0018t}$
	20°C	$\% = 7.55e^{-0.0583t} + 22.1e^{-0.00125t}$

Figure 4-10 and 4-11 give examples of GC fingerprints. Included is day 1 and day 121 for treatment flasks at 5°C and 20°C. Noticeable aliphatic degradation occurs at C_{15} and greater. The percentage of sample that was residual in the initial oil extract was 33% ± 3% standard error. The percentage of sample that was residual at day 121 in the 5°C and 20°C was 50% ± 10% and 43% ± 4% respectively. These data show that the percentage of the sample that was residual increased throughout the duration of the experiment. Since the residual portion of the sample is most likely not biodegradable (consisting of large, complex, long chain hydrocarbon molecules), the increase in residual is consistent with the occurrence of biodegradation of the other carbon fractions. Characterization of the increase during degradation is unavailable with these data. It is inadvisable to directly compare the rate constants between the GC data and the TEH degradation data because the GC data consists of percentages where changes may have been hidden because of the absence of the residual fraction of the sample. The GC carbon fraction data can be found in Tables B-18 and B-19 of Appendix B.

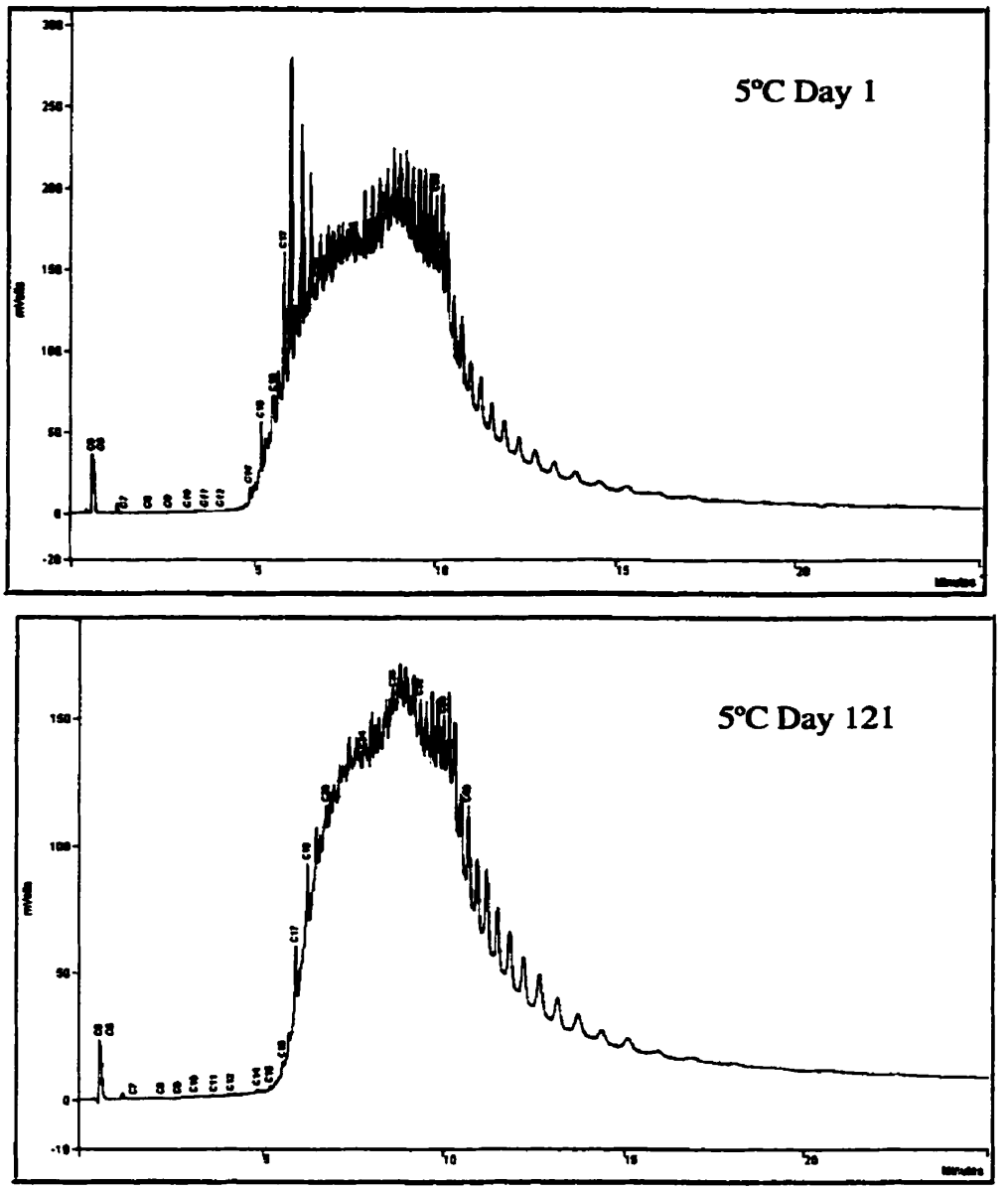
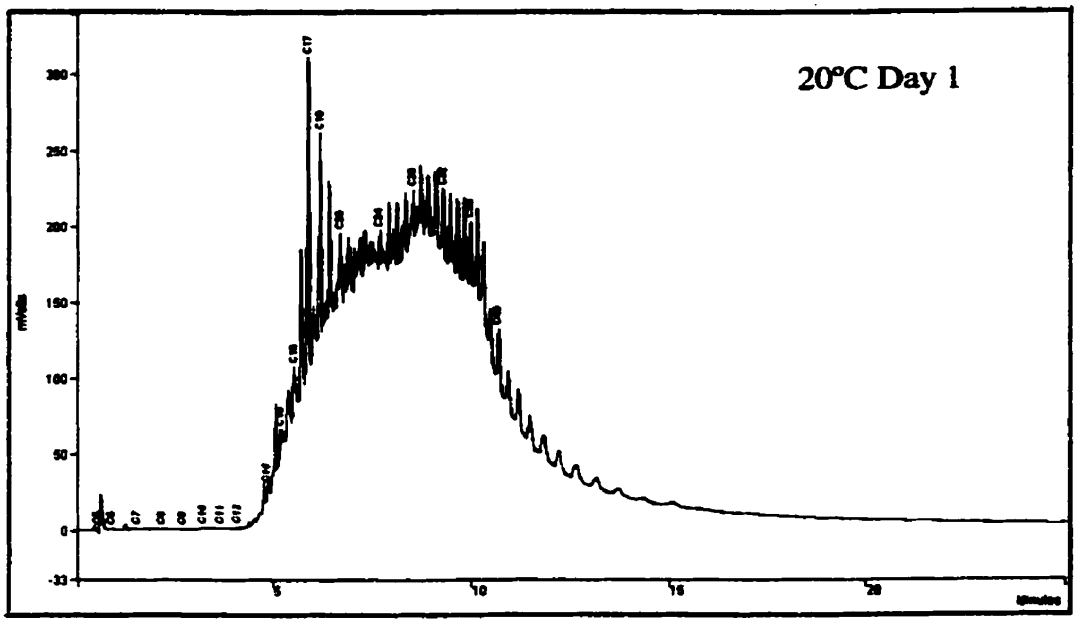


Figure 4-10: GC fingerprint of treatment at 5°C on day 1 and day 121



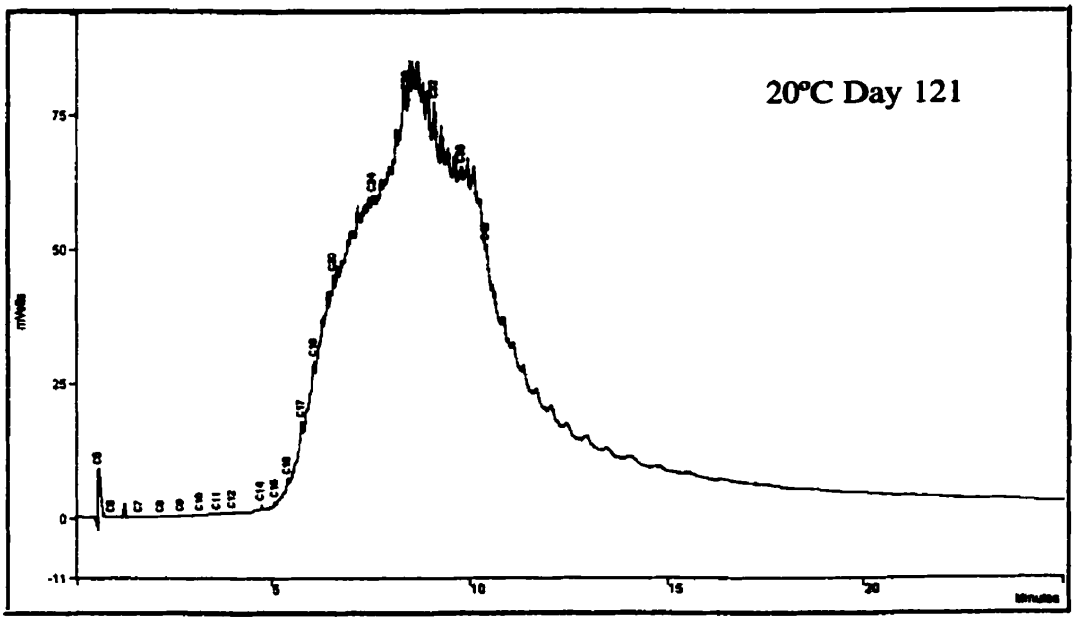


Figure 4-11: GC fingerprint of treatment at 20°C on day 1 and day 121

5.0 DISCUSSION

5.1 DISCUSSION OF RESULTS

There is no significant difference in the rate and extent of abiotic loss of TEH between 5°C and 20°C. Abiotic loss in the sterile medium was attributed to processes such as inorganic oxidation, photodegradation, sorption to glass walls of apparatus, volatilization and interaction with the soil medium. Some studies have found that abiotic loss increases with increasing temperature due to increased volatilization (Atlas 1975, Margesin and Schinner 1997b, Malina *et al.* 1999). However, these studies were conducted on freshly added diesel fuel and the abiotic loss results presented in this study are from a weathered crude oil that has less potential for volatilization (Alexander 1995). In addition, studies have found that abiotic loss has contributed little to loss of hydrocarbons within soil (Margesin and Schinner 1997a and 1997d). It is likely that the decrease in the abiotic control flasks is due to the adsorption to glass and centrifuge tubes. Over the duration of the experiment, small oil balls were formed in the slurry as time increased and this resulted and increased portion of the oil adsorbing to the lid of the centrifuge tube. This explanation is consistent with the low R^2 value for the regression analysis indicating high variability in abiotic TEH loss.

Numerous studies have shown that the extent of hydrocarbon biodegradation at colder temperatures is less than biodegradation at warmer temperatures. Mulkins-Phillips and Stewart (1974) looked at the biodegradation of Bunker C oil by mixed cultures in a mineral salts medium and found that the disappearance of oil at 15°C was between 41-85% and the disappearance of oil at 5°C was between 36-52%. Atlas (1975) investigated the degradation of various crude oils in seawater and found that the loss due to biodegradation at 20°C was between 26-50% compared to 11-28% at 10°C. Margesin and Schinner (1997b) investigated the biodegradation of diesel fuel in soil and found that the loss due to degradation at 10°C was 43% compared to a loss at 25°C of 55%. The results presented here, a loss of 44% at 20°C compared to a loss of 19% at 5°C, are consistent with the findings described above.

It has been well documented in the literature that an increase in temperature increases the rate of biodegradation (Atlas and Bartha 1972, Song *et al.* 1990, and Yeung *et al.* 1997). As an example, Zhou and Crawford (1995) measured gasoline vapour in the headspace above contaminated soils in closed microcosms. They observed that the rates increased from 62ppm/day to 114ppm/day to 135ppm/day at 11, 25 and 35°C respectively. How the effect of temperature on the rate of biodegradation changes over time has not been characterized in the literature.

The results of this study show that degradation at 5°C can be mathematically described by a single-stage first order degradation equation and the degradation at 20°C can be mathematically described by a two-stage first order degradation equation. A possible theoretical explanation for this model is that all the compounds degrade at the same rate at 5°C and all the hydrocarbon compounds at 20°C are either rapidly degraded or slowly degraded. In reality, each hydrocarbon compound may degrade at a different rate, and a hydrocarbon mixture should not be categorized into only a rapidly degraded portion and slowly degraded portion of hydrocarbons. For example, some compounds such as asphaltenes, are unable to be degraded at all. However, for the purposes of this discussion, these two hydrocarbon portions will be used to simplify explanations.

The results from the linear regression and the non-linear regression show that the rate of degradation at 5°C and 20°C are the same after an initial rapid stage of degradation at 20°C. The linear regression results show no significant difference in TEH treatment degradation rate between 5°C and 20°C after 21 days. The nonlinear regression results show, using the derivative graph, that the rate of degradation at 5°C and 20°C is the same at approximately 42 days. The 42 days is considered to be more representative than 21 days because the linear regression can only use predetermined time intervals based on the sampling day. The model derivative allows for a more detailed comparison of the rate changes at 5°C and 20°C.

Unlike most observed trends (Maliszewska-Kordybach 1993, Yeung *et al.* 1997, and Malina *et al.* 1999), the treatment degradation data at 20°C could not be described by a single-stage first-order kinetics model. Degradation occurred in two stages, one that was between 0 and approximately 42 days and the second that was approximately 42-121 days. These two stages can be explained by breaking the TEH into a rapidly degraded fraction and a slowly degraded fraction that is described by the two-stage first-order kinetics model given in section 3.2.2. The rapidly degraded fraction describes the first stage and the slowly degraded fraction describes the second stage. The treatment degradation at 5°C did not consist of two stages, but was only described by a single-stage first-order degradation model. The treatment degradation at 5°C from 0-121 days and the treatment degradation at 20°C from 21-121 days can be well approximated by a linear equation that has the same degradation rate (52.0mg/kg/day) for both temperatures. The nonlinear regression analysis shows that after the initial rapid degradation stage at 20°C, ending at approximately 42 days, the rates of degradation are the same at 5°C and 20°C.

Gibb (1999) found similar results when looking at a laboratory bioventing study of the degradation of a topped crude oil added to soil. She showed that there was an initial stage during which the rate of CO_2 production was greater at 20°C than at 5°C. This initial stage was followed by a second stage during which the rate of CO_2 production was similar at 20 and 5°C.

Coover and Sims (1987) who looked at the biodegradation of 16 PAHs in soil microcosms at 10, 20 and 30°C found similar results. They found that phenanthrene at 20 and 30°C showed two stages of degradation between 0-60 days and 60-240 days. During the initial stage, degradation was rapid and during the final stage rates were slow and approximately linear. They found that increasing the soil temperature improved the rate and extent of apparent loss of low molecular weight PAHs but had little effect on the loss of five and six-ring compounds. It is likely that a similar phenomenon is occurring during the degradation at 5°C and 20°C presented in this study.

Because the GC data do not include the residual fraction, the GC rate constants can not be conclusively compared to the TEH degradation rate constants. However, speculation as to the relationship between these two sets of data would be the following. The results of the nonlinear regression on the GC fraction C_{16}-C_{24} at 20°C show that the degradation of the C_{16}-C_{24} fraction does not explain the total degradation of TEH; the rate constants for the C_{16}-C_{24} fraction are less than the rate constants for TEH degradation. There is most likely degradation occurring in GC fractions other than the C_{16}-C_{24} at 20°C that is not accounted for. Degradation of the other fractions can be seen in the GC fingerprint examples (figure 4.12). However, at 5°C, the rate constants of the TEH degradation data are not significantly different than the rate constants of the GC fraction C_{16}-C_{24}. These relationships suggest that at 5°C almost all of the degradation occurs in the C_{16}-C_{24} fraction, but at 20°C most degradation occurs in the C_{16}-C_{24} with additional degradation occurring in other fractions.

The microbial enumeration data are consistent with and provides further support for the single-stage degradation at 5°C and two-stage degradation at 20°C. The 20°C oil degraders (Figure 4-8B) begin to decrease in MPN between approximately 20 and 40 days. This decrease is not seen in the oil degraders at 5°C (Figure 4-7B). Possible explanations for the GC data and microbial data will discussed in section 5.2.

Now that the degradation curves at 5°C and 20°C have been characterized as single-stage first order and two-stage first order respectively, a question remains unanswered. What mechanism, describing the effect of temperature, produces single-stage first order degradation at low temperatures and two-stage first order degradation at higher temperatures? The following discussion (section 5.2) proposes explanations to the above question. However, this question is complex and most likely involves numerous mechanisms acting simultaneously. Conclusively answering this question is beyond the scope of this project because the experimental design and resulting data were not originally designed to determine or distinguish the proposed mechanisms.

5.2 DISCUSSION OF MECHANISMS DESCRIBING THE EFFECT OF TEMPERATURE ON THE RATE OF BIODEGRADATION

There are five possible mechanistic explanations for the effect of temperature on the rate and extent of biodegradation of hydrocarbon biodegradation in soil. The five mechanisms are categorized into three groups that are represented visually in Figure 5-1. The groups are labeled biodegradability, bioavailability, and the Q_{10} effect. These mechanisms will help explain why there is two-stage first order degradation of TEH at 20°C and single-stage first order degradation of TEH at 5°C. The mechanisms within each group are described in detail below.

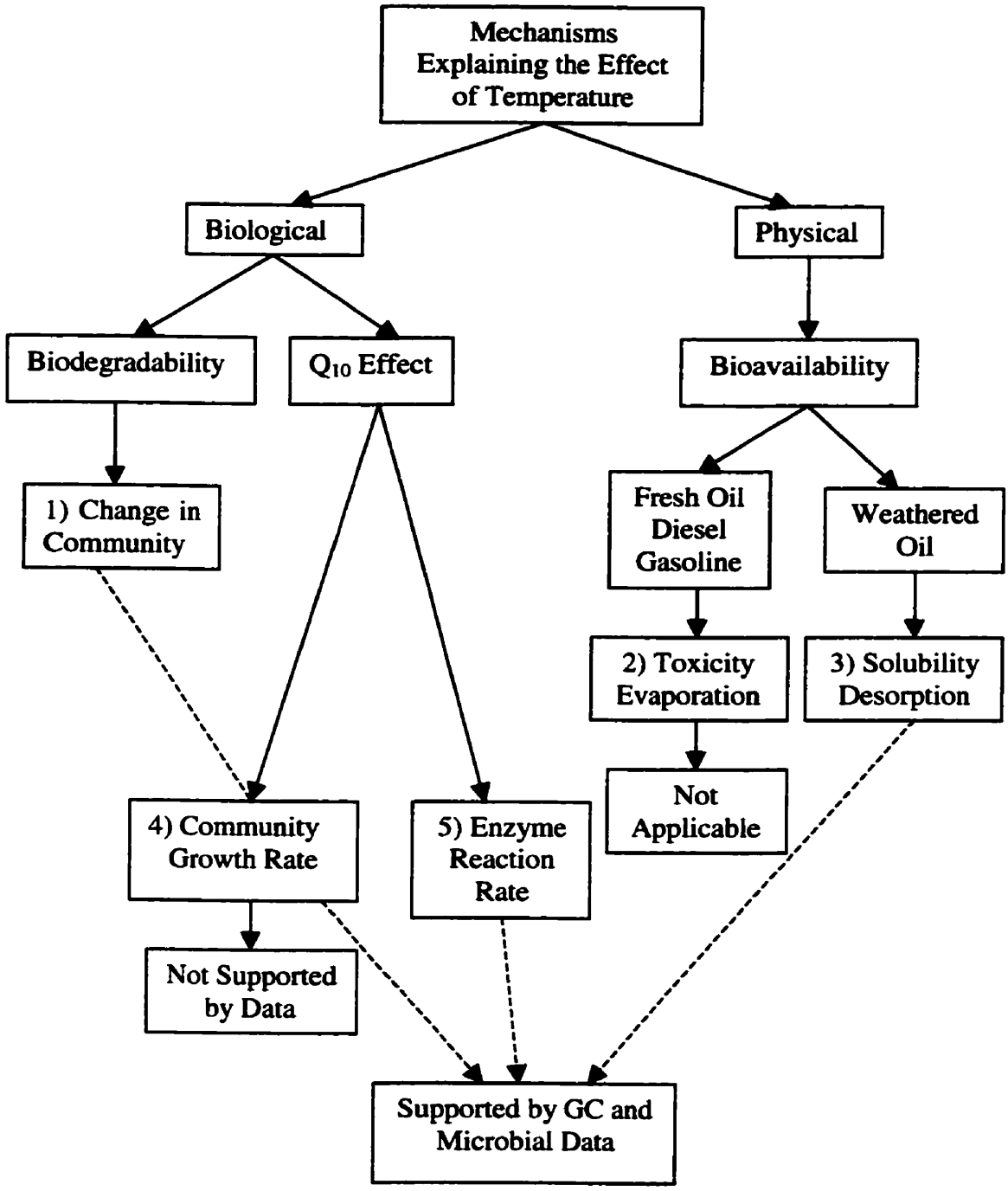


Figure 5-1: Summary of the mechanisms describing the effect of temperature on the rate of biodegradation. Boxes labeled 1-5 are the mechanisms described in the test

5.2.1 Biodegradability

The first mechanism is that temperature indirectly affects the biodegradability of a hydrocarbon mixture by changing the composition of the microbial community. This mechanism would occur in the absence of soil thereby eliminating the influence of bioavailability. It is possible that a microbial community made up of mostly psychrotrophs (as would be found in cold climates) may be able to degrade a different portion of a crude oil mixture than a microbial community consisting mostly of mesophiles (as would be found in warm climates). Different temperatures result in different species composition or different proportion of species already present within the microbial community and different species have different capabilities to degrade hydrocarbon compounds within an oil mixture. This mechanism involves the effect of temperature on community structure and is labeled box 1 in Figure 5-1. As a result, the history of the microbial community with respect to temperature is important. The length of time that a community is exposed to a specific temperature will determine the community composition and therefore affect the biodegradation rate.

Laboratory experiments in literature most often do not consider or describe source soil site conditions like temperature fluctuations and soil pretreatment conditions. Site conditions and pretreatment conditions will affect the results of biodegradation experiments run on the same soil at different temperatures because these conditions influence the microbial community. For this experiment, the soil was stored at 5°C or less for at least two years. It is likely that the microbial community is adapted to this temperature, therefore there will not be a lag phase in microbial growth which is shown to be true. Based on the pretreatment of the soil for this experiment it may be expected that if the microbial community was made up of psychrophiles the bacterial numbers in the 20°C treatment would show an increase as the population shifts towards mesophiles. However, this observation was not seen suggesting the presence of psychrotrophs. The actual community shifts that occurred during this experiment are unable to be characterized because it is beyond the scope of this project.

Many biodegradation rate constant measurements are determined for pure chemicals and not mixtures. Oil is a complex but largely biodegradable mixture of hydrocarbon compounds. To theoretically explain the single-stage and two-stage degradation in terms of biodegradability, the crude oil present in the soil can be classified into a readily degradable fraction (to be distinguished from rapidly degraded because rapid degradation can occur for reasons other than the compounds being readily degradable such as bioavailability) and a more difficult to degrade fraction (again, different from slowly degraded for the same reason). These fractions are dependent on the microbial community present and therefore change if the community changes. If temperature changes the microbial community and different communities have different biodegradability capabilities, then the biodegradability of the hydrocarbon will change. In relation to the TEH degradation results, two-stage first order degradation is seen at 20°C because the 20°C microbial community is capable of biodegrading a fraction rapidly and an additional fraction slowly. Single-stage first order degradation is seen at 5°C because there is only a more difficult to degrade fraction.

The microbial data and GC fraction data support this mechanism. The microbial data show a decrease at approximately 20-40 days of the 20°C oil degraders suggesting that there are fewer organisms capable of degrading the more difficult to degrade fraction once the readily degradable fraction has been degraded. The more difficult to degrade fraction likely consists of five and six ring hydrocarbon compounds. The GC data at 20°C shows a similar trend to the TEH degradation data (single-stage at 5°C and two-stage at 20°C). This suggests that a difference in degradability between the 5°C community and the 20°C community occurs within this fraction.

Literature supports the mechanisms of different biodegradability of the same hydrocarbon mixture at a different temperature because temperature affects the microbial community composition. Walker and Colwell (1974) found that slower but more extensive biodegradation occurred at 0°C than at 5°C and 10°C in Chesapeake Bay water and sediment. A study by Jobson *et al.* (1972) looked at the degradation of crude oil at

4°C and 30°C. The aromatic fraction of the crude oil was more rapidly metabolized at 30°C than at 4°C; very little of the aromatic fraction was used at 4°C. This suggests the pattern of compound utilization, within an oil mixture, varies depending on the microbial community present. However, the study by Jobson *et al.* (1972) did not characterize the microbial community and degradation takes place in the presence of soil so the distinction between the mechanism of bioavailability (to be discussed) and biodegradability cannot be made.

Westlake *et al.* (1974) found that temperature played a role in determining the metabolic capabilities of a microbial community. They looked at the relationship between the chemical composition of four northern and mid-Arctic crude oils and their biodegradability under psychrophilic and mesophilic conditions. Under psychrophilic conditions (4°C) the microbial community was consistently unable to degrade isoprenoids, phytane and pristane. However, at mesophilic conditions (30°C) the microbial community was able to degrade these compounds. Westlake *et al.* (1974) looked at the community composition by classifying species down to the genus level. They found that there were different community compositions at 4°C and 30°C. Eriksson *et al.* (1999) looked at degradation of fresh contamination of a hydrocarbon mixture by indigenous microorganisms in potting soil. They found that naphthalene and p-xylene were degraded down to levels below detection at 20°C but unaffected at 6°C. In 40 days Bromonaphthalene was 75% removed at 20°C but unaffected at 6°C.

Support for the biodegradability mechanism can also be found by characterizing individual microorganisms with respect to hydrocarbon degrading capacity. Whyte *et al.* (1998) found that a psychrotrophic *Rhodococcus* sp. was capable of mineralizing alkanes such as dodecane (C_{12}), hexadecane (C_{16}) and to a lesser extent, octacosane (C_{28}) and dotriacontane (C_{32}). However, this same bacterium was unable to mineralize toluene or naphthalene. Whyte *et al.* (1997) did isolate a *Pseudomonas* strain possessing both *alk* and *nah* pathways responsible for biodegradation of both alkanes and naphthalene. As data are accumulated on the capability of psychrotrophic and psychrophilic organisms to

degrade the many compounds present in hydrocarbon mixtures, the importance of biodegradability differences between psychrophilic/psychrotrophic and mesophilic communities to the effect of temperature on the rate of biodegradation can be determined.

The above studies demonstrate that a change in community composition resulting in a change in biodegradability of a hydrocarbon mixture could be the mechanism explaining the effect of temperature. However, the studies used soil, making it difficult to distinguish the effects of bioavailability and biodegradability. Liquid cultures or contaminated water samples are necessary to isolate bioavailability (described in section 5.2.2) from biodegradability.

Atlas and Bartha (1972) conducted a study measuring the rate and extent of crude oil biodegradation and mineralization at low temperatures in seawater with indigenous microbial communities. Carbon dioxide evolution was measured at 20, 15, 10 and 5°C. They found that the maximum carbon dioxide evolution rate increased with increased temperature. These results suggest that biodegradability is an important mechanism in describing the effects of temperature on the rate of biodegradation. Atlas and Bartha (1972) describe results that suggest that temperature influences the composition of the microbial community, further supporting the biodegradability theory. They collected seawater samples in September during which the mean surface water temperature was 17.5°C and in December during which the mean surface water temperature was 7.5°C. The September sample did not show any evidence of degradation (CO_2 evolution) in 60 days, however the December sample showed carbon dioxide evolution. In addition, higher CO_2 evolution rates were observed at 10°C and 5°C in the December sample than in the September sample. These results reflect a seasonal shift in microbial community composition.

5.2.2 Bioavailability

The second category of mechanisms is bioavailability. This category describes how a change in temperature affects the physical state of the oil in the soil making it more or less bioavailable for microorganisms to degrade. It is possible for a fraction of the hydrocarbon to be biodegradable but remain in the soil environment because it is not bioavailable. Temperature may affect the fraction that is biodegradable but not bioavailable.

Oil may be found in the unsaturated zone as a four-phase system consisting of a non-aqueous phase liquid (NAPL), adsorbed compounds to the surface of mineral grains or soil organic matter, dissolved compounds in soil water, and a vapour phase, as shown in figure 5-2 (Suthersan 1997). Under equilibrium conditions, the concentration of a compound in each phase may be described by mass balance and equilibrium equations. Increasing the temperature shifts the equilibrium towards the gas and liquid phases (i.e. towards the dissolved and volatile phases). As temperature decreases, the viscosity of oil increases and the volatilization of toxic short chain hydrocarbon compounds decrease, resulting in a higher fraction of the toxic short chain compounds in the soluble form (Margesin and Schinner 1999a). In this case the biodegradation in cold climates would be delayed due to the presence of toxic short chain compounds that would volatilize at higher temperatures (box 2 Figure 5-1). This second mechanism would most likely occur with fresh oil, diesel or gasoline contamination where toxic short chain compounds are still present. This is unlikely the case with a weathered crude oil, as in this study, therefore this mechanism will not be considered in further detail.

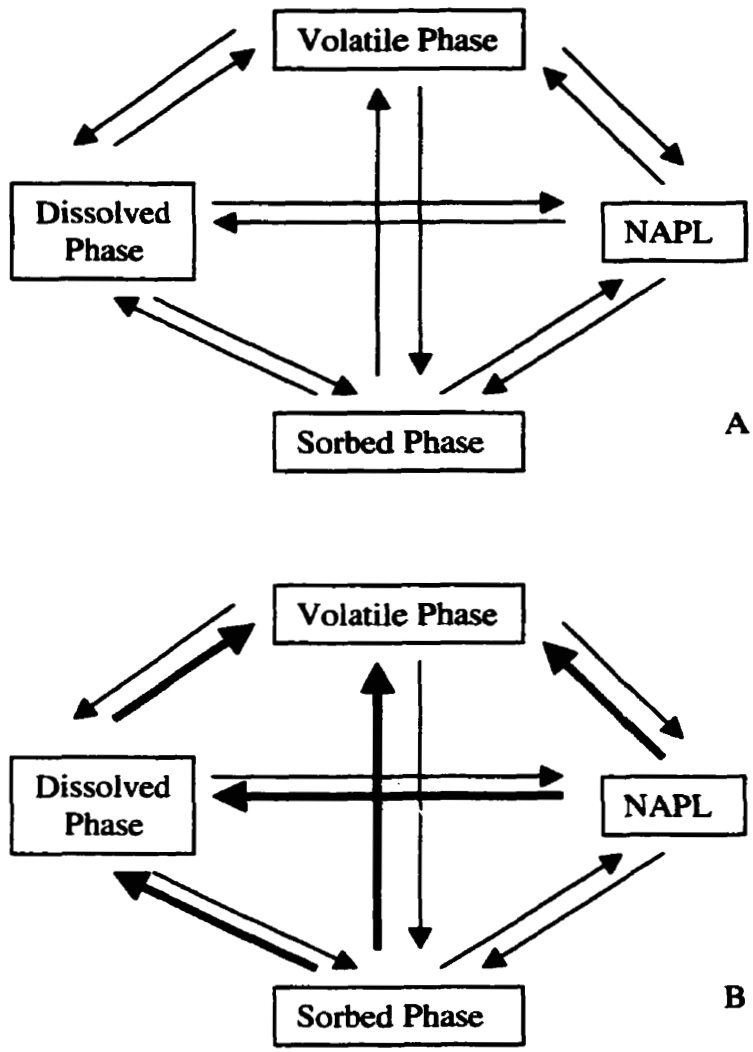


Figure 5-2: Four phases of oil found in an unsaturated zone soil A) Soil system at equilibrium B) Shift due to an increase in temperature (modified from Suthersan 1997)

Temperature can also affect bioavailability by affecting density, viscosity, solubility, vapour pressure and diffusivity, all of which influence desorption. The third mechanism for the effect of temperature is as temperature increases, solubility and diffusivity usually increase resulting in increased desorption of the hydrocarbon compounds from the adsorbed phase to the dissolved phase (box 3 Figure 5-1). Since

biodegradation of weathered crude oil is often limited by desorption (Hatzinger and Alexander 1995, Chung and Alexander 1998, Alexander 1999), lower degradation rates at lower temperatures may be due to decreased desorption because of decreased solubility. This mechanism is often used to describe the "hockey stick" curve described in section 2.3. During desorption of organic contaminants from soil, there is a rapid phase of desorption followed by a slow phase (Hatzinger and Alexander 1995, Alexander 1999, Gray *et al.* 2000). The slow stage of biodegradation is commonly attributed to the slow stage of desorption. Many studies have been done on the effects of sorption and desorption on bioavailability. However, few studies have been done on the effects of temperature on desorption of hydrocarbon contamination from soil.

The TEH, GC, and microbial data support the bioavailability mechanism. To theoretically describe the TEH results in terms of bioavailability, at 20°C there is a bioavailable fraction of hydrocarbons and a less bioavailable fraction of hydrocarbons. At 5°C temperature decreases desorption and diffusivity resulting in less bioavailable hydrocarbons. The GC data suggests that temperature produces a change in desorption and diffusivity of the C_{16}-C_{24} fraction of hydrocarbons. Finally, the oil degraders at 20°C decrease in MPN between approximately 20 and 40 days because the bioavailable fraction of hydrocarbon has been degraded. There is less hydrocarbon substrate available so the oil degrader numbers decrease.

Malina *et al.* (1999) examined the effects of temperature on the bioventing of soil contaminated with toluene and decane. They found that decreasing the temperature increased the soil-gas partitioning coefficient for toluene and decane, resulting in an increase in the mass of toluene and decane adsorbed to the soil. Whyte *et al* (1998) found that as temperature decreased, the mineralization of longer-chain alkanes (C_{28}-C_{32}) was lower than the mineralization of shorter chains (C_{12}-C_{16}). Octacosane (C_{28}) and dotriacontane (C_{32}) form relatively large crystals at 0 and 5°C thus decreasing the bioavailability of the compounds. Under cold conditions, the precipitation from crude oil of certain alkanes as waxes would greatly diminish their availability to oil-degrading

organisms (Margesin and Schinner 1999a).

If bioavailability is the mechanism explaining the effects of temperature on biodegradation rates, then the effect of temperature would be different depending on the soil characteristics because soil characteristics affect sorption and adsorption of compounds. Maliszewska-Kordybach (1993) examined whether soil properties influence the effect of temperature on extent and rate of polyaromatic hydrocarbon (PAH) disappearance from soil. Fluorene, anthracene, pyrene, and chrysene were added to a light loam soil and a loam sand soil, and were incubated at 10, 20 and 25°C for 180 days. Maliszewska-Kordybach (1993) found that a lower temperature decreased the extent of disappearance for all four compounds with the greatest temperature effect found on pyrene and the lowest temperature affect being on chrysene. Increasing temperature increased the degradation rate of only fluorene and anthracene in the light loam soil, but increased the degradation rate of all four compounds in the loam sand soil. In addition, the difference in half-lives of the compounds between the light loam soil and the loam sand soil was greatest at 10°C. These results support the bioavailability theory because the effect of temperature changes with different soils. It is likely that desorption is easier from a loam sand soil than a light loam soil because a loam sand contains less soil organic matter and has a smaller clay fraction. A smaller clay fraction has less surface area for adsorption therefore hydrocarbon compounds have the potential to be more bioavailable. In relation to this study, the soil used was a coarse textured soil. Results may differ if the same experiment was performed on a fine textured soil.

Support for the bioavailability theory can come from bioaugmentation studies mentioned previously. If decreased solubility and increased adsorption is responsible for the decreased rate of biodegradation at low temperatures than bioaugmentation would not be successful in increasing the degradation rate. Keeping in mind that bioaugmentation may be unsuccessful for other reasons (described in section 2.1.2.3 and 2.4) this is consistent with most of the bioaugmentation laboratory and field studies found in literature (Margesin and Schinner 1997a and 1997b, Wilson 1999).

If sorption and solubility play major roles in the effect of temperature then the use of an appropriate surfactant should result in similar degradation rates for both temperatures. Margesin and Schinner (1999b) found that the presence of the surfactant sodium dodecyl sulfate at concentrations between 50 and 100 mg/L increased the extent of diesel oil biodegradation in a liquid culture. However, when this same surfactant was added to soil, biodegradation of diesel oil was reduced. No explanation was presented for the different effect of the surfactant in liquid culture and soil.

Finally, Mohn *et al.* (2001) studied degradation of Arctic diesel fuel contaminating an Arctic tundra soil and looked at the effect of freeze-thaw cycles. They found that the treatment that cycled from 7°C to –5°C degraded to a greater extent and at a faster rate than the treatment at 7°C. They suggest that this is due to the effect of the freeze-thaw cycle on bioavailability. It is possible that the freeze-thaw cycle releases sorbed hydrocarbon compounds allowing for more rapid and extensive degradation.

5.2.3 Q_{10} Effect

The third category of explanations is called the Q_{10} effect and is separated into two possible mechanisms both of which predict that the rate of biodegradation in cold climates is much slower than in warmer climates. Mechanism four is that a decrease in temperature decreases the growth rate of microbial communities and therefore reduces the rate of biodegradation reactions (box 3 Figure 5-1). This mechanism assumes that the oil degrading community is in the exponential growth phase. In this project, this assumption is shown to be untrue because the microbial numbers do not show an exponential growth phase. The numbers are most likely not increasing because the contaminated soil is weathered and the microbial community is acclimated to soil conditions. Because the numbers of bacteria are not increasing, it is unlikely that the fourth mechanism is an acceptable explanation for the effect of temperature on the rate of biodegradation in this project.

The fifth mechanism looks at degradation at the level of enzyme reactions. If temperature is decreased, the rate of biodegradation reactions are decreased because of the effect of temperature on enzymatic reaction rates (box 5 Figure 5-1). The rate of change and the direction of change of the enzymatic reactions due to temperature changes depend on the species of organisms present. The species composition and the proportion of each species present depend on the previous temperature exposure of the soil.

Literature shows that microbial communities and microorganisms adapt to their surroundings. Cold-adapted strategies include molecular adaptation of membrane lipid composition and synthesis of cold shock proteins (Margesin and Schinner 1994). Bradley and Chapelle (1995) re-examined the assumption that rates of microbial hydrocarbon degradation in low-temperature groundwater systems are depressed relative to those found in more temperate systems. They compared aerobic toluene mineralization in sediments from an aquifer with an annual temperature range of 4-6°C to aerobic toluene mineralization in sediments from an aquifer with an annual temperature range of 19-22°C. Using the indigenous microbial community for degradation at each site and incubating within the site's annual temperature range, Bradley and Chapelle (1995) found that the degradation rate in the 5°C aquifer was approximately twice that of the degradation rate of the 20°C. These data show that different microbial communities will respond differently to an upshift and downshift in temperature. As described in section 2.2.2, cold-adapted organisms show greater heat sensitivity and warm-adapted organisms show greater cold-sensitivity.

The microbial data and GC data do not exclude the fifth mechanism as a possible explanation for the effect of temperature on the rate of degradation in this project. Because the effect of temperature on enzymatic reactions depends on the species of microorganism, it would be beneficial to explore the characterization of and changes in the microbial community in greater detail.

In summary, it is likely that a combination of three of the five mechanisms described above influence the effect of temperature on the rate of degradation in this experiment. The complexity of the soil environment, the complexity of the hydrocarbon substrate, the effect of environmental parameters and the influence of dynamic microbial populations make the mechanistic interpretation and modeling of soil degradation kinetics difficult. In the case of this study, which included a weathered crude oil, the initial effect of temperature in producing a two-stage first order degradation curve at 20°C, compared to a single-stage first order degradation curve at 5°C is best supported by literature and presented data in a combination of the bioavailability and biodegradability mechanisms. However, none of the above three mechanisms can be conclusively isolated as the primary mechanism with the data presented. An important question that can not be addressed with the presented data is the degree to which the microbial community is changed by temperature.

To address the effectiveness of cold-climate bioremediation, the mechanism of the effect of temperature must be determined. If the cold-climate site conditions can be manipulated to initiate the initial rapid stage of degradation observed at higher temperatures, cold-climate bioremediation can be very effective. Depending on the primary mechanism responsible for the effect of temperature, the use of heating, surfactants, or bioaugmentation may be beneficial. To follow are recommendations on how to determine the primary mechanism of the effect of temperature on the rate of biodegradation of crude oil in soil.

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The following is a summary of the conclusions from the results of this project. It should be noted that changing the soil characteristics (i.e. organic matter content, particle size distribution etc.), hydrocarbon characteristics (i.e. crude oil, pentane insolubles) and treatment conditions (soil slurry vs. solid phase) will likely affect this results.

1) The rate and extent of abiotic loss of TEH at 5°C and 20°C is the same. The rate of abiotic TEH loss is 17.2 mg/kg/day and the extent of TEH loss is 4%.

2) The extent of TEH loss over 121 days at 5°C is 19.0% and the extent of TEH loss over 121 days at 20°C is 44%. After 121 days of degradation the extent of TEH loss over 121 days at 20°C is greater than the extent of TEH loss over 121 days at 5°C.

3) The rate of biodegradation of TEH at 5°C and the rate of biodegradation at 20°C after approximately 21 days can be approximated linearly and is the same. This rate is 52.0 mg/kg/day.

4) A two-stage first order kinetics model can approximate the rate of degradation of TEH at 20°C. A single-stage, first-order kinetics model can approximate the rate of degradation of TEH at 5°C. The rate of change of TEH at 5°C and at 20°C is the same after approximately 42 days.

5) Since the microbial enumeration data do not show an exponential growth phase, it is unlikely that the initial effect of temperature is due to the different community microbial growth rates at the two temperatures.

6) The GC fraction showing the most relative change at both 5°C and 20°C was C_{16}-C_{24}. The C_{16}-C_{24} loss at 5°C was well approximated by a single-stage first order model and the C_{16}-C_{24} loss at 20°C was well approximated by a two-stage first order model. This suggests that the effect of temperature most likely occurs within the C_{16}- C_{24} fraction.

7) As a summary statement, it is most likely that the mechanisms explaining the effect of a decreased temperature on the rate of degradation of a weathered crude oil in soil are a combination of the following:

   - decreased degradability because it is very likely that the microbial community changes in response to different temperatures,
   - decreased solubility of hydrocarbon compounds,
   - and decreased enzymatic rates of degradation reactions.

6.2 RECOMMENDATIONS

The following recommendations consist of experimental steps necessary to help distinguish the primary mechanism explaining the effect of temperature on the rate of degradation of a weathered crude oil in soil. Since the GC results presented here suggest that the C_{16}-C_{24} fraction is the most acted upon fraction, it is advisable that the following experimental recommendations use this fraction of hydrocarbon compounds when possible. The following experiments done individually will not definitively isolate the mechanism tested by a single experiment. It will be necessary to conduct all of the experiments to define the primary mechanism after comparison of all the results. No one experiment eliminates any other of the mechanisms.

6.2.1 Test of Biodegradability Mechanism

The objective of a test of the biodegradability mechanism is to answer the question: 1) Does a community of psychrophiles and psychrotrophs have the capability to degrade the same hydrocarbon compounds within a crude oil mixture at 5°C as a community of mesophiles at 30°C? 2) If there is a difference in the degradation capability of the two communities, does this difference explain the initial degradation rate difference between biodegradation at 5°C and 20°C? This test must be completed in the absence of soil and with compounds that are completely or reasonably soluble to remove the influence of bioavailability. A possible experimental design would include the following.

Obtain an uncontaminated field soil from a site that has an ambient temperature between 20 and 30°C. Enrich the mesophilic community within the soil at 30°C using a soil slurry and then liquid medium. Obtain an uncontaminated field soil from a site that has an ambient temperature between 0 and 10°C. Enrich the psychrophilic and psychrotroph community within the soil at 5°C using a soil slurry and then liquid medium. To a set of flasks with mineral salts liquid media, add a known high number of microbes (10^8-10^9) from the mesophilic community and an artificial hydrocarbon mixture and incubate at 30°C. The artificial hydrocarbon mixture should contain representative compounds from aliphatic, aromatic and asphaltic groups of hydrocarbon. To a different set of flasks containing mineral salts liquid media, add the same high number of microbes (10^8-10^9) from the psychrophilic and psychrotrophic community and the same artificial hydrocarbon mixture. Monitor the degradation of each hydrocarbon compound over time in both the mesophilic community and the psychrophilic and psychrotrophic community.

Careful consideration should be given to the enrichment procedure because choice of enrichment substrate may affect the community and subsequently biodegradation. Compounds within the C_{16}-C_{20} fraction should be monitored. If biodegradability is important in producing the initial effect of temperature, than different compounds will be degraded at different rates by the different communities.

An additional recommendation to aid in the above experiment is to use a modification of the 96-well-MPN technique that enumerates aromatic and aliphatic hydrocarbon degrading bacteria separately (Wrenn and Venosa 1996). Separate 96 well plates are used to estimate the size of the aromatic and aliphatic degrading communities by using hexadecane as a growth substrate for aliphatic degraders and a mixture of phenanthrene, anthracene, fluorene, and dibenzothiophene as the growth substrate for aromatic degraders (Wrenn and Venosa 1996). This technique along with incubation at both 5 and 30°C will help monitor the composition of the microbial community.

6.2.2 Test of Bioavailability Mechanism

The objective of a test of the bioavailability mechanism is to answer the question: 1) Does a decrease in temperature decrease the desorption rate of a weathered crude oil in soil? 2) If a temperature decrease does decrease the desorption rate, then does the desorption rate explain the initial degradation rate difference between 5°C and 20°C. When considering the relationships between the four phases of oil in soil (figure 5.2), it is very difficult to isolate and measure desorption of oil from soil. Considerations for possible experiments include measuring the liquid phase extractable hydrocarbon content and measuring volatilization using headspace analysis. One possible technique to measure both the liquid phase and volatilization is solid-phase microextraction (SPME). SPME is a technique that integrates sampling, extraction, concentration, and sample introduction into a single step (Zhang, Yang and Pawliszyn 1994). The SPME apparatus contains a fine, fused silica fiber coated with a polymeric stationary phase that is used to concentrate the analytes directly from a sample. Considerations of mass balance between the four phases described in figure 5.2 could be included. Any further description of an experimental design to test the effects of temperature on desorption are beyond the scope of this project.

Additional recommendations for lines of experimentation include considerations of surfactants, effect of soil type, solid phase studies, and composition of microbial communities. A method of indirectly testing this mechanism is to conduct more extensive testing of the effects of a cold-active solubilizing agent (surfactant) to increase bioavailability as suggested by Whyte *et al* (1998). Biodegradability of surfactants is affected by temperature which changes the effect of the surfactant on the microbial community (Takamatsu *et al.* 1996). As previously mentioned, the same experiment could be conducted on a fine textured soil to examine whether the observed kinetic differences due to temperature are the same as presented here for a coarse soil. Since slurries decrease diffusion distances by dispersing mineral-organic matter aggregates so the effects of temperature may be different in solid phase systems. Finally, bioavailability depends on the types of microbial species present because some species are more mobile, produce biosurfactants or possess adsoprtion techniques to increase hydrocarbon bioavailability (Bastiaens *et al.* 2000).

6.2.3 Test of Q_{10} Effect Mechanism

As mentioned previously, the Q_{10} effect will depend on how the microbial community changes as a result of a shift in temperature. This is because as the community changes with a decrease in temperature, possibly shifting from a dominantly mesophilic community to a dominantly psychrotrophic community, the response of the enzymes to a change in temperature will change. Therefore, characterizing how a community changes with temperature is the next step in testing the Q_{10} effect mechanisms. This area of research is currently in progress by Rowsell (1999). If a community is characterized, specific degradation enzymes can be identified and the effect of temperature on the rate of degradation by specific enzymes can be examined.

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APPENDIX A – CALUCULATION OF NUTRIENT SOLUTION

NITROGEN CONCENTRATION

Ammonium nitrate was added to attain 400mg of nitrogen per kilogram of soil.

$$\frac{400mg\ N}{kg\ \text{soil}} * 0.020\ kg\ \text{soil} = 8.0\ mg\ \text{N to each slurry}$$

Mass of nitrogen in ammonium nitrate = 28.02 g/mole

Fraction of nitrogen in ammonium nitrate = 0.35

$$0.0080g\ N * {}^{1}\!/_{0.35} = 0.023g\ NH_4NO_3\ \text{per slurry}$$

Molar mass of ammonium nitrate = 18.05 g/mole

Fraction of ammonium = 0.225

Mass of ammonium added = 0.023g * 0.225 = 0.0052g

$$\text{Concentration of ammonium} = \frac{5.2mg}{0.060L} = 86\ {}^{mg}\!/_{L}$$

Molar mass of nitrate = 62.01 g/mole

Fraction of nitrate in ammonium nitrate = 0.774

Mass of nitrate = 0.023g * 0.774 = 0.01780

$$\text{Concentration of nitrate} = \frac{17.80mg}{0.060L} = 296.7\ {}^{mg}\!/_{L}$$

PHOSPHORUS CONCENTRATION

Phosphorus was added to maintain a concentration of 50 mg/L PO_4. To account for potential precipitation of phosphorous the concentration of phosphorous added was 100mg/L. Monobasic and Dibasic potassium phosphate was used.

The desired buffering ratio of HPO_4:H_2PO_4 for a pH of 7 is 0.62. Therefore 38 mg/L of HPO_4 and 62 mg/L of H_2PO_4 was added.

Molecular mass of KH_2PO_4 = 138.11 g/mole

Fraction of H_2PO_4 = 0.55

Molecular Mass of K_2HPO_4 = 174.18 g/mole

Fraction of HPO_4 = 0.70

$$\text{Mass of } K_2HPO_4 \text{ in 1L} = 38mg\ of\ HPO_4 \times \frac{1}{0.55} = 69.09mg$$

$$\text{Mass of } KH_2PO_4 \text{ in 1L} = 62mg\ of\ H_2PO_4 * \frac{1}{0.70} = 88.57mg$$

PREPARATION OF NUTRIENT SOLUTION

In a 2L volumetric flask add 0.138g of K_2HPO_4, 0.1823g of KH_2PO_4, and 0.7868g of NH_4NO_3. Fill the flask to volume with chlorine free tap water, and check pH using a digital pH/mV meter with a combination electrode and automatic temperature compensator.

SODIUM AZIDE

The effective concentration of sodium azide is 1mmole/L. To ensure a completely abiotic system fifty times this concentration will be used (50 mmole/L).

Molecular Weight of NaN_3 = 65.02g/mole

Mass of NaN_3 in each abiotic control flask:

$$= \frac{50mmole}{L} \times \frac{65.02g}{mole} \times \frac{1mole}{1000mmole} \times 0.0606L = 0.195g$$

APPENDIX B – RAW DATA

Table B-1: Initial Moisture Content of Source Soil

Sample	Replicate	Weight of Tray (g)	Weight of Wet Soil (g)	Weight of Tray and Dry Soil (g)	Weight of Dry Soil (g)	Gravimetric Soil Moisture (g/g)	Moisture Content (%)
Beginning	1	2.52	10.41	12.41	9.89	0.053	5.29
Beginning	2	2.53	10.22	12.26	9.73	0.050	5.03
Beginning	3	2.53	9.68	11.73	9.20	0.053	5.29
Beginning	4	2.50	10.63	12.60	10.10	0.052	5.25
Beginning	5	2.51	12.36	14.24	11.73	0.054	5.36
Beginning	6	2.44	11.64	13.49	11.05	0.053	5.30
Beginning	7	2.45	10.01	11.97	9.51	0.053	5.27
Beginning	8	2.44	12.08	13.94	11.50	0.051	5.10
Beginning	9	2.50	10.84	12.81	10.31	0.052	5.16
Beginning	10	2.45	11.49	13.36	10.91	0.052	5.24
End	1	1.58	11.00	12.14	10.56	0.042	4.20
End	2	1.59	10.17	11.33	9.75	0.043	4.34
End	3	1.59	13.61	14.62	13.03	0.045	4.45
End	4	1.59	13.29	14.34	12.76	0.042	4.23
End	5	1.58	12.53	13.59	12.01	0.044	4.38
End	6	1.58	13.33	14.34	12.76	0.045	4.45

Table B-2: Initial Particle Size Analysis Data: Hydrometer Method

Sample	Time (min)	R	c (g soil colloids/litre)	P	y (Sedimentation Parameter)	y (temp adjust)	Particle Size X(um)
1	0.5	18	12	31.57	44.5	49.35	69.79
	1	16	10	26.31	45	49.91	49.91
	3	13	7	18.42	45.9	50.90	29.39
	10	12	6	15.79	46.2	51.24	16.20
	30	10	4	10.52	46.7	51.79	9.46
	60	9.5	3.5	9.21	46.85	51.96	6.71
	90	8.5	2.5	6.58	47.1	52.23	5.51
	120	8.5	2.5	6.58	47.1	52.23	4.77
	270	8	2	5.26	47.2	52.34	3.19
	720	7	1	2.63	47.4	52.57	1.96
	1260	6	0	0.00	47.7	52.90	1.49
2	0.5	17	11	28.97	44.8	49.68	70.26
	1	16	10	26.34	45	49.91	49.91
	3	12	6	15.80	46.2	51.24	29.58
	10	10.5	4.5	11.85	46.6	51.68	16.34
	30	9	3	7.90	47	52.12	9.52
	60	8	2	5.27	47.2	52.34	6.76
	90	8	2	5.27	47.2	52.34	5.52
	120	7.5	1.5	3.95	47.3	52.46	4.79
	270	7.5	1.5	3.95	47.3	52.46	3.19
	720	7	1	2.63	47.4	52.57	1.96
	1260	6.5	0.5	1.32	47.6	52.79	1.49

	Trial 1	Trial 2
R_L	6	6
Total Sample Weight	40.09	40.05
Moisture Content	0.052	0.052
Oven Dry Weight (Co)	38.01	37.97
Temp adjust value	1.109	1.109

Table B-3: Initial Particle Size Analysis Data: Manual Sieving

Sample Desciption	Trial 1	Trial 2	*Adjusted 1	*Adjusted 2
Total Weight of Sample (g)	38.01	37.91	34.38	33.39
Weight of Paper (g) No. 30	0.94	0.95	0.94	0.95
Weight of Paper and Dry Soil (g) No. 30	4.54	4.53	4.54	4.53
Weight of Dry Soil (g) No. 30	3.6	3.58	3.6	3.58
Weight of Paper (g) No. 60	0.93	0.94	0.93	0.94
Weight of Paper and Dry Soil (g) No. 60	10.49	10.68	10.49	10.68
Weight of Dry Soil (g) No. 60	9.56	9.74	9.56	9.74
Weight of Paper (g) No. 120	0.92	0.95	0.92	0.95
Weight of Paper and Dry Soil (g) No. 120	7.35	7.38	7.35	7.38
Weight of Dry Soil (g) No. 120	6.43	6.43	6.43	6.43
Weight of Paper (g) No. 200	0.94	0.94	0.94	0.94
Weight of Paper and Dry Soil (g) No. 200	3.73	3.58	3.73	3.58
Weight of Dry Soil (g) No. 200	2.79	2.64	2.79	2.64
Weight of Day Sample <75um	15.63	15.52	12	11
Percent of Mass Retained on No. 30 Sieve	0.09	0.09	1.47	0.11
Percent of Mass Retained on No. 60 Sieve	0.25	0.26	0.28	0.29
Percent of Mass Retained on No. 120 Sieve	0.17	0.17	0.19	0.19
Percent of Mass Retained on No. 200 Sieve	0.07	0.07	0.08	0.08

* Adjusted to account for mass lost during manual sieving

Table B-4: Initial Organic Matter Content Data: Loss On Ignition Method

Sample	Replicate	Weight of Crucible (400°C) (g)	Weight of Soil and Crucible (105°C) (g)	Weight of Soil and Crucible (400°C) (g)	Weight of Soil (105°C) (g)	Weight of Soil (400°C) (g)	LOI (%)	LOI (mg/kg)
Sieved	1	7.47	10.00	9.85	2.53	2.38	6.02	60184
Sieved	2	7.82	10.68	10.51	2.86	2.68	6.11	61079
Sieved	3	6.26	9.12	8.94	2.86	2.68	6.13	61270
Sieved	4	6.94	9.48	9.33	2.55	2.39	5.98	59780
Sieved	5	7.46	9.98	9.82	2.51	2.36	6.04	60425
Sieved and Extracted	1	7.47	10.28	10.19	2.81	2.72	3.10	31005
Sieved and Extracted	2	7.82	10.73	10.64	2.90	2.81	3.06	30586
Sieved and Extracted	3	6.26	9.55	9.45	3.29	3.19	3.18	31813
Sieved and Extracted	4	6.94	10.02	9.93	3.08	2.99	3.01	30122
Sieved and Extracted	5	7.46	10.81	10.71	3.35	3.25	3.03	30251

Table B-5: Initial Hydrocarbon Content Data

Sample Description	Replicate	Moisture content (g/g)	Wet Soil extracted (g)	Dry Soil Extracted (g)	Empty Tray (g)	Tray and Extract (g)	Hydrocarbon Weight (g)	Hydrocarbon content (mg/kg)
Beginning	1	0.052	20.18	19.13	2.51	3.31	0.80	43370
Beginning	2	0.052	21.13	20.03	2.51	3.34	0.83	43209
Beginning	3	0.052	22.40	21.24	2.50	3.38	0.88	43156
Beginning	4	0.052	20.15	19.10	2.51	3.30	0.79	43241
Beginning	5	0.052	20.40	19.34	2.53	3.34	0.81	43521
Beginning	6	0.052	20.88	19.79	2.54	3.37	0.82	43403
Beginning	7	0.052	21.02	19.93	2.52	3.36	0.83	43528
Beginning	8	0.052	23.91	22.67	2.47	3.42	0.95	43531
Beginning	9	0.052	23.51	22.28	2.51	3.45	0.94	43981
Beginning	10	0.052	21.12	20.02	2.50	3.33	0.83	43060
Beginning	11	0.052	25.53	24.20	2.51	3.53	1.01	43692
Beginning	12	0.052	20.32	19.26	2.49	3.29	0.81	43621
End	1	0.04	19.95	19.08	1.59	2.37	0.77	42066
End	2	0.04	21.00	20.08	1.60	2.40	0.81	41921
End	3	0.04	21.45	20.52	1.59	2.42	0.83	42054
End	4	0.04	22.41	21.43	1.60	2.47	0.87	42366
End	5	0.04	21.70	20.76	1.58	2.45	0.87	43776
End	6	0.04	21.38	20.45	1.58	2.41	0.83	42437
End	7	0.04	20.36	19.48	1.59	2.38	0.79	42438
End	8	0.04	21.83	20.88	1.60	2.45	0.85	42620
End	9	0.04	20.85	19.94	1.58	2.40	0.82	42893
End	10	0.04	23.40	22.39	1.58	2.48	0.90	42049
End	11	0.04	21.36	20.43	1.58	2.41	0.83	42454
End	12	0.04	21.17	20.25	1.58	2.41	0.83	42478

Table B-6: Initial Asphaltene Content of Oil in Soil Data

Sample	1	2	3
Air Dry Weight of Soil (g)	20.58	20.27	22.74
Moisture Content (g/g)	0.05	0.05	0.05
Oven Dry Weight of Soil (g)	19.51	19.21	21.56
Weight of Beaker (g)	50.74	50.48	50.44
Weight of Beaker and Extract (g)	51.57	51.29	51.36
Weight of Maltenes Beaker (g)	50.81	50.74	50.81
Weight of Pentane Insolubles and Beaker (g)	50.80	50.53	50.51
Weight of Beaker and Maltenes (g)	51.57	51.50	51.64
Mass of Oil	0.83	0.81	0.92
Mass of Pentane Insolubles	0.06	0.05	0.06
Mass of Maltenes (g)	0.76	0.76	0.83
Hydrocarbon Content (mg/kg)	44313	43887	44330
Percent Asphaltenes (%)	6.93	5.70	6.97

Table B-7: Slurry Reproducibility Test

Sample	Weight of Oven-Dry Solids Extracted (g)	Hydrocarbon Weight (g)	Hydrocarbon content (mg/kg)
1	19.80	0.78	41055
2	19.45	0.77	40965
3	19.86	0.76	40019
4	19.66	0.77	40951
5	19.57	0.76	40182

Table B-8: Initial Autoclaved and Extracted Soil Data

Sample Description	Moisture content (g/g)	Wet Soil extracted (g)	Dry Soil Extracted (g)	Empty Tray (g)	Tray and Extract (g)	Hydrocarbon Weight (g)	Hydrocarbon content (mg/kg)
1	0.05	20.17	19.11	1.58	2.38	0.80	43814
2	0.05	20.06	19.01	1.57	2.37	0.80	43661
3	0.05	20.53	19.45	1.60	2.44	0.83	44769

Table B-9: Water Loss Data for Trial Run

Flask	Initial Weight (g)	Final Weight (g)	Water Loss (g)	Average Loss (g)
TT20-D1-R1	194.02	193.72	0.296	0.42
TT20-D1-R2	209.94	209.45	0.485	
TT20-D1-R3	210.60	210.17	0.430	
TC20-D1	211.56	211.08	0.480	
TT20-D3-R1	180.31	179.53	0.775	1.02
TT20-D3-R2	206.91	206.03	0.879	
TT20-D3-R3	214.67	213.10	1.570	
TC20-D3	207.54	206.69	0.858	
TT20-D7-R2	207.44	205.37	2.072	2.09
TT20-D7-R3	199.35	197.21	2.141	
TC20-D7	201.63	199.59	2.046	
TT20-D14-R1	225.75	217.65	8.100	
TT20-D14-R2	206.70	202.12	4.583	4.76
TT20-D14-R3	207.74	203.03	4.714	
TC20-D14	205.62	200.63	4.985	

Table B-10: Liquid-liquid Extraction Data from Trial Run

Flask	Weight of Empty Tray (g)	Weight of Tray and Extract (g)	Weight of Oil (g)	Weight of Oil (mg)	Hydrocarbon Content of Soil (mg/kg)	Combined Liq-liq with soil (mg/kg)
TT20-D1-R1	1.58	1.59	0.004	4	38007	38011
TT20-D1-R2	1.59	1.59	0.0036	3.6	37240	37244
TT20-D1-R3	2.54	2.54	0.0036	3.6	38668	38672
TC20-D1	2.53	2.53	0.0029	2.9	38446	38449
TT20-D3-R1	1.59	1.59	0.0041	4.1	39574	39578
TT20-D3-R2	1.58	1.59	0.0048	4.8	39078	39083
TT20-D3-R3	1.59	1.59	0.0018	1.8	38641	38643
TC20-D3	1.58	1.58	0.0021	2.1	39729	39731
TT20-D7-R1	2.51	2.51	-0.0003	-0.3	43312	43312
TT20-D7-R2	2.54	2.54	0.001	1	35093	35094
TT20-D7-R3	2.53	2.53	0.0047	4.7	35220	35225
TC20-D7	2.49	2.49	0.0018	1.8	40429	40431
TT20-D14-R1	1.59	1.59	0.0022	2.2	32068	32070
TC20-D14	1.59	1.60	0.0024	2.4	46289	46291

Table B-11: Nutrient Data

Treatment 20ºC								
	Nutrient							
Time Interval (days)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)	SO_4^{2-} (mg/L)	Cl^- (mg/L)	Na^+ (mg/L)	NH_4^+ (mg/L)	Mg_2^+ (mg/L)	Ca_2^+ (mg/L)
21 (rep1)	101	<det	48	N/A	N/A	N/A	N/A	N/A
21 (rep2)	76	<det	195	N/A	N/A	N/A	N/A	N/A
45 (rep1)	92	<det	84	N/A	N/A	N/A	N/A	N/A
45 (rep2)	137	<det	113	N/A	N/A	N/A	N/A	N/A
45 (rep3)	105	<det	101	N/A	N/A	N/A	N/A	N/A
60 (rep1)	54	<det	77	N/A	N/A	N/A	N/A	N/A
60 (rep2)	119	<det	98	N/A	N/A	N/A	N/A	N/A
90 (rep1)	230	<det	93	N/A	N/A	N/A	N/A	N/A
90 (rep2)	245	<det	98	54	N/A	N/A	N/A	N/A
121 (rep1)	432	<det	171	55	275	<det	27	139
121 (rep2)	369	<det	99	212	163	<det	20	111
121 (rep3)	480	<det	162	42	234	<det	25	131
Treatment 5ºC								
	Nutrient							
Time Interval (days)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)	SO_4^{2-} (mg/L)	Cl^- (mg/L)	Na^+ (mg/L)	NH_4^+ (mg/L)	Mg_2^+ (mg/L)	Ca_2^+ (mg/L)
7 (rep1)	266	12	83	0.74	N/A	N/A	N/A	N/A
7 (rep1)	262	11	79	7	N/A	N/A	N/A	N/A
10 (rep1)	268	5	90	N/A	80	28	25	96
10 (rep2)	262	4	81	N/A	N/A	N/A	N/A	N/A
21 (rep1)	281	2	88	N/A	N/A	N/A	N/A	N/A
21 (rep2)	283	<det	85	N/A	N/A	N/A	N/A	N/A
30 (rep1)	280	<det	90	N/A	N/A	N/A	N/A	N/A
60 (rep1)	250	<det	107	N/A	N/A	N/A	N/A	N/A
60 (rep2)	213	<det	148	N/A	N/A	N/A	N/A	N/A
90 (rep1)	284	<det	103	N/A	N/A	N/A	N/A	N/A
90 (rep2)	266	<det	94	N/A	N/A	N/A	N/A	N/A

Table B-12: Total Extractable Hydrocarbons Remaining in Control Flasks

Time	TEH Remaining at 5°C	TEH Remaining at 20°C
1	40511	41353
1	39294	41524
3	40842	40173
3	40879	40229
3	40839	41459
3	40786	outlier
5	39834	41626
5	41526	40025
7	40500	38715
7	40424	39589
7	39306	42653
7	38233	44847
10	40069	39472
10	40226	40283
14	39111	39592
14	39155	39010
14	39770	40526
14	39931	40162
21	38593	38640
21	39156	40078
30	40276	37883
30	40903	39344
30	40942	39049
30	outlier	38857
45	38781	39150
45	39713	41076
60	38247	39764
60	38805	38867
60	40073	38796
60	40091	39279
75	37757	39089
75	37840	39393
90	39154	38168
90	39670	38676
90	38404	38328
90	38327	37275
121	39012	39202
121	38340	37643
121	39037	39122
121	39511	outlier

Table B-13: Total Extractable Hydrocarbons Remaining in Treatment Flasks

Time	TEH Remaining at 5°C	TEH Remaining at 20°C	Time	TEH Remaining at 5°C	TEH Remaining at 20°C
1	39433	40459	30	39640	27966
1	41065	40758	30	39489	28733
1	40732	41533	30	39742	29318
3	39835	39940	30	39677	28047
3	39948	39519	30	39627	28136
3	40313	39676	30	40351	28577
3	40930	39855	30	40504	28501
3	40687	39973	30	40222	27968
3	40487	38626	30	40108	27338
3	39191	37967	30	40405	outlier
3	40236	38130	45	37141	28069
3	39614	38359	45	35837	28617
3	40582	38171	45	37017	26568
5	40669	36914	60	36032	24846
5	40347	37444	60	35178	25065
5	39985	37010	60	35360	25702
7	40333	34793	60	35130	26288
7	41294	33604	60	34744	26606
7	41385	34367	60	36051	26572
7	40542	34534	60	35609	25881
7	42425	33543	60	35982	25974
7	41382	34490	60	35982	26168
7	40682	34519	60	35480	26766
7	40959	36111	75	33290	24835
7	39795	35385	75	33170	25096
7	39780	34389	75	32920	23912
10	39210	32987	90	34032	24041
10	39274	34308	90	33882	24091
10	39676	32957	90	33315	23216
14	38240	32896	90	34305	23428
14	38350	32657	90	33684	23656
14	38291	31956	90	35085	23648
14	39585	32923	90	33834	23226
14	39219	32691	90	33888	23404
14	39523	32908	90	33450	23418
14	39945	32544	90	34745	24432
14	40148	32921	121	33012	23912
14	39793	33111	121	32815	21761
14	40053	33393	121	33228	22849
21	38430	31486	121	32694	22130
21	38647	30480	121	33086	23026
21	38210	30882	121	32934	22090
			121	31710	22523
			121	32754	22849
			121	31980	22583
			121	34638	22357

Table B-14: 20°C Treatment Most Probable Numbers (20°C Incubation)

Heterotrophs						
Day	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Average**	**Standard Deviation**	**Standard Error**
1	2883827	788302	3108432	2260187	1279627	738793
3	6767546	5076058	3552820	5132141	1608097	928435
5	57267838	31084323	28838369	39063510	15805355	9125226
7	67675457	38235089	42122256	49344267	15993808	9234030
10	79347898	31084323	42122256	50851492	25288196	14600147
14	79347898	67675457	110180333	85734563	21960379	12678831
21	23343422	22111096	31084323	25512947	4864137	2808311
30	7934790	21201452	23343422	17493221	8346839	4819050
45	143632858	50760581	91807945	95400461	46540247	26870024
60	4212226	9299413	3823509	5778383	3055490	1764088
75	1101803	5076058	N/A	3088931	2810223	1987128
90	3823509	7934790	3823509	5193936	2373649	1370427
121	4212226	2883837	3108432	3401498	711035	410516
Oil Degraders						
Day	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Average**	**Standard Deviation**	**Standard Error**
1	1808240	929941	793479	1177220	550722	317960
3	3823509	1808240	2334342	2655364	1045284	603495
5	5076058	18082395	12117066	11758506	6510578	3758884
7	14363286	4212226	18082395	12219302	7179338	4144993
10	5076058	6767546	4212226	5351943	1299808	750444
14	42122256	3552820	1808240	15827772	22788392	13156884
21	18082395	9102116	173874	9119462	8954273	5169752
30	1808240	4639620	N/A	3223930	2002088	1415690
45	793479	928573	707250	809767	111557	64407
60	310843	676755	1436329	807976	574103	331458
75	233434	1808240	N/A	1020837	1113556	787403
90	110180	572678	676755	453204	301591	174124
121	355282	1436329	310843	700818	637359	367979

Table B-15: 20°C Most Probable Number (5°C Incubation)

Heterotrophs						
Day	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Average**	**Standard Deviation**	**Standard Error**
1	233434	N/A	N/A	233434		
3	507606	1101803	310843	640084	411785	237744
5	1198930	310843	929941	813238	455400	262925
7	11989	921223	929941	621051	527481	304541
10	119893	143633	507606	257044	217317	125468
14	288384	382351	382351	351029	54252	31322
21	1436629	2334342	N/A	1885486	634779	448857
30	11989	180824	180824	124546	97477	56278
45	2334342	1808240	1808240	1983607	303745	175367
60	5727	4212	N/A	4970	1071	758
75	18	120	N/A	69	72	51
90	144	180	N/A	162	26	15
121	120	120	120	120	0	0
Oil Degraders						
Day	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Average**	**Standard Deviation**	**Standard Error**
1	233434	233434	180824	215897	30374	17537
3	288384	421223	352694	354100	66431	38354
5	3108	1808	1808	2241	751	433
7	57268	272381	928573	419407	453878	262047
10	288384	929941	676755	631693	323144	186567
14	572678	221111	507606	433798	187044	107990
21	1436	234216	2211110	815587	1214150	700990
30	221111	507606	54078	260932	229371	132428
45	42122	46396	31084	39867	7901	4562
60	6768	7935	11018	8574	2196	1268
75	4212	1102	N/A	2657	2199	1555
90	2211	221	507	980	1076	621
121	2211	221	507	980	1076	621

Table B-16: 5°C Treatment Most Probable Number (20°C Incubation)

Heterotrophs						
Day	Replicate 1	Replicate 2	Replicate 3	Average	Standard Deviation	Standard Error
1	929941	N/A	916271	923106	9666	6835
3	567606	233434	463962	421667	171054	98758
5	421223	221111	221111	287815	115535	66704
7	3823509	1082492	1808240	2238080	1420164	819932
10	5014067	9299413	5076058	6463179	2456446	1418230
14	2334342	1738740	2723814	2265632	496118	286434
21	4329620	4212226	N/A	4270923	83010	58697
30	5726784	7934790	3823509	5828361	2057522	1187911
45	50760581	35307678	79823723	55297327	22602126	13049344
60	91807945	148959834	92994126	111253968	32659623	18856042
75	143632858	50760581	105604161	99999200	46689149	26955993
90	57267838	70089660	57267838	61541779	7402682	4273941
121	42122256	42122256	N/A	42122256	0	0
Oil Degraders						
Day	Replicate 1	Replicate 2	Replicate 3	Average	Stardard Deviation	Standard Error
1	310943	173874	233434	239417	68730	39681
3	110180	57268	57268	74905	30549	17637
10	3108432	5076058	1738740	3307743	1677563	968541
14	5726784	1808240	2211110	3248711	2155507	1244482
21	676755	91702	604983	457813	319086	184224
30	916271	793479	676755	795502	119771	69150
45	5726784	11018033	1808240	6184352	4621915	2668464
60	5076658	3108432	5726784	4637291	1363350	787131
75	6049829	4212226	1808240	4023432	2127088	1228075
90	1808240	3108432	2883837	2600170	694964	401238

Table B-17: 5°C Treatment Most Probable Number (5°C Incubation)

Heterotrophs						
Day	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Average**	**Standard Deviation**	**Standard Error**
1	18082	23343	23343	21589	3037	1754
3	31084	18082	N/A	24583	9194	6501
5	180824	N/A	46396	113610	95055	54880
7	310843	1202298	929941	814361	456828	263750
10	1198930	1198930	14363286	5587049	7600444	4388119
14	92994	38235	143633	91621	52712	30434
21	233434	180824	9299413	3237890	5249499	3030799
30	180824	793479	310843	428382	322797	186367
45	119893	233434	929941	427756	438595	253223
60	1808240	1775185	3108432	2230619	760388	439010
75	7982372	5076058	11989305	8349245	3471195	2004095
90	67675	119893	42122	76563	39640	22886
121	1199	11989	N/A	6594	7630	5395
Oil Degraders						
Day	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Average**	**Standard Deviation**	**Standard Error**
1	50.76	79.35	18.08	49	31	18
3	2334	5076	9299	5570	3509	2026
7	5497	6768	4212	5492	1278	738
10	3108432	929941	138543	1392305	1537984	887956
14	233434	805745	929941	656373	371503	214487
21	35528	N/A	604983	320256	402665	284728
30	23343	5727	23343	17471	10171	5872
45	4212226	1730578	2334342	2759049	1294189	747201
60	1808240	793479	2334342	1645354	783239	452203
75	572678	676755	1436329	895254	471465	272201
90	18082	119893	676755	271577	354568	204710

Table B-18: Percentage of Total Sample for GC Fractions at 20°C

Time (days)	C_{10}-C_{16} (%)	C_{16}-C_{24} (%)	C_{24}-C_{36} (%)	C_{36}-C_{40} (%)
1	4.2	29.4	32	13.6
1	4.2	29.9	32.7	13.9
1	4.1	28.9	31.8	13.8
3	2.7	28.8	32	13.5
3	2.3	29.3	34.1	14.5
3	2.6	28	32.5	14.2
5	2.2	26.8	30.7	13.2
5	2	27.4	32.3	13.9
5	1.8	26.9	31.5	13.5
7	1.7	27.7	33.6	14.3
7	1.8	26.2	32.4	14
7	2.1	26.4	31.9	13.5
10	1.8	25.8	32.1	13.9
10	1.9	25.6	31.9	13.7
10	1.8	25.1	31.6	13.7
14	3.2	25.1	30.7	13.4
14	3.1	24.9	30.7	13
14	3.6	25.7	31.3	13.2
21	2.5	25.5	33.7	14.4
21	2.5	23.4	31.3	13.6
30	1	22.5	33.4	14.7
30	2	22.5	31.7	13.6
30	1.6	23	32.7	13.8
45	1.6	21.5	32	14.2
45	1.5	21.4	32	14
45	1.4	20.7	30.8	13.5
60	1	21.5	35.1	15.2
60	1.2	20.8	32	13.7
60	1.2	21.2	32.5	13.7
75	1.1	20.1	32.5	14.6
75	0.4	18.4	31.3	13.5
75	0.6	19.9	31.5	13.6
90	1.3	20.5	33.2	13.7
90	1.1	19.9	33.2	14
90	1.3	19.2	31.2	13.8
121	0.3	20.4	35.7	15.1
121	0.3	18.9	34.3	14.5
121	0.5	18.1	32.9	14.2

Table B-19: Percentage of Total Sample for GC Fractions at 5°C

Time	C_{10}-C_{16} (%)	C_{16}-C_{24} (%)	C_{24}-C_{36} (%)	C_{36}-C_{40} (%)
1	3.3	29	32.7	14
1	3.4	30.2	33.4	13.9
3	3.7	27.2	30.7	13.6
3	3.9	29.8	33.6	14.1
3	1.6	29.2	33.8	14.4
5	3.2	27.1	30.3	13.3
5	1.1	28.5	33.7	14.4
5	1.2	28.3	33.2	14.2
7	2.1	28.6	33.5	14.3
7	2.5	29	33.1	14.1
7	2.1	28.6	33.2	14
10	1.8	27.3	31.8	13.9
10	2	27.5	32.2	13.9
10	1.9	27.8	32.6	14
14	1.4	25.9	31.9	13.9
14	1.8	27.8	33.1	14.2
14	2	28.3	32.7	14
21	1.8	28.6	33.9	14.5
21	1.9	27	31.9	13.8
21	2.2	27.3	32.1	13.7
30	2.8	27.5	31.3	13.9
30	3.6	27.7	30.9	13.5
30	2.9	27.8	31.4	13.6
45	3.2	27	31	13.6
45	2.9	26.4	30.9	13.6
45	3.3	27.1	31	13.6
60	1.6	27.5	33.5	14.3
60	2.6	26.9	31.8	13.6
60	1.7	26.4	32	13.7
75	1.4	24.6	31.7	14.1
75	1.3	24.6	30.7	13.4
75	1.7	25.2	31.5	13.7
90	1.2	23.7	30.2	13.6
90	1.4	25.4	32.2	13.8
90	1.5	26	32.5	13.9
121	0.2	22.5	30.8	13.5
121	0.1	22.1	31.4	13.8
121	0.2	22.5	31.9	13.8