THE INFLUENCE OF COLD CLIMATE SEASONAL TEMPERATURE REGIMES ON BIOREMEDIATION OF PETROLEUM HYDROCARBON-CONTAMINATED SOILS

by

Wonjae Chang

Department of Civil Engineering and Applied Mechanics
McGill University, Montreal

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Abstract

Cold-adapted hydrocarbon-degrading bacteria are able to survive and grow in petroleum-contaminated soils in Arctic and sub-Arctic sites, and have been the basis for consideration of bioremediation technologies for clean-up of these sites. Several laboratory studies have also shown that petroleum hydrocarbon biodegradation rates by cold-adapted hydrocarbon-degrading bacteria are influenced by incubation temperatures, and those studies have employed constant incubation temperatures. However, temperatures in cold climates are dynamic due to diurnal, periodic or seasonal temperature variations and variability in incoming solar radiation, and these temperature changes may influence on-site soil microbial activity. To date there has been very little research focused how variations in site temperatures and various seasonal temperature regimes influence biodegradation performance.

The overall objective of this research was to investigate the rate and extent of total petroleum hydrocarbon (TPH) biodegradation in contaminated soils from a sub-Arctic site under site-relevant temperature regimes in cold climates and to assess the effect of different temperature regimes on TPH biodegradation activity. A series of pilot-scale biodegradation experiments was conducted using field-aged petroleum-contaminated soils shipped from a former military Distant Early Warning (DEW) line site in Resolution Island, Nunavut, Canada (61°30'N 65°00'W).

Pilot-scale landfarming experiments were performed in a laboratory in soil tanks under site temperature profiles representative of the 3-year site air temperatures in July and August where temperature varied uniformly between 1 °C to 10 °C over 10 days. The rates and extent of biodegradation of the non-volatile, higher molecular weight hydrocarbons (F3) was significant and comparable to the rates and extent of biodegradation observed for semi-volatile, lower molecular weight fraction (F2). The first-order biodegradation rate constants for the F2 and F3 hydrocarbon fractions were similar to each other in both low initial TPH and high initial TPH landfarms and estimated to be 0.011 to 0.024 day^{-1} and 0.016 to 0.019 day^{-11}, respectively. Changes in ratios of residual concentrations of C14, C16 and C18 alkanes with progressive biodegradation was tracked, and showed that as TPH levels declined, the relative abundance of the higher-molecular weight alkanes declined. Soil aggregates with diameters ranging from 0.6 mm to 2 mm contained residual TPH that was not bioavailable and thus controlled the effective endpoint of biodegradation.

Variable temperatures representative of site temperature changes between 1 and 10 °C over two months dramatically influenced the rates and extent of biodegradation of petroleum hydrocarbons compared to the rates and extent obtained under a constant average incubation temperature of 6 °C. Under the variable site temperature condition, more rapid biodegradation of both semi- and non-volatile hydrocarbons occurred by over a factor of two due to accelerated bioactivity and growth of indigenous hydrocarbon-degrading microbial populations. Preferential biodegradation of semi-volatile hydrocarbons over non-volatile hydrocarbons was significant in

the constant average temperature mode, but not under the variable temperature regime. The biodegradation rates determined by the variable site temperature approximation were in better agreement with those determined by an on-site experiment at the same site.

A study was undertaken to quantitatively assess biodegradation of petroleum hydrocarbons and microbial respiration and response during the seasonal transition periods preceding and following summer where freezing and thawing of the surface soil layers occurs at the sub-Arctic site. During the freezing phase, a statistically significant extent of biodegradation of 13% of semi-volatile hydrocarbons occurred, which was correlated with the emergence of *Corynebacterineae*-related hydrocarbon-degrading bacteria and growth of heterotrophic microbial populations. Petroleum hydrocarbon biodegradation, microbial respiration and changes in the size and composition of microbial community occurred under sub-zero temperatures but only when there was substantial liquid, unfrozen pore waters coexisting with pore ice. A rapid rate of temperature increase caused a burst of microbial respiration activity as previously observed in several studies, and it was found that this microbial activity also resulted in significant reductions of semi- and non-volatile hydrocarbon concentrations of up to 25% and 11%, respectively.

Résumé

Les bactéries capables de dégrader les hydrocarbures adaptées au froid sont en mesure de survivre et de croître dans les des sols contaminés au pétrole dans les sites arctiques et subarctiques, et ont été la base de l'examen des technologies de biorestauration pour le nettoyage de ces sites. Plusieurs études de laboratoire ont également démontré que les taux de biodégradation des hydrocarbures pétroliers par bactéries d'hydrocarbures biodégradables adaptées au froid sont influencés par la température d'incubation, et ces études ont employé des températures d'incubation constante. Toutefois, les conditions de température de terrains dans les climats froids sont dynamiques dues aux variations de température diurnes, périodiques ou saisonnières et de la variabilité du rayonnement solaire entrant, et ces changements de température peuvent influencer l'activité microbienne du sol sur le site. À date, très peu de recherches ont été axées sur comment le régime des variations des températures du site et des températures saisonnières variées influent le rendement de la biodégradation.

L'objectif global de cette recherche était d'étudier le taux et l'étendue de la biodégradation des hydrocarbures pétroliers totaux (HPT) dans les sols contaminés à partir d'un site subarctique, sous des régimes de température de sites pertinents touchés par des climats froids, et d'évaluer l'effet de différents régimes de température sur la biodégradation des HPTs. Une série d'expériences de biodégradation à échelle pilote a été réalisées en utilisant du sol âgé contaminé au pétrole expédié à partir d'un ancien site militaire de réseaux de radars, le Distant Early Warning (DEW), dans l'île Resolution, au Nunavut, Canada (61° 30' 1"N 65° 0' 1"O).

Des expériences d'épandage contrôlé à une échelle pilote ont été réalisées dans un laboratoire dans des réservoirs contenant du sol représentant des profils de température du site durant 3 années en juillet et août, où la température varie de manière uniforme entre 1 °C à 10 °C pendant 10 jours. Les taux et l'étendue de la biodégradation des hydrocarbures non volatils de masse moléculaire plus élevés (F3) a été significative et comparable aux taux et à l'importance de la biodégradation observés pour les semi-volatils, de masse moléculaire moins élevés (F2). Les constantes de vitesse de biodégradation du premier ordre pour les fractions d'hydrocarbures F2 et F3 étaient semblables les unes des autres. Cela était vrai dans l'épandage contrôlé avec des nivaux de HPTs initiaux bas, ainsi qu'avec les nivaux de HTPs initiaux élevés, et cela est estimée de 0,011 à 0,024 jour^{-1} et de 0,016 à 0,019 jour^{-1}, respectivement. Les modifications de ratios des concentrations résiduelles des alcanes C14, C16 et C18 avec une biodégradation progressive a été suivie à la trace, et cela a démontré qu'une réduction des niveaux de HTPs entraîne une diminution de l'abondance relative des alcanes à haut poids moléculaire. Les agrégats de sols avec des diamètres allant de 0,6 mm à 2 millimètres contenaient des HPTs résiduels qui ne sont pas donc biodisponibles, ont contrôlé le point d'aboutissement efficace de la biodégradation.

Les variables de températures représentatives des changements de température du site entre 1° et 10 °C entre une période de deux mois ont considérablement influencé la vitesse et l'étendue de la biodégradation des hydrocarbures pétroliers par rapport aux taux et l'étendue obtenus sous une température d'incubation moyenne constante de 6 °C. Sous la température

variable du site, une biodégradation plus rapide d'à la fois les hydrocarbures semi-volatils et non volatils s'est produite à un facteur de deux, près en raison d'une bioactivité accélérée, et d'une croissance des populations microbiennes indigènes qui prospèrent en décomposant les hydrocarbures. La biodégradation préférentielle des hydrocarbures semi-volatils par rapport aux hydrocarbures non volatils fut significative dans le mode avec température moyenne constante, mais pas sous le régime de température variable. Les taux de biodégradation déterminés par l'approximation des variables de température du site ont été en meilleur accord avec celles déterminées par les expériences sur place du même site.

Une étude a été entreprise afin d'évaluer quantitativement la biodégradation des hydrocarbures pétroliers, ainsi que la respiration et réponse microbienne au cours des périodes de transition saisonnière précédant et suivant la saison d'été, où le gel et le dégel des couches superficielles du sol se produisent au site subarctique. Pendant la phase de congélation, une étendue statistiquement significative de la biodégradation de l'ordre de 13% des hydrocarbures semi-volatils s'est produite, ce qui a été corrélé avec l'émergence des bactéries d'hydrocarbures biodégradables *Corynebacterineae* connexes et la croissance des populations microbiennes hétérotrophes. La biodégradation des hydrocarbures pétroliers, la respiration microbienne et les changements dans la taille et la composition des communautés microbiennes sont apparus sous des températures au-dessous de zéro, mais seulement ou il y avait du liquide non gelées substantiel dans les pores du sol, coexistant avec de la glace interstitielle. Un taux rapide d'augmentation de température a provoqué une poussée d'activités de respiration microbienne comme précédemment observée dans plusieurs études. Il a été constaté que cette activité microbienne a également entraîné de manière significative des réductions de concentrations d'hydrocarbures semi-volatils et non volatils allant jusqu'à 25% et 11%, respectivement.

Dedication

This thesis is dedicated to my family

They encouraged all my enthusiasm

And

They believed in me

Without you

This thesis would never have been completed

Thank you

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Contribution of Authors

The Methods, Results, Discussion and Conclusion sections of the thesis is presented in Chapters 3-5 as three journal manuscripts, 1 of which is accepted and 2 are to be submitted.

Authorships of three articles are explained as below:

Chapter 3
Biodegradation of semi- and non-volatile petroleum hydrocarbons in aged, contaminated soils from a sub-arctic Site: laboratory pilot-scale experiments at site temperatures. (2010). Accepted in *Chemosphere*

W. Chang, M. Dyen, L. Spagnuolo, P. Simon, L. Whyte, S. Ghoshal

Experimental design and execution, including operation of pilot-scale reactors, as well as all the analysis of the data and the writing of the manuscripts were conducted by W. Chang. Supporting data on microbial characterization was provided by M. Dyen. L. Whyte and S. Ghoshal provided supervision of the research and experimental design, and editing the manuscript. L. Spagnuolo and P. Simon provided input on relevant experimental conditions that were representative of field implementation and provided soil samples for the study.

Chapter 4:
Comparison of the effects of time-varying site temperatures and constant incubation temperatures on the biodegradation of petroleum hydrocarbons in pilot-scale experiments with contaminated soils from a cold regions site. To be submitted to Chemosphere, March 2010.
W. Chang, L. Whyte, S. Ghoshal

Experimental design and work were conducted by W. Chang as well as all the analysis of the data and the writing of the manuscripts. L. Whyte and S. Ghoshal provided supervision of the research and editing the manuscript.

Chapter 5:
Hydrocarbon biodegradation under sub-zero seasonal freeze-thaw and post thaw soil temperature regimes in contaminated soils from a sub-Arctic site. To be submitted to *Environmental Science and Technology*, March 2010.

W. Chang, S, Klemm, L. Whyte, S. Ghoshal

Experimental design of the long-term freeze-thaw experiments, temperature program design and execution of pilot-scale reactors, petroleum hydrocarbon, soil respiration, and soil moisture data, data analyses and the writing of the manuscripts was done by W. Chang. All the microbial assessments of soil sample were conducted by S. Klemm. L. Whyte and S. Ghoshal provided supervision of the research and experimental design, and editing the manuscript.

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Chapter 1

Introduction

1.1. Background: significance of petroleum hydrocarbon-contaminated soils in cold regions

The challenge of environmental protection within global cold regions, such as the Arctic and Antarctica, is further complicated as a result of accelerated environmental change and the significant need for the development of energy resources. Petroleum contamination of soils in cold regions has received significant attention since the natural environment of cold regions is particularly susceptible to human impacts. To date, many petroleum-contaminated sites identified in cold regions require active remediation and efficient site management [1] .

Hydrocarbon fuels, including arctic-grade diesel, lubricating engine oils, JP-5 fuels, and kerosene and motor oils, have been used extensively in cold regions for heating, transportation, and electricity generation, and within mining, oil and gas production facilities and equipment and military base installations. Due to the ubiquity of the above processes, soil contamination by petroleum has become a widespread issue, resulting from accidental losses, process inefficiencies, and infrastructure shortcomings within human activities. Accidental oil spillage, chronic leakage from oil transportation pipelines and on-ground or buried fuel storage tanks, the illegal disposal of fuel wastes, drilling activity, and infrastructure failure within human settlements have all contributed to increased levels of soil contamination [2]. While current practices are more controlled and less likely to be a major source of petroleum hydrocarbon contamination, the effects of previous environmentally destructive activities remain today. Site assessments for contamination revealed that presently inoperable sites of previous mining and military activity have significant petroleum contamination present within the soil [3, 4].

In the Canadian Arctic, it was estimated that there are as many as 377 contaminated sites in the Northwest Territories, Yukon, and Nunavut, many of which are contaminated with petroleum hydrocarbons. One major point-source of petroleum hydrocarbon contamination was

identified in abandoned military radar sites, specifically Distant Early Warning (DEW) Line sites, which were constructed across the Arctic during the Cold War (Fig. 1.1). In Alaska, the Exxon Valdez Oil spill of 1989 released approximately 35,500 tons of oil into Prince William Sound (PWS); it was estimated that 40% of this spilled volume directly impacted the PWS coastline [5, 6]. Approximately 6,400 contaminated sites and leaking underground storage tanks have been recorded in Alaska, of these sites, around 3,400 were cleaned up by 2006 [7].

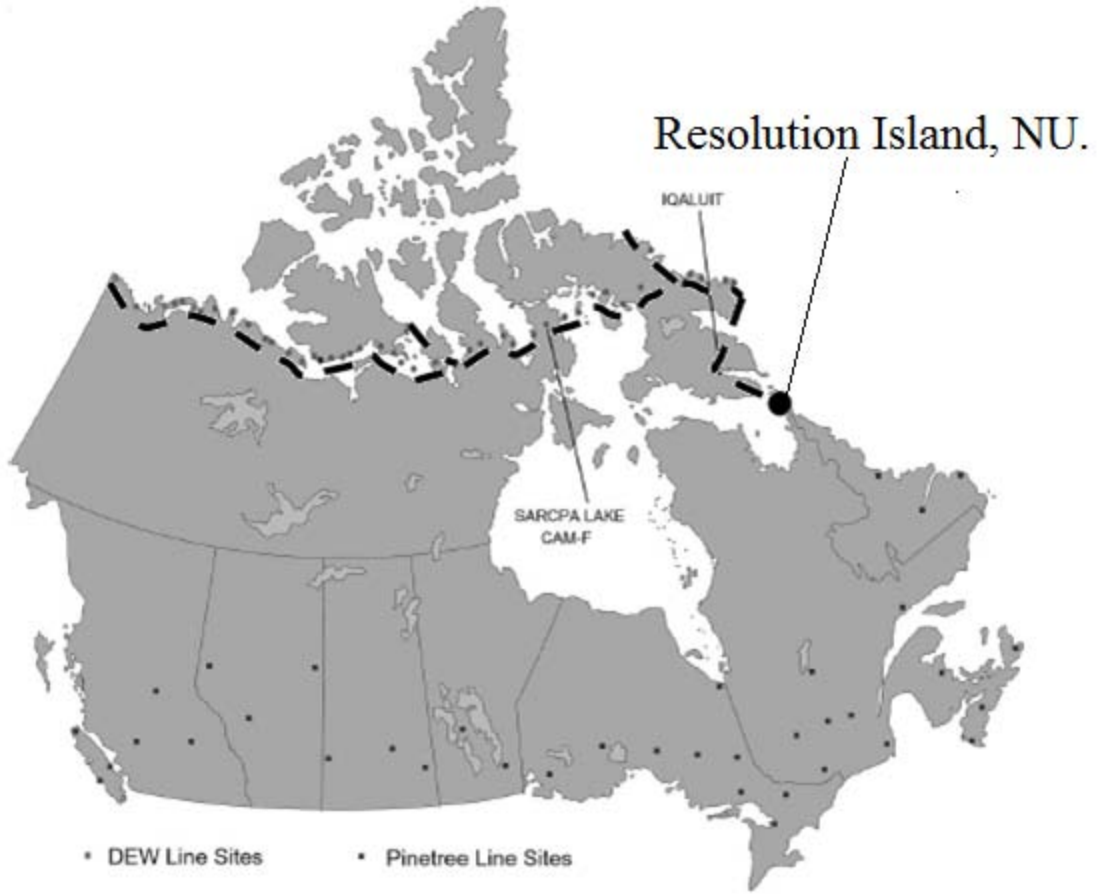

Figure 1.1 Distant Early Warning (DEW) lines served as former military radar stations in Canada during the Cold War. The dotted line refers to DEW lines. The contaminated soils for the present study were shipped from the Resolution Island site (61°30'N 65°00'W) located on the DEW lines [3].

In Antarctica, petroleum hydrocarbon soil contamination is relatively localized to drilling sites and scientific research stations, where chronic spills occurred at the abandoned research stations for over 30 years. At McMurdo Station alone, nearly 385 spills of JP8 were recorded between 1991 and 2000. It was estimated that 100,000 to 1 million m^3 of soil are contaminated to levels of >100 mg fuel/kg soil. Site investigations and preliminary studies have been conducted at close to 200 sites in Antarctica [4]. A significant number (> 100 sites) of potentially contaminated sites were also identified in Iceland, Greenland, Sweden, Norway, and Finland [4]. In Russia, the Komi oil spill of 1994, one of largest environmental disasters of the last century and due to the rupture of an aged pipeline, caused approximately 37,000 to 44,000 tonnes of oil to pour across the Siberian tundra regions and absorb into the soil [4].

Previous studies regarding the fate of petroleum hydrocarbons spilled into soils and sediments from chronically contaminated sites have indicated that residual hydrocarbons persist in the ground for long periods of time (> 10 years), and serve as a source of long-term environmental contamination. Prior studies have also suggested that the process of natural attenuation, resulting from abiotic weathering, evaporation, dissolution, and intrinsic biodegradation, is generally limited in colder regions due to cold temperature stresses (e.g. subzero temperatures and freeze-thaw cycles), nutrient deficiency, water limitation, and low indigenous microbial populations [8]. The penetration and migration of petroleum liquids to groundwater and through fractured permafrost bedrock has been reported, and the resulting environmental impacts have been documented [9, 10].

The restoration of contaminated sites is necessary to protect human health, ecosystems, and land and water resources in cold regions. Growth in public awareness concerning the presence of petroleum contamination, and the consequences for the northern areas, coupled with the commitment of governments at various levels to remedy the situation, has created a strong demand for site remediation in the broad cold regions.

The nations responsible for the regulation and protection of Antarctica and the Arctic have established a number of domestic guidelines and various legislation focused on petroleum hydrocarbon contaminated sites in cold regions. Currently, soil quality guidelines and clean-up levels vary at both the regional and nation levels, and there is no globally specialized guideline to address the characteristics of cold region contamination [4].

Active site remediation in cold regions is typically only feasible during the short thawing seasons (2-3 months per year), and, in many cases, the site conditions are challenging with respect to temperature and remoteness. On-site experiments, site characterizations, field implementations, and transportation to the sites are often prohibitively expensive. Nonetheless, significant efforts have been made to efficiently remediate petroleum contaminated soils at low temperatures. Bioremediation through the facilitation of indigenous cold-adapted microorganisms in contaminated cold soils (e.g. landfarming and biopile techniques) has been the most frequently evaluated treatment in cold environments. Currently, it is generally accepted that bioremediation is a cost-effective and minimally destructive remedial option for petroleum hydrocarbon contaminated cold soils [11].

1.2. Motivation of the study

1.2.1. Approaches

The present study focuses on the bioremediation of petroleum hydrocarbon contaminated sub-Arctic soils under temperature conditions. The emphasis of the study examines how site temperature profiles for site-relevant summer and seasonal freeze and thaw conditions during seasonal transitional periods impact the biodegradation performance of petroleum hydrocarbons in sub-Arctic soils.

Several cubic meters ~20 tons of field-aged petroleum hydrocarbon contaminated and uncontaminated (pristine) soils were excavated and shipped from a site on Resolution Island (RI; 61°30'N 65°00'W), located off the southern tip of Baffin Island in the Canadian province of Nunavut (Fig. 1.1). The RI site is situated on the former DEW lines, which were officially abandoned in 1974. The detailed site history and site characteristics are described in a study conducted by Poland et al. [3] of this and several other DEW line sites. The RI site is categorized as a sub-Arctic zone, defined as the region between the 50°N and 70°N lines of latitude where mean monthly temperatures are above 10 °C for one to three months any given year.

In general, the site soil characteristics play a key role in developing bioremediation strategies for contaminated soils. Prior to launching the main body of this study, the soil characterization study for physicochemical, microbiological and mineralogical properties was

conducted, in which smaller quantities of contaminated and uncontaminated soil (~30 kg) samples were collected aseptically at the site and shipped frozen, overnight, for use during the initial site soil analyses (results included in Chapter 3).

1.2.2. Rationale for bioremediation of petroleum hydrocarbon contaminated sites

It was initially recognized that the field-contaminated soils were highly weathered in cold climates for over thirty years. Volatile petroleum hydrocarbons (PHCs) in the range of <C10 were lacking in the weathered site soils, whereas semi-volatile PHCs (>C10-C16), non-volatile PHCs (>C16-C34), and unresolved complex mixtures (UCM), which refers to a variety of structurally complex hydrocarbon compounds such as branched, cyclic, and unsubstituted alkyl chains fractions, were the dominant hydrocarbon fractions in the site contaminated soils. A significant number of viable cold-adapted hydrocarbon-degrading microbial population (>10^5 CFU/g) were enumerated from the contaminated soils at both 4 °C and 20 °C. In addition, key catabolic genes encoding degradation enzymes for a variety of hydrocarbons were also detected in the soils. The initial characterization of site soils thus led to the evaluation of biostimulation feasibility. Biostimulation is typically considered a less destructive and more cost-effective method for the remediation of petroleum-contaminated cold region soils due to the use of indigenous microbial populations, as opposed to other remediation techniques, such as bioaugmentation, soil washing, surfactant-enhanced remediation, and thermally-enhanced remediation, which have produced mixed results in cold regions for enhancing biodegradation activity in field-contaminated soils. Landfarming was selected for this study as an implantation technique. In cold region sites, landfarming has been popularly employed and was technically feasible for the RI site of this study [12].

1.2.3. Influence of cold temperature variation in active seasons

Drastic changes in the soil temperature of the active layers and near-surface soils often occur at polar sites during summer months. Periodic and diurnal temperature changes characteristic of cold region soils are linked to fluctuations in air temperature and surface albedo response resulting from cyclic patterns of incoming solar radiation [13]. It was observed that soil

temperatures at the Resolution Island site fluctuated greatly, occasionally reaching highs of 20 °C, and that landfarm soil temperatures were also correlated with fluctuations in air temperature [3]. Generally, the majority of *ex situ* soil bioremediation in cold climates were influenced by natural temperature fluctuation with various degrees of temperature gradients.

Previous studies have demonstrated through laboratory microcosm experiments conducted at constant average temperatures (e.g. 2, 5, or 10 °C) that significant biodegradation of petroleum hydrocarbons can be achieved in contaminated soils from cold region sites, at temperatures less than 10 °C.

To develop a realistic understanding of achievable biodegradation rates and extents in the field, further research is needed to determine the effect of variable site temperatures on bioremediation performance. Very few studies have addressed the influence of site relevant summer temperatures, which vary between 1 and 10 °C, upon the rate and extent of petroleum hydrocarbons and changes in microbial respiration activity in field-contaminated soils.

The rate of petroleum hydrocarbon biodegradation is a scale-dependent variable. Since bioremediation systems are scale-up, which means mass transport (e.g. oxygen and nutrient diffusive transport), contaminant desorption-dissolution, and spatial heterogeneity become rate-controlling steps [14]. Larger-scale studies that incorporate heterogeneity both at the pilot-scale and microcosm-scale are better able to understand potential field-scale bioremediation outcomes. In this study, a series of biodegradation experiments was performed in scaled-up systems at the site temperature cycles and constant average temperature of the site.

1.2.4. Hydrocarbon composition during biodegradation: semi- and non-volatile petroleum hydrocarbons

Spilled hydrocarbons persist for long periods of time (e.g. over 30 years) in cold climates [5, 15, 16]. Generally, the process of long-term weathering (or aging) occurs on petroleum hydrocarbons in the field, and as a result low molecular weight (LMW) hydrocarbons (e.g. volatile fractions) are often lacking or at minimal levels in aged contaminated soils [17, 18]. In contrast, hydrocarbons with higher molecular weights (HMW), including alkanes (>nC16), branch, cyclic, and aromatic compounds (e.g. unresolved complex mixtures) are persistent and

often become the dominant fraction of the total petroleum hydrocarbon (TPH) in field-aged contaminated soils [19].

Previous studies have indicated that indigenous cold-adapted hydrocarbon-degrading microbial populations prevail in contaminated cold soils [20]. Some cold-adapted microorganisms are capable of the degradation of relatively persistent HMW petroleum hydrocarbons. It was reported that *Pseudomonas* spp. B17 and B18, isolated from petroleum-contaminated Arctic soils, were able to degrade several LMW alkanes (nC5-nC12) and HMW aromatic hydrocarbons (toluene and naphthalene) at both 5 °C and 25 °C [21]. Whyte et al. [22] also indicated that a psychrotrophic *Rhodococcus* sp. Q15 was able to mineralize a wide spectrum of aliphatics (C10 to C21 alkanes, branched alkanes, and a substituted cyclohexane) present in diesel fuel at 5 °C, and observed that the growth rate of the isolate increased with increasing temperatures ranging from 5 to 30 °C.

The majority of findings from relevant previous research were obtained, however, through the addition of radio-labelled and unlabelled target compounds to contaminated site soils, and thus demonstrate the biodegradability of HMW target compounds that have been recently added to the soils, rather than aged within the soils. Low bioavailability conditions due to aging may not be reflected in this biodegradation activity [18]. Sotsky et al. [17] reported that the majority of bacterial populations in field-aged contaminated soils more frequently express *xylE* genes as opposed to *alkB* genes, presumably due to the selective pressures of the more abundant residual aromatic hydrocarbons compared to lower levels of alkane hydrocarbon fractions in the composition of residual petroleum hydrocarbons found in the soils at the time of sampling. Little information is available that documents the change in petroleum hydrocarbon composition of relatively lower and higher molecular weight petroleum hydrocarbon fractions present in field-aged contaminated soils during bioremediation under cold temperature regimes.

1.2.5. Hydrocarbon biodegradation during seasonal freeze-thaw conditions

The rate of metabolic activity of cold-adapted bacteria often deviates from the Arrhenius relationship at or below freezing temperatures due to the expression of cold-active enzymes and cold stress-induced proteins (e.g. cold-shock, cold-acclimation, and anti-freezing proteins). Thus, metabolic activity of freezing-tolerant bacteria continues in cryo-environments [23]. In

uncontaminated permafrost soils, metabolic activity in indigenous cold-adapted bacteria was detected at levels as low as -20 °C [24]. Regardless of these findings, little attention has been paid to the extent of petroleum hydrocarbon biodegradation beyond the summer season, which here refers to the seasonal transitional period after the summer months and before the following thawing season.

During the seasonal transitional period, ground temperatures are dominated by seasonal freeze-thaw temperature regimes. Soil temperature profiles, obtained through soil depth analysis during field monitoring in a number of polar sites, indicate that active soil layers are slowly freezing and thawing [25-27] during this time. Previous efforts to examine the effect of soil freeze-thaw on petroleum hydrocarbon biodegradation have been based on rapid freeze-thaw cycles (relatively short-term effect) that presumably approximate diurnal or short-term freeze-thaw effects at the surface layers. These studies have shown that the repeated freeze-thaw cycles stimulated hydrocarbon biodegradation at the microcosm-scale [28, 29].

The rate of freeze-thaw cycling plays a key role in controlling the redistribution of soil solute concentrations between unfrozen water and pore ice phases within the soil [30], and influencing microbial survivability associated with the formation of intracellular ice [31]. This is an important role to consider when attempting to demonstrate hydrocarbon biodegradation activity under freeze-thaw conditions, since the rate of soil freeze and thaw essentially controls multiple stress conditions such as cold temperatures, unfrozen water availability, and osmotic stresses.

In a study conducted by Rike et al. [25] *in situ* O_2-CO_2 soil gas was measured in the active layer and permafrost soils in oil-contaminated and less contaminated areas during both the seasonally transitional and winter frozen periods in the Arctic [25]. The results of the study indicated that bioactivity within the oil-contaminated sites was extended to below subzero temperatures during the seasonal freezing period after the summer months, and suggested that *in situ* hydrocarbon biodegradation, on the basis of predicted TPH biodegradation rates, can be potentially extended to subzero temperature regimes during the seasonal freezing and thawing period. However, a quantitative measure of petroleum hydrocarbon biodegradation in aged-contaminated soils impacted by freeze-thaw was lacking from the study. There is currently little knowledge on petroleum hydrocarbon biodegradation feasibility and microbial responses under seasonal freeze-thaw conditions.

1.3. Specific objectives

This study focused on pilot-scale bioremediation of petroleum hydrocarbon contaminated soils shipped from the Resolution Island site under site temperature conditions. The overall goal of this study was to evaluate the feasibility of site soil bioremediation through the application of biostimulation under cold temperature conditions similar to those used in field landfarming operations.

The specific objectives of this study are:

1. To compare the rates and extent of biodegradation of relatively lower molecular weight (semi-volatile) and higher molecular weight (non-volatile) petroleum hydrocarbon fractions present in the field soils during biostimulation at representative time-varying site temperatures profiles;

2. To characterize the residual, non-degraded TPH and determine its distributions in soil aggregate size fractions;

3. To compare the rates and extent of biodegradation of total petroleum hydrocarbons, semi-volatile, and non-volatile hydrocarbon fractions obtained from a variable site temperature profile and a constant average temperature;

4. To assess the extent of petroleum hydrocarbon biodegradation and associated microbial activity (respiration, population size, and community changes) and its relationship to changes in temperature and unfrozen liquid water content in historically diesel-contaminated soils impacted by seasonal freeze-thaw conditions.

1.4. Scope and structure of the thesis

This thesis is a manuscript-based thesis. The submitted and/or accepted manuscripts are presented in the thesis. The co-authorships and connection texts are presented on the first page of each chapter. Chapter 2 presents a critical review of previous studies related to the relevant research topics. Chapter 3 presents the rates and extents of biodegradation of lower molecular

weight (semi-volatile) and higher molecular weight (non-volatile) petroleum hydrocarbon fractions present in the field soils during biostimulation at representative site temperatures profiles and the characterization of the residual, non-degraded TPH and its distribution in soil aggregate size fractions. Chapter 4 presents the comparison of the biodegradation performance obtained from a variable site temperature profile and a constant average temperature. Chapter 5 presents the extent of petroleum hydrocarbon biodegradation and associated microbial activity and its relationship to changes in temperature and unfrozen liquid water contents in historically diesel-contaminated soils impacted by seasonal freeze-thaw conditions. Intellectual contributions are summarized in Chapter 6.

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Chapter 2

Literature review

2.1. Hydrocarbon biodegradation under cold temperatures

Generally, psychrotolerant bacteria are metabolically active at a wide range of temperatures from subzero temperatures up to ~30 °C [1]. By definition, psychrotolerants (also referred to as psychrotrophs) grow minimally at temperature of 0-5 °C and optimally at temperatures around 20-25 °C and may have upper limits of growth at 40 °C. On the other hand, psychrophiles are specifically adapted to low-temperature growth and their minimum, optimum and maximum temperatures for growth are ≤ 0 °C , ≤ 15 °C and ≤ 20 °C, respectively [1]. The cardinal temperatures of culturable, psychrotolerant hydrocarbon-degraders generally fall within the average summer temperatures, and typically range broadly from ≥0 °C to 20 °C, or fluctuate widely (e.g. freeze/thaw or periodic fluctuation) in sub-Arctic and Antarctica sites [2-7]. On the other hand, it has been generally accepted that the growth of psychrophiles is inhibited at temperatures of greater than 15 °C [1]. In addition, psychrophiles generally exhibit greater activity in consistently cold habitats [1, 8, 9]. It has also been proposed that psychrotolerant hydrocarbon-degrading microorganisms may be more suitable than psychrophiles for the active summer treatment seasons when temperatures fluctuate [10-13].

A variety of psychrotolerant hydrocarbon-degraders isolated from oil-contaminated cold region soils were characterized. Whyte et al. [10] reported that an isolate, *Rhodococcus* sp. Q15, was able to degrade alkanes up to n-C21, as well as some branched alkanes in diesel, and, furthermore, was able to grow on dotriacontane (n-C32) at both 5 °C and 25 °C. Bej et al. [7] reported that alkane-degrading bacteria previously isolated from oil-contaminated soil from around Scott Base, Antarctica were able to grow on n-alkanes from hexane (C6) through to eicosane (C20), and on the branched alkane pristane. Representative isolates, identified in the laboratory as *Rhodococcus spp.* strains 5/14 and 7/1, were able to grow at -2 °C, but the growth rate was greatest at 15 °C, compared to -2 °C, 4 °C, and 37 °C. Aislabie et al. [11] isolated

aromatic-degrading bacteria from oil-contaminated soil in an Antarctica site. These representative strains, either *Sphingomonas spp.* or *Pseudomonas spp.*, are psychrotolerant hydrocarbon-degraders that grow rapidly at temperature up to 25 °C. The authors implied that these isolated strains can possibly grow at site ambient summer temperatures, at which surface temperatures often reached as high as ~20 °C. Specifically, *Sphingomonas sp. Ant 17* isolated from the contaminated site soils degraded various aromatic hydrocarbons including C1-, C2-, and C3-substituted naphthalenes and phenanthrene, grew rapidly at >15 °C, and, albeit at a slow rate, were able to grow at temperatures as low as 1 °C, thus categorizing them as psychrotolerant [14].

In uncontaminated soils, metabolic activity of cold-adapted bacteria in permafrost soil samples was detected at temperatures as low as -20 °C [15]. In cryoenvironments, the presence of continued microbial respiration activity (e.g. below the snow pack or ice-water interface) during winter periods was linked to special freezing-tolerant microorganisms – eutectophiles, living at the interface of ice and liquid water [8, 16].

For petroleum-contaminated soils, it has not been reported how slow freeze-thaw rates mimicking field seasonality affect petroleum hydrocarbon biodegradation in contaminated cold soils. Leszkiewicz [17] reported that freeze-thaw cycles inhibited petroleum hydrocarbon biodegradation activity. In direct contrast, other microcosm-scale studies showed that the rapid freeze-thaw cycles stimulated hydrocarbon biodegradation at the microcosm-scale [17-19]. The authors speculated that the causes of the stimulatory effects were related to the increased bioavailability, or availability of nutrients from dead freezing-intolerant cells, but the exact cause-effect of freeze-thaw on biodegradation activity remains uncertain [20].

Rike and coworkers measured *in situ* O_2-CO_2 soil gas though the active layer and permafrost in both oil-contaminated and less contaminated areas during seasonally transitional and winter frozen periods in the Arctic [21]. This study firstly suggested the potential of extended biodegradation activity during seasonal freezing and frozen soils. Biodegradation activity by O_2-CO_2 soil gas was continued up to temperatures of -2 °C in late October at the site. Microbial activation during the seasonal thawing period was detected at -6 °C; however, biodegradation activity was observed between -1 °C and -3 °C during the soil thawing period.

2.2. Nutrient requirements for bioremediation in cold regions

Optimization of nutrient amendments has been a central issue in many cold-region bioremediation studies for petroleum-contaminated soils. Previous studies have reported that, in many cases, petroleum-contaminated soils collected from Arctic, Antarctic and alpine sites were typically deficient in soluble inorganic nitrogen (N) and phosphorous (P) [22, 23]. Nutrient deficiency in polar soils correspondingly results in a significant imbalance of C: N: P ratios and limits the rate of hydrocarbon biodegradation [24].

Water-based inorganic nutrient amendments have been frequently employed and have generally achieved a positive effect in enhancing petroleum hydrocarbon biodegradation or mineralization activity in both laboratory- and field-scale trials; however, in some cases, this process either inhibited or only marginally affected biodegradation [2, 12, 22, 25-27]. Organic nutrient amendment strategies, such as water soluble urea solutions and oleophilic urea-based fertilizer (i.e. Inipol EAP22®), were also effective in stimulating indigenous hydrocarbon biodegradation activity in laboratory and field trials [6, 12].

The suggested optimal nutrient requirements assessed by C: N: P (or C: N) ratios were highly varied in literatures [28]. Although numerous C: N: P (or C: N) ratios based on total carbons (or target hydrocarbons) have been suggested and have estimated nutrient demands in many cases, there has been no general consensus in the definitive optimum ratios for maximizing the rate of biodegradation [29]. It is important to note that there are many factors causing significant uncertainties in determining optimal nutrient dosage, including non-bioavailable hydrocarbon (e.g. adsorbed), N or P losses (e.g. complexation with clays, volatilization, or denitrification), microbial properties, contamination levels and specific environmental conditions [28].

The effect of amended N concentrations on the basis of soil water and/or dry soil weight (e.g. N_{H20} or N mg/Kg, [26]) have been extensively evaluated to determine the optimal ranges of N in some coarse grained, contaminated, cold-regions soils [12, 23, 25, 27, 30, 31]. Braddock et al. [25] reported that a lowered N concentration of 100 mg N/Kg generally resulted in the greater biodegradation activity, when compared to the higher N level of over 200 mg N/Kg. The authors suggested that salt concentrations in pore water may be toxic at the N levels of over 200 mg N/Kg.

Similarly, Walworth et al. [23] reported that a modest nitrogen level of 125 mg N/kg soil (equivalent to 604 mg N/kg soil H_2O) resulted in maximum oxygen consumption and the most significant biodegradation at 6 °C, compared to biodegradation activity at 250, 375, 500 and 625 mg N/Kg. Børresen and Rike [31] reported that the higher nitrogen concentrations (600 – 1000 mg NH_4-N /kg) tended to be correlated with longer microbial acclimation periods compared to the low N (50 – 200 mg NH_4-N /kg) in ^{14}C-hexadecae microcosms at 5 °C

As evidenced by the above, excess nitrogen supply adversely affects microbial growth, respiration activity, and biodegradation rates due to the depression of osmotic soil water potential related to increased salt concentrations in pore waters or the possibly toxicity of ammonium and nitrite [23, 25]. The effects of N concentration were subsequently interacted with soil moisture [32]. Compared to fine and clayey soils, coarse-textured soils have been shown to be more susceptible to excess N-loading, due largely to the limited water-holding capacity of these soils [33]. To avoid osmotic stress, Walworth et al. [32] suggested that water-soluble N concentration should be maintained below 250 mg N/kg (equivalent to 1202 mg N/Kg soil H_2O or a maximum of 2000 mg N/kg) in soil moisture ranges of 5 to 10% of coarse grained cryic soils.

Phosphorous concentrations, in contrast, have received relatively less attention than the N response. It has, however, been shown that the co-addition of P with N is critical in maximizing biodegradation activity, evidenced by comparison to the exclusive addition of N or P [25]. Due to the low solubility of P and complexation on the mineral surface, excess P has not been significantly associated with the inhibition effect. However, large P loading-inhibited hydrocarbon biodegradation and the possibility of precipitation of P during the aqueous phase should be considered, depending on the pH conditions [34]. The use of trimetaphosphate, polyphosphates, or phosphoric acid (i.e. 20: 20: 20 commercial fertilizer), increases P- solubility and reduces adsorption [28].

2.3. Utilization of high molecular weight hydrocarbons by cold-adapted hydrocarbon-degrading bacteria

Intrinsic microbial uptake and degradation of non-volatile or high molecular weight (HMW) hydrocarbon(s) at low temperatures may be a prerequisite in effective bioremediation of aged contaminated sites in cold regions, such as DEW-lines in the Canadian Arctic or old spill

sites. Previous studies have indicated that cold-adapted hydrocarbon degraders are able to utilize a broad range of hydrophobic hydrocarbon compounds, including especially HMW hydrocarbons. Some examples of microbial characterization for the utilization of HMW hydrocarbons are presented below.

Sotsky et al. [39] revealed that a significant proportion of hydrocarbon-degrading bacterial populations within Alaskan sediment impacted by the *Exxon Valdez* oil spill possessed both *xylE* genes, for the degradation of aromatic hydrocarbons (e.g. xylenes), and *alkB* genes, used for alkane degradation. More importantly, the authors indicated that a greater portion of the bacterial populations also possessed more *xylE* genes than *alkB* genes, presumably due to the selective pressures of aged residual hydrocarbons, in which low molecular weight aliphatic hydrocarbons were relatively lacking in contaminated soils shipped from the site. Whyte et al. [35] reported that *Pseudomonas* spp. B17 & B18, isolated from petroleum-contaminated Arctic soils (Baffin Island, NT, Canada), degraded low molecular weight alkanes (C5-C12) as well as aromatic hydrocarbons (toluene and naphthalene) at both 5 °C and 25 °C in microcosm systems, and revealed that B17 and B18 possessed both *alk* and *nah* catabolic pathways. Thomassin-Lacroix et al. [18] characterized microbial consortium of enrichment cultures (7 °C) from petroleum-contaminated Arctic soils (Ellesmere Island, Nunavut, Canada). In this study, the enrichment cultures were grown on Jet A-fuel, which provided a diverse range of carbons: C10 to C18. The majority of the sequences of members in consortiums and clones were closely related to *Rhodococcus erythropolis*, *Sphingomonas* spp., and *Pseudomonas synxantha*. Some isolates in the microbial consortiums grew on different chain length of pure dodecane, hexadecane, a branched pentadecane, and 2,6,10,14-tetramethylpentadecane [18].

Hydrocarbons fuels persist for long periods of time (e.g. over 30 years) in Arctic and sub-Arctic sites [25, 36, 37]. During this time, weathering of hydrocarbons may occur in the old sites [38], and low molecular weight hydrocarbons (<C10) in contaminated soils are often lacking or at significantly reduced levels [39]. Residual medium- and long-chain hydrocarbons (>C16), including branch, cyclic, and aromatic compounds, may be a dominant fraction of the total petroleum hydrocarbons (TPH) in field-aged contaminated soils. Changes in bioavailable hydrocarbon fractions may select specialist and/or versatile hydrocarbon-degrading microbial populations that were exposed to or contacted liquid or solid phase hydrocarbons at low temperatures. DNA-based methods using specialized catabolic gene probes for different classes

of hydrocarbon compounds have confirmed the prevalence of cold-adapted hydrocarbon-degrading microbial populations in hydrocarbon-contaminated cold soils, as compared to pristine soils [40, 41].

Previous laboratory and field biodegradation experiments conducted in field-contaminated soils in cold regions have shown mixed results. Some previous studies have reported that lower molecular weight n-alkanes and un-substituted aromatic hydrocarbons are biodegraded preferentially over the relatively HMW alkanes, isoalkanes, alkylated aromatic hydrocarbons, isoprenoids, and branched and cyclic hydrocarbons [6, 42, 43]. The cause of the observed preferential biodegradation was not clearly revealed in the studies.

In aged, contaminated soils, Sanscartier et al. [44] reported that preferential biodegradation occurred between <nC16 and >nC16 hydrocarbons in both laboratory (~22 °C) and field experiments. In the study, biodegradation of the heavier hydrocarbon fractions was limited until <nC16 hydrocarbon fractions were below ~200 mg/Kg, after 134 days, and at 22 °C. However, the on-site biodegradation experiments for aged petroleum-contaminated soils conducted by Braddock et al. [25] showed that significant biodegradation of hexadecane (nC16, a linear alkane), 2, 6-dimethylnaphthalene (an aromatic), and phytane (a branched alkane) occurred after 6.5 weeks. Zytner and coworkers [45] reported that only modest preferential degradation of the lower molecular weight straight chain alkanes occurred in field-contaminated soils. In the study, the first order rate constants of dodecane (C12) and eicosane (C20), relative to the TPH concentrations, were similar to each other.

An increasing body of literature has suggested that other microbial uptake mechanisms of hydrophobic hydrocarbons interact with the cell surface: (i) biosurfactant production or the use of cell surface components for emulsifying; (ii) uptake system with high substrate affinity for reducing concentrations of substrates close to the cell surface and thereby increasing the diffusive substrate flux; and (iii) reduction of distance between cells and substrates by enhancing adhesion to hydrophobic surface [46]. Eriksson et al. [47] observed that significant growth of a biofilm, enriched from Arctic soil samples, occurred on pyrene and phenanthrene in solid phase (crystal forms), and that the biofilm cultures were able to degrade both pyrene and phenanthrene at 22 °C.

2.4. Temperature variations and its significance for bioremediation in cold regions

Summer temperatures in active layers or surface soil treatment are highly dynamic and vary largely from near 0 °C to occasionally above 20 °C in many sites [2-7]. Field temperatures play a significant role in controlling the growth of indigenous hydrocarbon-degrading populations, the nature and extent of hydrocarbon metabolism, and the physical nature and chemical composition of hydrocarbons [2, 3, 10]. The impacts of site temperatures on the rate and extent of petroleum hydrocarbon biodegradation is not clearly understood.

Table 2.1 summarizes the temperature regimes and temporal formats of the set-up temperatures that have been employed to detect biodegradation activity and/or to measure the rate and extent of biodegradation of petroleum hydrocarbons in previous laboratory experiments. The summaries provided in Tables 2.1 and 2.2 below, include the laboratory, on-site biodegradation experiments, or field operations using aged contaminated soils that were all collected from old petroleum-contaminated sites in cold climates.

The selection of the incubation temperatures was presumably based on average temperatures of the site soils (typically at 5 or 10 °C) taken from the available temperature records or a representative average temperature of cold region sites. Table 2.1 displays all the previous laboratory experiments that have been performed at constant low temperatures, with the exception of several freeze-thaw studies. Previous freeze-thaw induced biodegradation experiments have been conducted under the general assumption that diurnal freeze-thaw effect may take place at the sites. The freeze-thaw conditions considered in the previous laboratory experiments employed relatively rapid freeze-thaw rates representative of daily freeze-thaw cycles between -5 and 7 °C for 48 days [53] or 4, 8 and 16 day freeze-thaw cycles between -5 and 5 °C for 48 days [19] or 3 cycles per day between -5 and 10 °C for 100 days [17]. These studies were performed at the small scale with 40, 60 or 1000 g soil [17, 19, 53]. Typically, previously reported long-term on-site experiments have focused on petroleum hydrocarbon bioremediation under summer temperature regimes, and bioremediation operations are closed during the seasonal transitional period and winter but are reactivated for the following summer season. Thus the extent of petroleum hydrocarbon biodegradation under seasonal freeze-thaw conditions and during seasonal transitional period is unknown. Moreover, natural fluctuations in

site temperatures of air and/or soils were reported during on-site experiments or during field implementation (Table 2.2). The influence of natural variations of site temperatures on petroleum hydro carbon biodegradation has not been directly investigated. The previous approaches and findings under the constant temperature or freeze-thaw conditions are indispensable for understanding the effects of low temperature and rapid freeze-thaw on biodegradation, and in evaluating bioremediation feasibility. To date, however, little information is available regarding the effect of time-varying temperature profile representing site temperature variation including seasonal soil freeze-thaw events during bioremediation.

Table 2.1. Previous laboratory biodegradation experiments for petroleum hydrocarbons in soils from cold climate soils. Adapted from Rike et al., [20].

	Field conditions		Laboratory experimental conditions			Ref.
	Site	Air or soil temperature (presented in study)	System-scale	Incubation temperatures	Temporal temperature changes	
1	Barrow, US	a.m.t. July: 2.9 ±2.4˚C; a.m.t. Aug: 1.8 ±1.9˚C (near site)	Microcosm (400g)	10˚C	Constant (6 weeks)	[25]
2	Barrow, US	August: 7.3˚C (soil 0.1 m)	Microcosm (400g)	5, 10, 15, & 20˚C	Constant (40 days)	[32]
3	Ft. Wainwright, US	August: 8˚C (soil 1 m); October: 3˚C (soil 3m)	Microcosm (400g)	1, 11, & 21˚C	Constant (40 days)	[32]
4	Ellesmere Island, Canada	a.d.t. in July: 3.6˚C; ~5˚C (4-6 weeks for summer)	Microcosm (20 g or 40 g)	5˚C & 23˚C	Constant (16 weeks)	[22] & [49]
5	Inn Valley, Austrian Alps	Not reported	Microcosm (100 g)	10˚C	Constant (30 days)	[50]
6	Ellesmere Island, Canada	a.d.t. in July: 6.4˚C	Microcosm (80 g)	7˚C	Constant (92 days)	[51]
7	Longyearbyen, Norway	m.a.a.t: -6˚C; In July: <5˚C (1 m)	Microcosm (40 g)	5˚C	Constant (32 or 128 days)	[42]
10	Mackenzie River, Canada	Not reported	Bench-scale (2 kg)	10˚C	Constant	[52]
11	Ellesmere Island, Canada	Summer: fluctuated occasionally above/below 0˚C; Fall: diurnal freeze/thaw cycle	Microcosm (60 g)	-5, 0, 7, 7& -5˚C (daily)	Freeze-thaw (48 days)	[53]
12	Longyearbyen, Norway and Central Alaska, US	Fall/spring: fluctuated above/below 0˚C	Microcosm (40 g)	-5 to 5˚C: cycle/4 days, 8 days, or 16 days	Freeze-thaw (48 days)	[19]

Table 2.1 (*cont*.)

	Field conditions		Laboratory experimental conditions			Ref.
	Site	Air or soil temperature (presented in study)	System-scale	Incubation temperatures	Temporal temperature changes	
13	McMurdo Station, Antarctica	< 0 to 20˚C	Bench-scale (1 kg)	-5 to 10˚C (3 cycles/day)	Freeze-thaw (100 days)	[17]
14	Resolution Island, Canada	Ave. soil temp: 9.3˚C; Ave. air temp: 3˚C (summer)	Bench-scale (1.2 kg)	5, 8, and 18˚C	Constant (5 months)	[5] & [4]
15	Tanquary Fiord, Canada	m.a.a.t: -15˚C; daily temp. >0˚C (summer)	Bench-scale (18 kg)	22˚C	Constant (313 days)	[44]
16	Old Casey Station, Antarctica	Ave. soil temp: 3˚C; surface soil temp: > 20˚C or >30˚C	Microcosm (20 g)	10˚C	Constant (95 days)	[27]
17	8 DEW-lines	Highly dynamic; occasionally 20˚C	Microcosm (3 g)	7, 15, 22, 30˚C	Constant	[12]
18	Northern Ontario, Canada	Not reported	Microcosm (200 g)	2, 5, 15, 25˚C	Constant	[45]

a.d.t. - Average daily temperature (air)
a.m.t. - Average monthly temperature (air)
m.a.a.t. - Mean annual air temperature (air)

Table 2.2. Previous field implementation or on-site biodegradation experiments for petroleum hydrocarbons in soils from cold climate soils. Adapted from Rike et al. [20].

	Field conditions		Field experimental conditions			Ref.
	Site	Air or soil temperature (presented in study)	System-scale	Temperature regime	Temporal temperature changes	
1	Barrow, US	a.m.t. July: 2.9 ±2.4˚C; a.m.t. Aug: 1.8 ±1.9˚C (near site)	Pilot-scale (20 kg)	Not reported (summers)	Natural fluctuation (6.5 weeks)	[25]
2	Tyrolean Stubai Alps	a.m.t. 0.6, -1.3, -1.8˚C Fluctuated 0-20˚C in summers	Pilot-scale (150 kg)	Fluctuated >0˚C to 20˚C	Natural fluctuation (3 seasons)	[3]
3	Prudhoe Bay, US	Aug: approx. 5˚C; Nov: approx. -25˚C	Biopile (49 m × 40 m ×2.4 m H)	0.5 to 7.8˚C	Stable thermal insulated (22 months)	[54]
4	Cambridge Bay, Canada	a.d.t. July: 7.6˚C; a.d.t. Aug: 6.1˚C	Biopile (0.25 $m^{3)}$	2.7 to 10˚C in covered biopile 1.7-6.7˚C uncovered biopile	Stable (2 season)	[55]
5	Ellesmere Island, Canada	a.d.t. in July: 6.4˚C	Pilot-scale (750 kg)	a.d.t. 10 to14˚C	Natural fluctuation (65 days)	[51]
6	Saviktok Pt. Canada	a.d.t. ~10˚C	Wind-powered Biopile (17000 m^3)	Not reported (summers)	Natural fluctuation (2 seasons)	[56]
7	Ellesmere Island, Canada	a.d.t. 5˚C in summer	Biopile (45 m^3)	Ave. core temp. 15˚C	Natural fluctuation (12 months)	[57]
8	Old Casey station, Antarctica	a.d.t. summer: 10˚C	Natural attenuation & controlled release nutrients	Not reported	Natural fluctuation (3 years)	[58]

Table 2.2 (*cont.*)

	Field conditions		Field experimental conditions			Ref.
	Site	Air or soil temperature (presented in study)	System-scale	Temperature regime	Temporal temperature changes	
9	Ellesmere island, Canada	a.d.t. in July: 3.6˚C; ~5˚C (4-6 weeks for summer)	*In situ* treatment (fertilizer)	Not reported (summers)	Natural fluctuation (3 seasons)- strongly varied	[59]
10	Resolution Island, Canada	Ave. soil temp: 9.3˚C; Ave. air temp: 3˚C (summer)	Landfarming trials (5 m × 5 m × 0.3 m H)	Fluctuated >0 - 22˚C	Natural fluctuation (2 seasons)	[4] & [5]
11	Northern Ontario, Canada	Not reported	Landfarming trials (9 m × 6 m W × 0.3 m H)	Not reported (summers)	Natural fluctuation (2 seasons)	[45]
12	Barrow, US	1.3-4.8˚C for summers	Landfarming (3600 m^3)	Not reported (summers)	Natural fluctuation (70 days)	[60]

a.d.t. - Average daily temperature (air)
a.m.t. - Average monthly temperature (air)
m.a.a.t. - Mean annual air temperature (air)

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Chapter 3

Biodegradation of semi- and non-volatile petroleum hydrocarbons in aged, contaminated soils from a sub-Arctic site: Laboratory pilot-scale experiments at site temperatures

Wonjae Chang[1], Michael Dyen[2], Lou Spagnuolo[3], Philippe Simon[4], Lyle Whyte[2], Subhasis Ghoshal[1]

[1]Department of Civil Engineering, McGill University, Montreal, Canada
[2]Department of Natural Resource Sciences, McGill University, Montreal, Canada
[3]Indian and Northern Affairs Canada, Ottawa, Canada
[4]Qikiqtaaluk Environmental, Montreal, Canada

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3.1. Introduction

Field bioremediation studies conducted in Arctic and Antarctic regions have demonstrated the reduction of petroleum hydrocarbons, and at least part of the reduction is attributable to biodegradation [1-6]. Bioremediation has been proposed to be a cost effective and non-disruptive remediation technology for polar sites contaminated with petroleum hydrocarbons [7-9].

Given that the ground is frozen for most of the year, and that soils usually encountered in these regions often contain low levels of nitrogen (N) and phosphorous (P) nutrients to support biological growth, the implementation of bioremediation appears to be challenging. However, the presence of indigenous cold-adapted hydrocarbon degrading microorganisms at many northern sites makes biostimulation through addition of N, P nutrients, oxygen and moisture a feasible approach for implementation of bioremediation [5, 10-12]. The presence of significant populations of aerobic, cold-adapted bacteria in petroleum-contaminated soils from polar and alpine regions has been reported [10, 13-15].

Some cold-adapted microorganisms are capable of degradation of relatively persistent high molecular weight petroleum hydrocarbons. Whyte et al., [16] reported that an isolate, *Rhodococcus* sp. Strain Q15 was able to degrade alkanes up to n-C21 as well as some branched alkanes in diesel, and was able to grow on dotriacontane (n-C32). *Rhodococcus* strains capable of growth on eicosane (n-C20) have been reported by Bej et al. [15]. The above studies were conducted by addition of radiolabelled and unlabelled target compounds to contaminated site soils, and thus demonstrate the biodegradability of the high molecular weight target compounds that have been freshly added rather than aged in the soils. Studies on petroleum biodegradation in soils from cold regions have reported that lower molecular-weight n-alkanes and unsubstituted aromatic hydrocarbons are biodegraded preferentially over the relatively higher molecular weight n-alkane compounds, isoalkanes, alkylated aromatic hydrocarbons, isoprenoids and the branched and cyclic hydrocarbons [4, 17-19]. These studies have generally relied on biodegradation experiments with oils dispersed in liquid cultures or soils to which oil has been freshly added. Controlled studies on changes in the composition of TPH from aged, contaminated soils in cold climates are lacking.

The rates of petroleum hydrocarbon biodegradation have been shown to be strongly dependent on temperature. Although cold-adapted hydrocarbon-degrading microorganisms can degrade significant amounts of petroleum hydrocarbons at temperatures around 0 °C, several studies have reported that the rates of biodegradation are enhanced at or above 10 °C [1, 12, 20, 21].

In this study, the biodegradation rates of several lower molecular-weight hydrocarbons (boiling points in the range of >nC10 - nC16) and higher molecular-weight hydrocarbons (boiling points in the range of >nC16 - nC34) in soils contaminated by Arctic diesel spills in the 1970s and earlier were evaluated. The soils were obtained from an old military radar site at Resolution Island , Nunavut, Canada (61°30'N 65°00'W) and details of the site are described elsewhere [2]. The objectives of the study were to (i) evaluate the feasibility of bioremediation of the site soils by biostimulation under conditions similar to field landfarming operations; (ii) compare the rates and extents biodegradation of relatively lower molecular weight and higher molecular weight petroleum hydrocarbon fractions present in the field soils, and (iii) to characterize the residual, non-degraded TPH and determine its distributions in soil aggregate size fractions.

Biotreatment experiments were carried out in 1 m long covered pilot-scale soil tanks. Soils were biostimulated by nutrient and pH buffer amendments and by periodic tilling as in landfarming operations, and an untreated control was maintained. Temperature conditions during the 60-day experiment were maintained at the historical daily average air temperature recorded over two summer months at the site. The soil tanks were periodically monitored for temperature, O_2 and CO_2 concentrations in the soil gas, and for petroleum hydrocarbon concentrations in soil samples periodically extracted from the tank. At the end of the 60-day period, soil samples were analysed for residual TPH in different particle aggregates of various sizes.

3.2. Materials and methods

3.2.1. Site soils

Contaminated soils and representative uncontaminated soil were obtained from the Resolution Island site. The soils were shipped frozen in sealed metal containers and thereafter

transferred and stored in sealed drums at -5 °C. Data from physicochemical and microbiological characterization of the contaminated soils is presented in Table 3S.1 (Supplementary Data). The petroleum-contaminated site soil contained low nitrate and ammonium levels typical of Arctic soils. The gravimetric moisture contents ranged from 10 % to 11 %. The soils were acidic with a pH of 4.3. Soil pH was measured by dissolving soil in 10 mM $CaCl_2$ at a soil/solution ratio of 1:2 (w:v) [22]. The petroleum-contaminated soils were classified as sand according to the USDA classification system (gravel: 27%; sand: 72%; silt and clays: 1%).

Indigenous heterotrophic and hydrocarbon-degrading bacteria (grown on Arctic diesel amended minimal agar plates) were between 2.1×10^2 to 1.0×10^5 CFU/g in contaminated and pristine soils aseptically sampled at the site. A variety of catabolic genes for degrading hydrocarbons: *alkB, ndoB, phnAc,* and *xylE,* were identified in the contaminated soils; but were not detected in the pristine soils [23].

Analyses conducted in accordance with Canadian Wide Standard-Tier 1 Method [24, 25] indicate that the petroleum hydrocarbon contaminants in the site soils consists of F2 fractions, (semi-volatiles with boiling points between those of >C10 to C16) and F3 fractions (non-volatiles with boiling points between those of >C16 to C34). The method detection limit (MDL) for the contaminated soils were 10 mg TPH/kg and 1 mg /Kg for o-terphenyl added as a spiked surrogate to the soils. Polycyclic aromatic hydrocarbons were not present at significant levels in the soils. Further details of the analytical procedure are provided in the Supplementary Data section.

3.2.2. Pilot-scale landfarming experiments

Stainless steel soil tanks which were 1.0 m long, 0.65 m wide and 0.35 m deep and filled with soil up to a depth of 22 cm were used for the pilot-scale landfarming experiments as shown in Figure 3S.1 (Supplementary Data). Compressed air (78% N_2, 21% O_2, and 1% Ar) was supplied into headspace of the landfarm tank at an air flow rate of 30 L/day. Air exited the closed headspace of the tank through activated carbon and moisture traps (Na_2SO_4). Soil gas was sampled for O_2 and CO_2 analysis using a hand-held infrared/electrochemical sensor (ATX 620, Industrial Scientific Co.) from horizontal ports at different depths as shown in Figure 3S.1. Soil gas sampling was performed every 10 days immediately before and after tilling.

An important aspect of the pilot-scale landfarming experiments was that they were subjected to varying temperatures similar to that at the site during summer months. Hourly temperature data for July and August of between 2001 and 2005 were acquired from Environment Canada. The ambient field temperatures of the site fluctuated approximately cyclically between 1 °C and 10 °C and the six-consecutive temperature cycles with uniform temperature changes in this range were maintained in a cold room where experiments were conducted that closely matched the July-August field temperature profiles as shown in Figure 3S.2 .

Approximately 800 kg of contaminated soils for the four landfarm tanks (Tanks A-H, A-L, B-L and U) were sieved through a sterilized standard sieve with opening size of 4.75 mm. Table 3.1 lists the soil treatments and initial TPH concentrations in soil tanks. Nutrients were provided in Tanks A-H, A-L and B-L by amending the soils with filter-sterilized solutions of a commercial water-soluble fertilizer (20% N: 20% P_2O_5: 20% K_2O, 20:20:20 Plant Prod®). Tanks A-H, A-L and B-L were amended with 2000 mg/Kg of $CaCO_3$ to yield a soil pH between 6.5 and 7.0. The extra volume of water for nutrient and pH buffering amendments enhanced the gravimetric soil moisture content by less than 1%. Tank U was operated as a control landfarm and did not receive any nutrients, $CaCO_3$ or tilling.

Five soil samples were collected from each layer of surface (0-5 cm), middle (5-10 cm) and bottom (15-22 cm) of each landfarm on Day 0, Day 20, Day 40 and Day 60. Soils samples from each layer were composited and used for measuring TPH, F2 and F3 concentrations, soil pH, soil moisture contents, and residual N and P. On Day 0 and Day 60, samples of Tank A-L landfarm soils were sieved gently without drying using ASTM standard sieves Nos. 10, 30, and 200, to segregate coarse (2-4.75 mm), medium (0.6-2 mm) and fine (0.075-0.6 mm) soil particles or aggregates. In these undried soils, there was notable adhesion of fine particles to the relatively larger particles, and these associated masses are referred to as aggregates in this study. TPH losses during the soil fractionation were negligible. Samples of the three soil fractions were analyzed for residual TPH. In addition the BET surface areas of these fractions were measured (Micromeritics Tristar 3000 Analyzer) and their mineral composition was determined by X-ray diffraction.

3.3. Results and discussions

3.3.1. TPH biodegradation activity

The results from all the pilot-scale landfarming treatments of the contaminated site soils showed significant reductions in TPH concentrations as shown in Figure 3.1. Approximately 64%, 55% and 49% of initial TPH was eliminated in Tank A-H, Tank A-L, and Tank B-L, respectively. In contrast, losses were insignificant in the untreated control (Tank U). There were no significant differences in TPH concentrations with depth in any of the tanks. One-way ANOVA indicated statistically significant changes ($\alpha = 0.05$) in the mean TPH concentration at successive time points in both Tank A-H and A-L, and in Tank B-L. The activated carbon traps fitted at the headspace outflow of each tank was extracted and analyzed for petroleum hydrocarbon concentrations, and only trace amounts were detected. Thus volatilization losses of TPH appear to be negligible.

As evident from Fig. 3.1, TPH biodegradation and soil gas CO_2 production patterns were significantly different between the two tanks with low- and high initial TPH concentrations. In Tank A-H, TPH concentrations decreased at a uniform rate and the TPH concentrations did not level off in the 60-day period. In contrast, in Tank A-L, TPH concentrations decreased rapidly between Day 0 and Day 20 and thereafter there was only a small decrease in the mean TPH. Overall, the low initial TPH concentrations significantly affected biodegradation time profiles and the extent of biodegradation was limited after reaching a TPH threshold level of ~500 mg/Kg. Soil gas phase CO_2 increased steadily over the treatment period after Day 10 in Tank A-H, whereas the CO_2 accumulation decreased after Day 10 in tanks A-L and B-L. The CO_2 production was much lower in control Tank U (< 0.32% CO_2).

The onset of TPH biodegradation and significant CO_2 production in Tank A-H was also associated with an increase in the hydrocarbon-degrading microbial populations in all the three soil depths sampled. Cold-adapted hydrocarbon-degrading microorganisms enumerated by viable plate counts at 5 °C in minimal agar plates exposed to Arctic diesel vapor, and were found to be 2.6×10^5 - 3.8×10^5 CFU/g soil at Day 0 in Tanks A-H and Tank U soils. The cold-adapted hydrocarbon degrading microbial population increased to 8.9×10^6 - 1.5×10^7 CFU/g soil in Tank A-H by Day 20 and these population levels remained approximately constant up until Day 60.

The control system, Tank U showed no significant change in the microbial population size during that time and ranged from 1.0 x 10^5 CFU/g soil to 6.0 x 10^5 CFU/g soil. The increases in viable hydrocarbon-degrading population concurrent with TPH reductions in the treated tanks are a clear indication of enhanced TPH biodegradation activity following nutrient addition, pH adjustment and tilling.

3.3.2. Biodegradation rates and extents of different petroleum hydrocarbon fractions

Substantial biodegradation of both semi-volatile (F2: >C10 to C16) and non-volatile, high-molecular weight fractions (F3: >C16 to C34) occurred in Tank A-H, and the F2 and F3 concentrations were reduced by 60% and 68%, respectively, as shown in Figure 3.2. It is interesting to note that during the test period, the F2 and F3 hydrocarbon fractions were biodegraded concurrently. This is in contrast to some reports in the literature of sequential degradation of lower and higher molecular weight petroleum hydrocarbons and/or limited biodegradation of the F3 fraction [4, 17, 19]. In Tank A-L, reductions of F2 and F3 were also comparable at 63% and 53%, respectively. However, as shown in Fig. 3.2B, the extent biodegradation of the F2 and F3 fractions was limited to approximately 200 mg/Kg. Biodegradation of the F2 fraction ceased after Day 20, whereas F3 biodegradation reached a plateau only after Day 40. The F2 and F3 fraction biodegradation patterns in Tank B-L were somewhat different because the initial F2 hydrocarbon concentration of 352±122 mg/Kg was lower than F3 concentration, and close to the observed threshold concentration for limiting biodegradation of ~200 mg/Kg. Although F2 biodegradation was negligible in Tank B-L, biodegradation of the higher molecular weight F3 fractions was significant, especially between Day 0 and 20.

The change in composition of the F2 and F3 hydrocarbon fraction during the 60-day biotreatment period is shown in a representative GC chromatogram in Figure 3S.3. Biodegradation of a wide spectrum of hydrocarbons, including saturated hydrocarbons (represented by individual peaks) and branched and cycloalkanes (represented by the baseline hump of the unresolved complex mixture) occurred over the entire range of F2 and F3 hydrocarbon fractions [26].

The first-order biodegradation rate constants, *K*, for the F2 and F3 petroleum hydrocarbon fractions were similar to each other in Tank A-H and estimated to be 0.011±0.002 day^{-1} and 0.019±0.001 day^{-1}, respectively (Table 3.2). For Tank A-L the first-order biodegradation rate constant for the F2 fraction was 0.024±0.005 day^{-1} and was in the range of the degradation rate constant of 0.016±0.002 $day^{-1}$for the F3 fraction. TPH biodegradation in the more frequently tilled landfarm, Tank B-L, resulted from the non-volatile F3 biodegradation rather than the semi-volatile F2 biodegradation. Thus, the TPH biodegradation in Tank B-L is attributable primarily to the F3 biodegradation rate of 0.011±0.002 day^{-1}. In these experiments, frequent tilling did not significantly influence the petroleum hydrocarbon biodegradation rates and extents.

Overall, the biodegradation rates for the F2 and F3 hydrocarbon fractions were of comparable magnitude in our study. Our results are similar to those reported by Bento et al. [27] where similar rates and extents of biodegradation of the lighter fraction (C12-C22) and heavier fractions (C23-C40) were observed in microcosm studies diesel contaminated soils conducted at 27 °C. However, preferential biodegradation of the lighter hydrocarbon fractions have been reported by other studies. Observations of Børresen et al. [19] who found that n-C11 to n-C18 alkanes were degraded faster than the n-C19 to n-C36 alkanes in oil dispersions in liquid cultures maintained at 5 °C and similar observations have been reported by Coulon et al. [17] in experiments conducted at a sub-polar site where diesel and crude-oil were added to surface soil test plots. In a study of biodegradation of weathered hydrocarbon-contaminated soils from an Arctic site, Sanscartier et al. [4] reported that hydrocarbons lighter than C16 (i.e., F2 fraction) were biodegraded and reduced below ~ 200 mg/kg during laboratory studies conducted at 22 °C although the F3 hydrocarbon fraction was degraded minimally and only after the F2 hydrocarbon fraction was depleted. In field studies with the same soil, those authors did not observe significant degradation of the F3 hydrocarbon fraction. The sequential or concurrent biodegradation of lighter and heavier hydrocarbon fractions may be influenced significantly by the abundance and viability of higher molecular weight hydrocarbon degrading microorganisms and microbial community dynamics.

Various studies have calculated first-order degradation rate constants for biodegradation studies with petroleum-contaminated soils. Paudyn et al. [2] performed an on-site pilot-scale landfarming experiment at the same Resolution Island site. In the field study, the first-order TPH

degradation rates ranged from 0.017 to 0.026 day^{-1} and is generally in good agreement with the TPH biodegradation rates of K = 0.01 to 0.018 day^{-1} observed in our study. Zytner et al. [28] reported the first order TPH biodegradation rate constants in the range of 0.022 to 0.0043 day^{-1}, from a landfarming experiment at a historically diesel-contaminated northern site. Our study was conducted in pilot-scale tanks containing 200 Kg soils each, a scale much larger than microcosms that are commonly used for assessment of biodegradation performance in laboratory studies. Because of the similarity in TPH biodegradation rate constants in this study and the field study at the same site. It appears that the pilot-scale tanks provided an environment for various processes that influence biodegradation rates such as oxygen mass transfer and mixing efficiency in a manner similar to the field. However, additional studies are required to confirm this.

The initial TPH concentration in the various treatment tanks appears to influence the sequence of degradation of C14, C16 and C18 alkanes. Figure 3.3 demonstrates the changes in the relative abundance of these alkanes as a function of TPH concentrations during the treatment period. In Tank A-H (Fig. 3.3A) the magnitude of alkane ratios was small, i.e., the C14 was more abundant, and the ratios were also relatively constant at TPH concentrations between 1000 to 2000 mg/Kg, indicating that all three alkanes were biodegraded concurrently. This phenomenon was also observed by de Jonge et al. [25] and suggests that biodegradation was not controlled by solubilization, volatilization or diffusion of the alkanes from the petroleum hydrocarbon mixture. As TPH concentrations decreased below 1000 mg/Kg, an increase in the alkane ratios was observed. Below 1000 mg/Kg, increasing alkane ratios indicated a preferential degradation of the longer C-chain alkanes, C16 and C18, over the relatively shorter chain alkane, C14. It appears that below 1000 mg/Kg, C18 and C16 alkanes were more bioavailable over C14 and/or microbial community shifts at around 1000 mg/Kg caused lower biodegradation rates of C14. This biodegradation pattern occurred in all the different treatment tanks. Thus, although F2 and F3 hydrocarbon fractions biodegraded concurrently, certain higher molecular weight hydrocarbons were degraded preferentially at low TPH concentrations.

3.3.3. Residual TPH distribution in soil aggregates of various sizes

The TPH concentration profiles from Tanks A-L and B-L suggest that at a TPH concentration of approximately 500 mg/Kg (170 to 340 mg/Kg for F2 and 260 to 300 mg/Kg for

F3 fractions), hydrocarbon biodegradation is essentially negligible. Nutrients, moisture and pH were not found to be limiting at the end of the 60-day treatment period. For example in Tank A-L, residual inorganic N and total P were 67 mg/Kg, and 280 mg/Kg, respectively. The final moisture content of 11.3 % was not significantly altered from the initial moisture content of 10-11%). Thus residual nutrients and moisture contents were sufficient for biodegradation. The soil pH was nearly unchanged at ~ pH 6.8. Oxygen concentrations were uniform at approximately 20.5% at each sampling port and thus oxygen depletion was not a limiting factor either.

The TPH distribution in the various soil particle size fractions were evaluated to investigate if the residual TPH and its limited bioavailability was attributable to a particular size fraction. The soil from Tank A-L was comprised of 39 wt.% coarse particles and macroaggregates (2-4.75 mm dia.), 59 wt.% medium, (0.6-2 mm dia.) and 2 wt.% fine (0.075-0.6 mm dia.) soil particles and aggregated particles on a wet-weight basis (11% moisture contents).

The majority of the initial TPH mass was associated with the medium sized particles and aggregates, and to a lesser extent to the coarser particles and macroaggregates, while the fines accounted for a neglible fraction of the TPH mass as shown in Figure 3.4A. The bulk of the TPH mass decrease during the treatment period is attributable to the hydrocarbons in the medium-size fraction, although this size fraction also had the highest residual TPH concentration. Examination of the TPH concentrations on the basis the TPH mass per unit weight of each size fraction, as shown in Figures 3.4B and 3.4C, provides additional insight on TPH biodegradation from the different size fractions. The TPH concentrations (TPH mass per unit weight of each size fraction) showed highest depletion in the coarse-sized particles and macroaggregates and lower depletion in the medium-sized particles and the fines. This suggests that particle characteristics were controlling the residual TPH levels in the pilot-scale landarming operations.

In soils contaminated with Arctic diesel, semi- and non-volatile petroleum hydrocarbons are partitioned pirmarily between the diesel non-aqueous phase, the soil organic matter, and the soil mineral phase. Semi- and non-volatile petroleum hydrocarbons can partition from the hydrocarbon non-aqueous phase liquid and bind onto mineral phases (clay) and soil organic matter phases [29-31]. However, there is little direct information available on the bioavaialbility or sequestration of hydrophobic compounds sorbed to mineral or soil organic matter phases in oil-contaminated soils. The contaminated soils used in this study had a low organic matter

content of 2.4%. The soil mineral composition of the coarse and medium size fractions are shown in Table 3.3. The fine size fraction was not analyzed because it was associated with a small amount of the total TPH mass, and its residual TPH concentration is similar to the medium size fraction. Both the coarse and medium size fractions are predominantly comprised of quartz and carbonate minerals and the clay (kaolinite) fraction is only slightly higher in the medium fraction (3% compared to 2%). Kaolinite is a non-swelling clay and has a surface area approximately only twice that of quartz and thus the slightly higher clay content in the medium size fraction cannot account for the higher residual hydrocarbon cocentrations in this fraction [32]. The soil organic matter content in the coarse and medium size fractions are similar, are thus not likely responsible for the higher residual hydrocarbons in the medium sized fraction.

Biodegradation rates of relatively insoluble, higher-molecular weight hydrocarbons from the oil phase is considered to be dependent on direct microbial contact at the oil-water interface [33]. The soil micropore structure may have a significant influence on biodegradation rates as some of the oil phase may not be accessible for direct contact with microorganisms. For example, Noordman et al. [34] found that hexadecane immobilized in silica gel particles with 6 nm pores was biodegraded significantly slower than hexadecane immobilized in silica gel with significantly larger pores. The measured BET surface area of the coarse fraction at 3.7 m^2/g was only slightly smaller than that of medium and fine fractions and does not reflect substantial differences in microporosity. However, these BET surfaces areas in wet, oil-contaminated soils cannot be considered to accurately reflect surface geometry of the size fractions, as pores or surface deformations covered in oil or bound water would not be accessed by the absorbing N_2 molecules. This is a well recognized hindrance in the BET surface area characterization [32, 34].

3.4. Conclusions

Inorganic nutrient amendments, soil neutralization and tilling at 10-day intervals were effective in rapidly stimulating indigenous, cold-adapted hydrocarbon-degrading microbial populations at temperature conditions similar to the site. The final hydrocarbon levels in pilot-scale landfarm tanks with low initial TPH (~1000 mg/Kg) after treatment for two months were just above or below the Canada-wide standards of 150 mg/Kg for the F2 hydrocarbon fraction and 300 mg/Kg for the F3 fraction applicable to the Resolution Island site. Soils with higher

levels of TPH will likely take considerably longer to attain treatment endpoints. The rates and extents of biodegradation of the non-volatile, higher molecular weight hydrocarbon fraction (>C16-C34) was significant and comparable to the rates and extents of biodegradation of the lower molecular weight (>C10-C16) fraction. Biodegradation of the higher molecular weight hydrocarbon fraction occurred even when there was a lack of biodegradation activity of the lower molecular weight fraction. Comparison of the biodegradation time profiles of C14, C16 and C18 alkanes showed that the relative abundance of C16 and C18 alkanes decreased more rapidly than that of C14 alkanes as TPH levels decreased when TPH levels were below 1000 mg/Kg. These observations of changes in petroleum hydrocarbon composition with biodegradation are different from the more commonly observed sequential degradation of lower molecular weight hydrocarbons followed by more persistent, higher molecular weight hydrocarbons.

The residual TPH concentration was approximately 500 mg/Kg. Nutrients, pH, oxygen or the population size of hydrocarbon degraders did not limit biodegradation. The majority of residual TPH mass remained in soil particle and aggregate sizes ranging between 0.6 to 2 mm rather than in larger or finer size fractions. The residual TPH mass per unit weight of the size fraction was similar for the medium and fine size fractions. The BET surface area of the medium size fraction was 1.4 times that of the coarse fraction, and this likely indicates that hydrocarbons were distributed differently in the two size fractions, leading to the different end points.

Table 3.1. Landfarming soil treatments for petroleum hydrocarbon contaminated site soils.

Landfarm I.D.	Initial TPH[a] (mg/Kg)	Nutrient amendment[b]		pH adjustment	Tilling
		N (mg/Kg)	P (mg/Kg)	$CaCO_3$ (mg/Kg)	
Tank A-H (high initial TPH)	2141 (±392)	250	55	2000	Every 10 days
Tank A-L (low initial TPH)	1010 (±103)	100	25	2000	Every 10 days
Tank B-L (frequent tilling)	1048 (±201)	250	55	2000	Twice till/week
Tank U (untreated:control)	1218 (±177)	NA	NA	NA	NA

[a] mean TPH (± standard deviation)

[b]20:20:20 commercial fertilizer (PlantProd®) is composed of 20% total nitrogen (N), 20% phosphoric acid (P_2O_5), 20% soluble potash (K_2O), 1% EDTA and trace metals (Cu, Fe, Mn, Mo, and Zn). Nutrient concentrations were adjusted based on the high and low TPH concentration to achieve final C_{TPH}: N: P molar ratio of 100: 9: 1 for Tanks A-H and A-L, and 100:18:2 for Tank B-L.

Table 3.2. First order rate constants for semi-volatile (F2), non-volatile (F3) and TPH biodegradation during 60-day treatment period.

CCME TPH fractions[a]	Landfarm I.D.	K (day^{-1})[b]	R^2
F2: >C10-C16	Tank A-H	0.011 (±0.002)	0.87
	Tank A-L	0.024 (±0.005)	0.81
	Tank B-L	0.0	NA
F3: >C16-C34	Tank A-H	0.019 (±0.001)	0.99
	Tank A-L	0.016 (±0.002)	0.93
	Tank B-L	0.011 (±0.002)	0.85
TPH	Tank A-H	0.014 (±0.001)	0.97
	Tank A-L	0.018 (±0.003)	0.86
	Tank B-L	0.011(±0.002)	0.78

[a] CCME TPH fractions are defined in CCME (2001).

[b]First order reaction equation: $\ln[C] = -KT + \ln[C]_0$ where, [C] = [TPH], [F2], or [F3] and K = rate constant (mean ± SE).

Table 3.3. Properties of aggregate sizes separated from soils in Tank A-L after the 60-day biotreatment period.

Aggregate size designation	Coarse	Medium	Fine
Particle size range	2.00 – 4.75 mm	0.60 – 2.00 mm	0.075 – 0.60 mm
% mass fraction	39%	59%	2%
BET surface area	3.7 ± 0.05 m^2/g	5.3 ± 0.04 m^2/g	6.5 ± 0.01 m^2/g
Mineralogy	Quartz: 60% Feldspar: 23% Calcite: 6% Dolomite: 8% Muscovite: 1% Kaolinite: 2%	Quartz: 63% Feldspar: 21% Calcite: 4% Dolomite: 8% Muscovite: 1% Kaolinite: 3%	NA

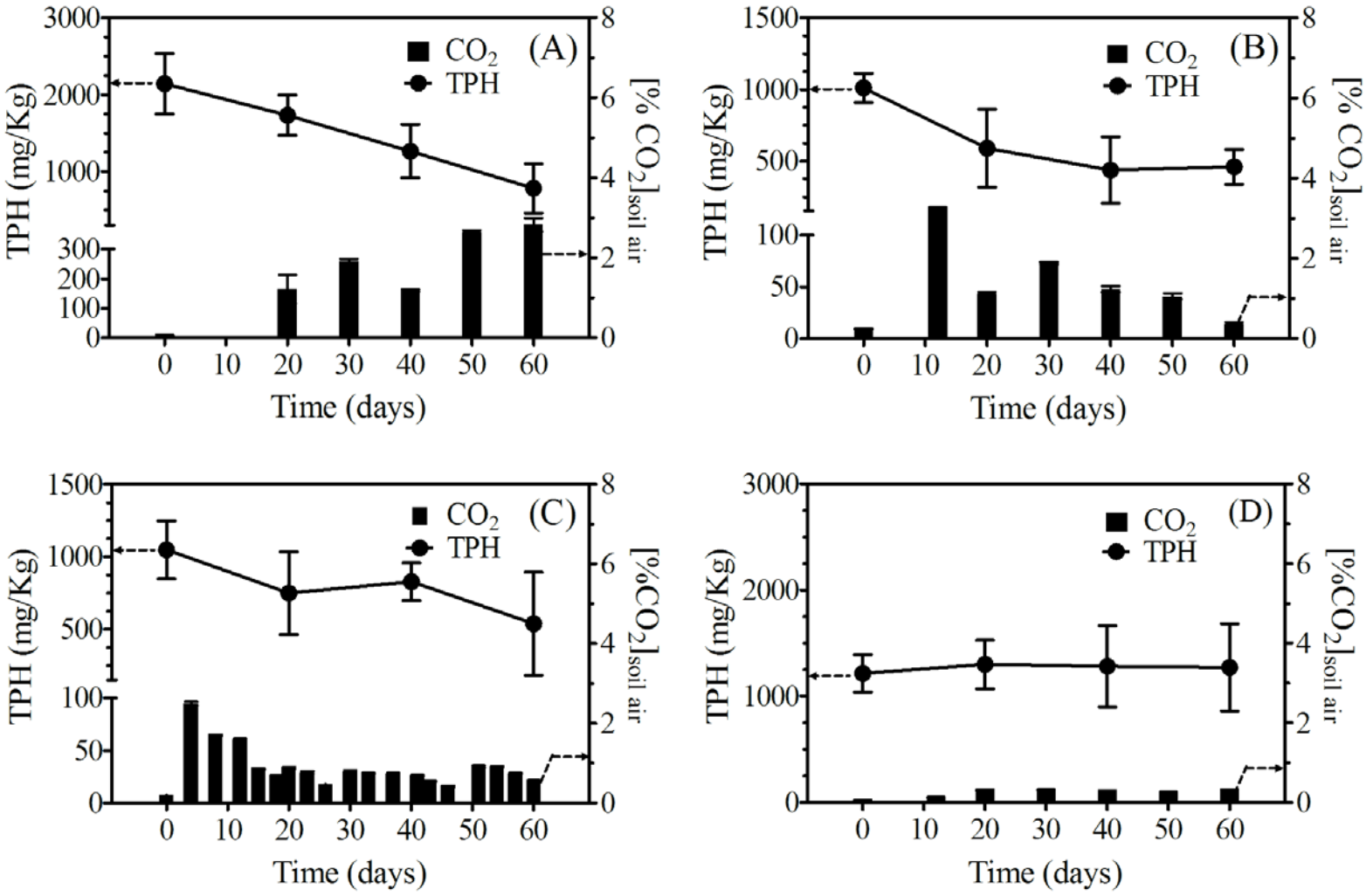

Figure 3.1. Changes in TPH concentrations and soil gas % CO_2 (v/v). Soil gas was retrieved from sampling ports of the pilot-scale tanks. (A): Tank A-H (high initial TPH), (B): Tank A-L (low initial TPH), (C): Tank B-L (frequently tilled) and (D): Tank U (control). Error bars indicate one standard deviation from the mean.

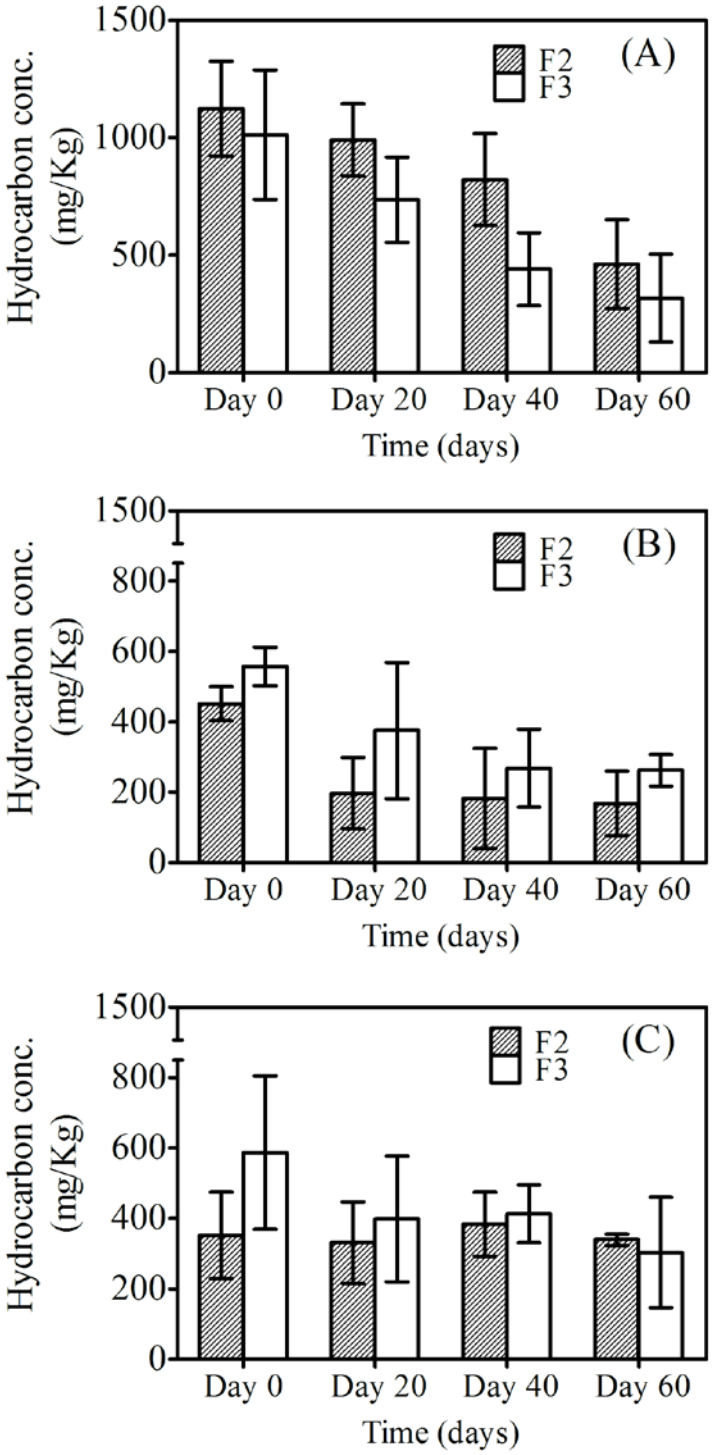

Figure 3.2. Changes in F2 and F3 concentrations (mean ± standard deviation) during the pilot-scale landfarming experiments. (A): Tank A-H, (B): Tank A-L and (C): Tank B-L. The set of F2 and F3 data from each tank is statistically significant (two-way ANOVA, $p < 0.05$).

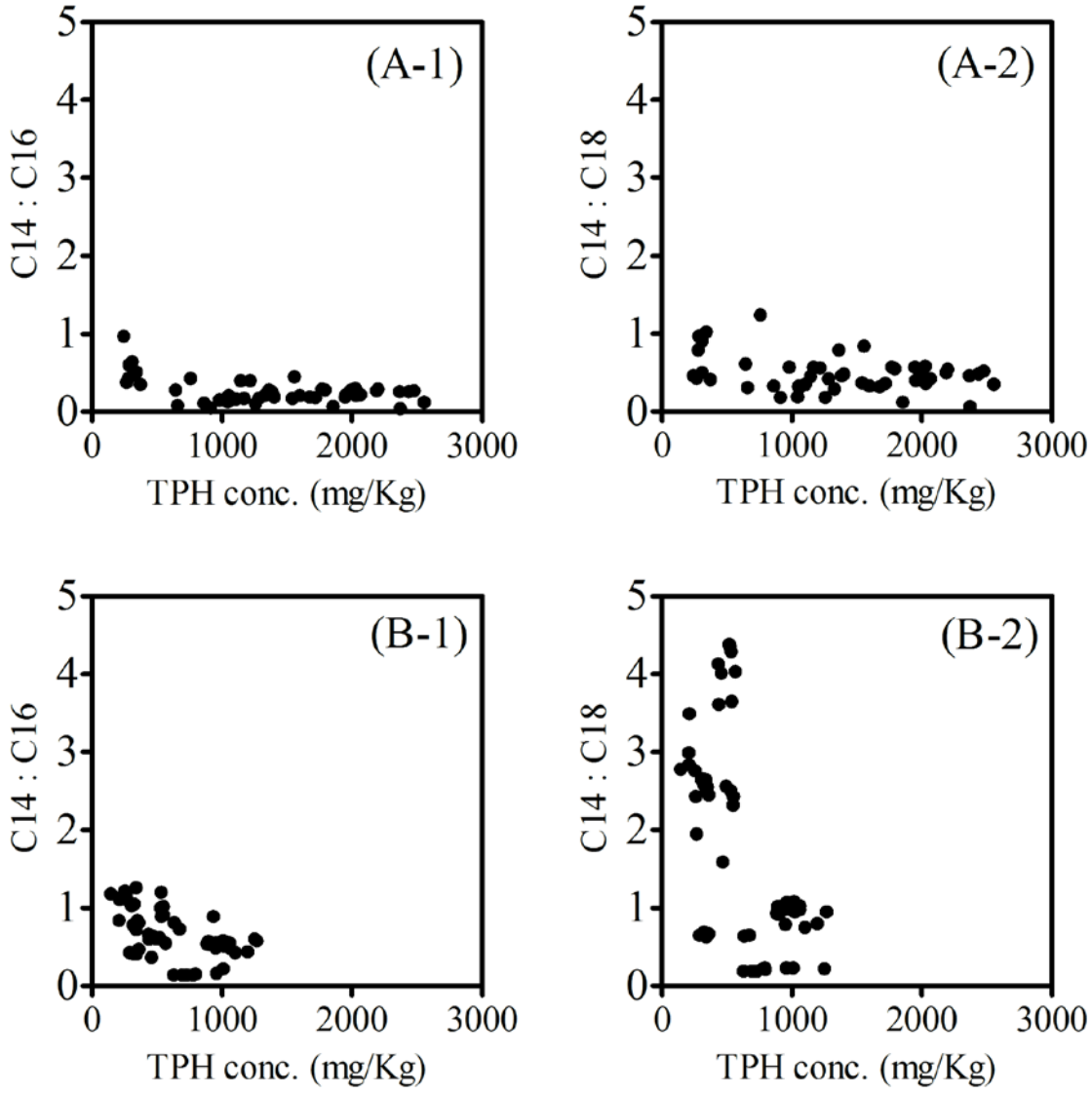

Figure 3.3. Redistributions of alkanes (C14 : C16 and C14 : C18) as a function of TPH concentration. (A-1 & A-2): Tank A-H and (B-1 & B-2): Tank A-L.

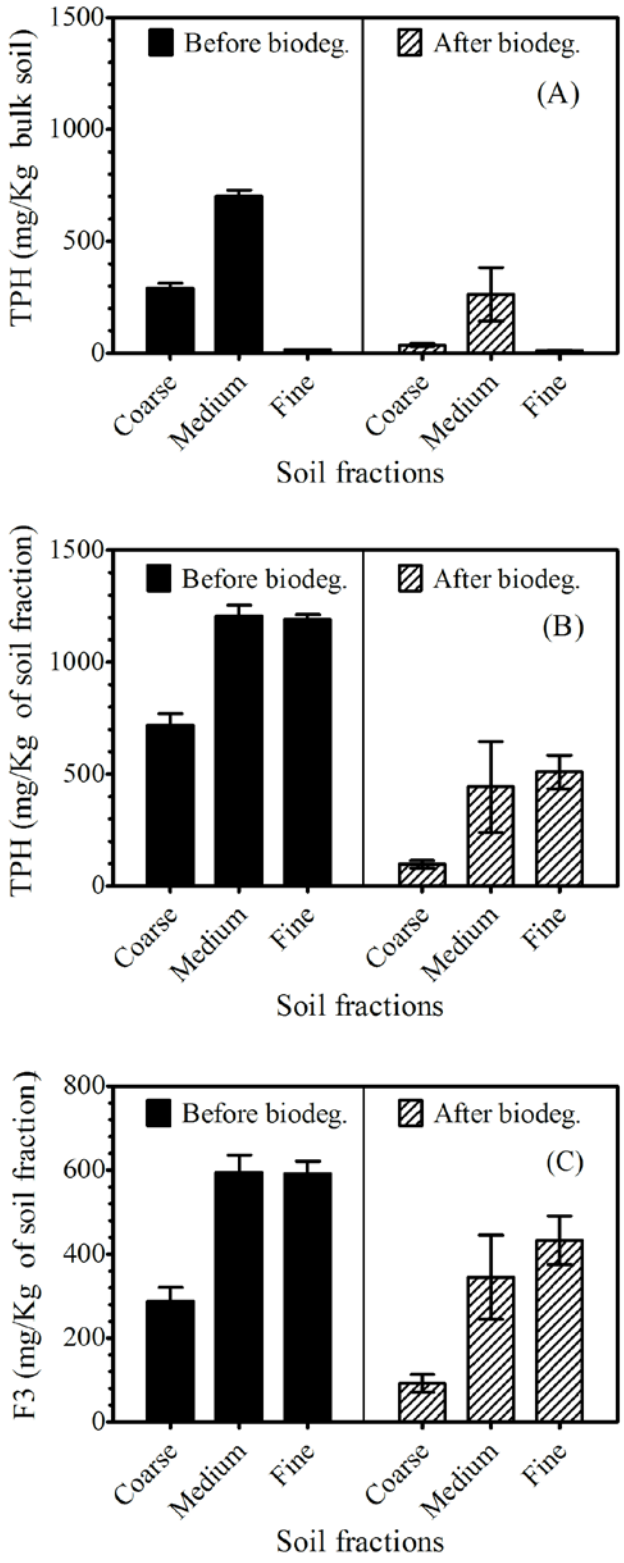

Figure 3.4. TPH and F3 concentrations before and after biodegradation in bulk soils and the different soil fractions. Coarse: 2-4.75 mm dia., Medium: 0.6-2 mm dia. and Fine: 0.075-0.6 mm dia.. Bulk soil refers to a non-fractionated soil. Further details on soil fractions is provided in Table 3.3.

Supplementary Data

Table 3S.1. Physicochemical and microbial characterization of the untreated Resolution Island site soils.

Soil grain size classification	Gravel (%)	27
	Sand (%)	71
	Silt & clays (%)	1
Gravimetric soil moisture	%	11
Soil pH	as $CaCl_2$ extract	4.3
Petroleum hydrocarbons (mg/Kg)	F1: >C6 – C10	ND
	F2: >C10- C16	352 - 1124
	F3: >C16 – C34	431 - 1013
	F4: >C34 – C40	< 10
	PAH 2- and 3 ring	< 0.1
Nutrients(mg/Kg)	Inorganic nitrogen	ND
	Total phosphorous	200 – 210
Hydrocarbon-degraders	CFU/g	$2.1 \times 10^2 – 1.0 \times 10^5$
Catabolic genes	Positive detection	*alkB, ndoB, phnAc, xylE*

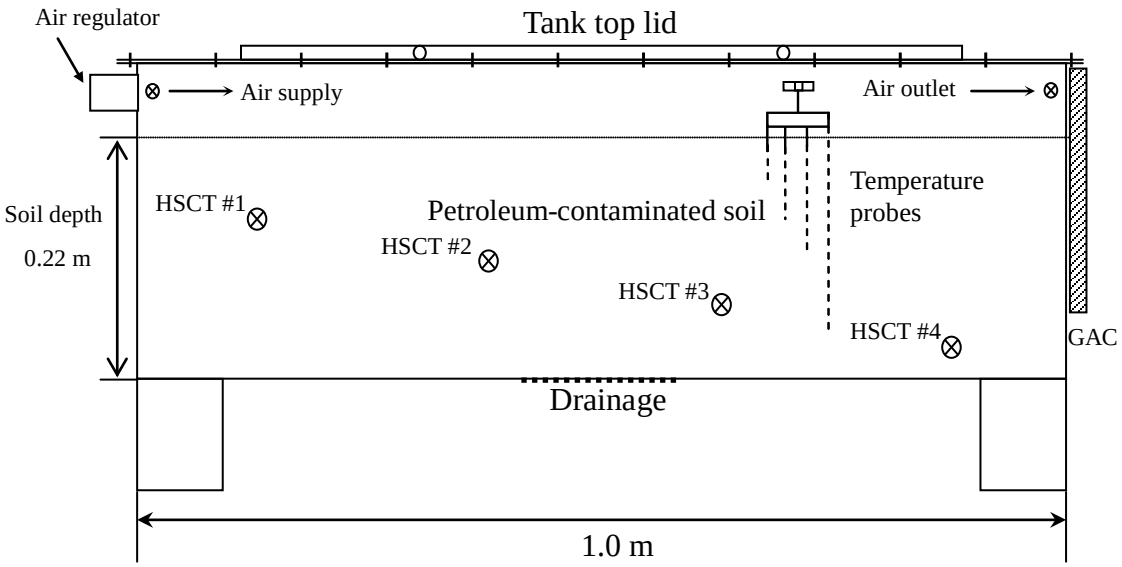

Figure 3S.1. Schematic diagram of stainless steel soil tank (1m long × 0.65 m wide × 0.35 m deep) for the pilot-scale landfarming experiment (not drawn to scale). Note: HSCT- Horizontal soil gas collection tube. GAC: granular activated carbon trap.

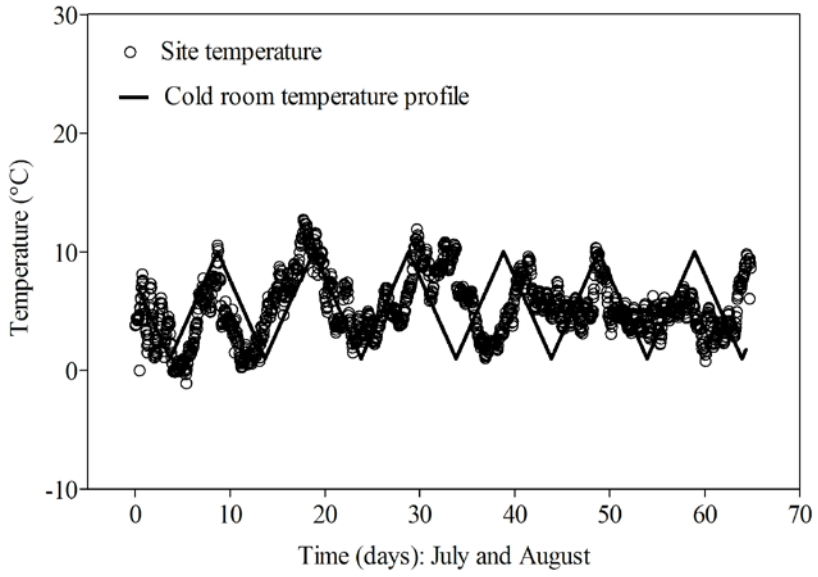

Figure 3S.2. Site temperatures at Resolution Island, Nunavut, Canada for July and August and an equivalent cold room temperature profile. The hourly temperature data for July and August between 2001 and 2005 were obtained from the climate database of Environment Canada.

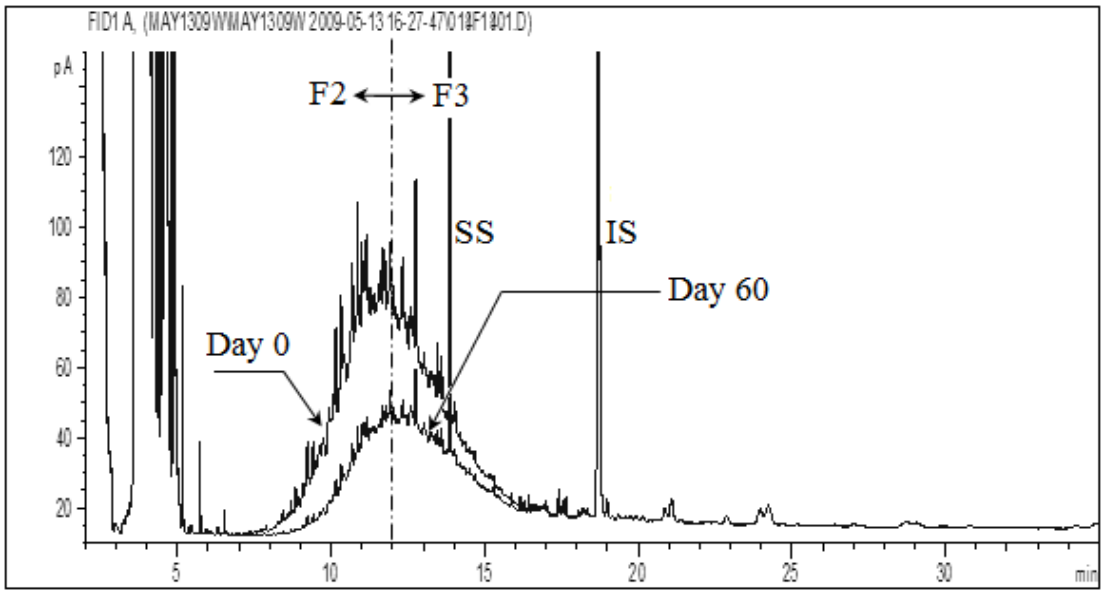

Figure 3S.3. Representative TPH chromatograms of medium size soil particle fractions of before and after biotreatment in Tank A-L. SS: o-Terphnyl (Surrogate Standard) and IS: squalene (Internal Standard).

Supplementary method

Procedure of Total Petroleum Hydrocarbon (TPH) analyses

The Canada-wide standard (Tier 1 Method) for petroleum hydrocarbon analyses in soils - was used to quantify TPH concentrations in soils [24]. Briefly, wet soil samples (10 g) was blended with anhydrous sodium sulfate (1:1) and placed in a cellulose extraction thimble. In an automatic Soxhlet extraction apparatus (Gerhardt Soxtherm, UK), 140 mL of the extraction solvent comprised of 50:50 (vol: vol) hexane: acetone was added to a set of extraction beakers containing clean boiling beads. Cooling water for condenser circulation was maintained at 4 °C for consistent extraction recovery efficiency (>85%) of the surrogate standard. TPH-extracts were passed through a silica gel bed with 50:50 (vol/vol) hexane: dichloromethane (DCM) and then concentrated by nitrogen blow-down. The volume of final TPH-extract was measured and filtered with a 1-µm Teflon filter. Calibration stocks were prepared for determining average response factors for F2, F3 and F4 fractions with the filtered TPH extracts. Alkane standards were spiked into final extract samples to analyze alkanes of interest. TPH was analyzed by a gas chromatograph equipped with flame ionization detector (Agilent, Model 6890, J&W DB-1 capillary column). The TPH concentration was quantified by integration (C10-C50) of the resolved and unresolved peaks after subtraction of a blank run, as described in de Jonge et al. [24].

Acknowledgements

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Connecting text: Chapter 3 and 4

In Chapter 3, pilot-scale landfarming experiments were performed in a laboratory under site temperature profiles representative of the 3-year site air temperatures in July and August where temperature varied uniformly between 1 °C to 10 °C over 10 days. To understand the effect of the variable site temperatures on the biodegradation performance, the rate and extents of biodegradation of petroleum hydrocarbons were compared between the variable site temperature and constant average temperature in Chapter 4.

Chapter 4

Comparison of the effects of time-varying site temperatures and constant incubation temperatures on the biodegradation of petroleum hydrocarbons in pilot-scale experiments with contaminated soils from a cold regions site

Wonjae Chang[1], Lyle Whyte[2], Subhasis Ghoshal[1]

[1]Department of Civil Engineering, McGill University, Montreal, Canada
[2]Department of Natural Resources, McGill University, Montreal, Canada

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4.1. Introduction

Bioremediation of petroleum-contaminated soils in cold regions can be achieved through biodegradation activity of indigenous hydrocarbon-degrading microorganisms that are able to survive and grow in cryic soils, historically contaminated with petroleum hydrocarbons [1, 2]. There appears to be a general consensus that biostimulation during on-site landfarming or biopile operations, is a feasible, cost-effective and less destructive remediation technique for the environmentally challenging conditions at such sites [3, 4].

Generally, field temperatures in cold regions sites are temporally dynamic and temperature variations may influence on-site soil microbial activity. The amplitude and frequency of temperature fluctuations in cold climates may be strongly dependent on geographical locations (e.g. high Arctic versus sub-Arctic regions), seasons, and the variability of incoming solar radiation at the site [5]. A number of on-site bioremediation field experiments and field operations during the short, non-frozen summer months have measured significant natural temperature fluctuations occurring in the ambient air, soil surface and in the shallow active layers at the sites [6]. For example, a strong positive correlation between soil temperature fluctuations and variations in incoming solar radiation flux was found in petroleum-contaminated soils at an Antarctic site, and the soil temperatures varied cyclically from near 0 to 20 °C over the summer season. Furthermore, the amplitude of soil temperature fluctuations were more significant in petroleum-contaminated soils than in pristine soils due to differences in surface albedo caused by petroleum contamination [7]. In the complex conditions found in the field, it is very difficult, if not impossible, to isolate the effect of temperature variability from the multitude of other rate-influencing environmental factors [8].

Site temperature variability may influence the growth and structure of indigenous cold-adapted hydrocarbon-degrading microbial communities in contaminated soils, and thus influence biodegradation rates. It has been widely recognized that psychrophiles prevail in permanently cold environments, such as the high Arctic, high altitudes or the deep seas whereas environments with periodic, diurnal or seasonal temperature fluctuations are favourable to psychrotolerant bacteria that are sensitive to temperatures but grow over a broad range of temperatures [9-12].

The rationale for the temperature selection in various laboratory biodegradation

experiments with soils from cold regions has not often been justified in the context of relevant site temperature regimes. The effect of site temperature variations on petroleum hydrocarbon biodegradation in cold regions soils has largely been evaluated through laboratory microcosm-scale experiments operated at different incubation temperatures (e.g. 5 or 10 °C).

The objective of this study is to compare the influence of the temporally variable site temperatures representative of summers at a sub-Arctic site with that of constant average temperatures on petroleum hydrocarbon biodegradation and microbial activity in pilot-scale landfarming experiments. Landfarming is a common bioremediation technique implemented in many Arctic and sub-Arctic sites [3]. Comparative sets of pilot-scale landfarming experiments employing a biostimulated tank with nutrient and pH buffering amendments and an unamended control tank, were conducted under two different temperature regimes: (i) a representative variable site temperature profile where temperatures varied uniformly and cyclically from 1 to 10 °C (VAR) over 10 days during a 60-day experiment; and (ii) a constant average temperature of 6 °C (CST) over the same duration of the experiment. Aged, petroleum-contaminated sub-Arctic soils were shipped from a site in Resolution Island (RI), Nunavut, Canada, which was a military radar station operated in the 1970s. The details and characteristics of the RI site are described elsewhere [13, 14]. In the pilot-scale experiments, the rates and extents of total petroleum hydrocarbons (TPH), semi- (F2: >C10-C16) and non-volatile hydrocarbon fractions (F3: >C16-C34) in the VAR- and CST- modes were evaluated. Biodegradation patterns of the different hydrocarbon fractions of TPH, F2, F3 and Unresolved Complex Mixtures (UCMs) were compared. Microbial activity under the two temperature modes was assessed by soil gas O_2 and CO_2 concentration measurements, and by culture-dependent and -independent analyses to determine changes microbial communities and microbial growth patterns.

4.2. Materials and Methods

4.2.1. Site soils

The petroleum-contaminated and uncontaminated (pristine) soils were shipped frozen in sealed sea-cans and stored at -5 °C as quickly as possible in a storage cold room before use. A

smaller quantity of both contaminated and pristine soils were collected aseptically at the site and shipped frozen, overnight for microbial characterization.

The detailed analytical methods and results of soil characterization are presented elsewhere [15]. Briefly, petroleum hydrocarbon analyses conducted in accordance with Canadian Wide Standard-Tier 1 Method [16] indicate that the majority of petroleum hydrocarbon contaminants in the site soils consists of F2 fractions (>C10 to C16, semi-volatiles) at 352 mg/Kg to 1124 mg/Kg, F3 fractions (>C16 to C34; non-volatiles) at 431 mg/Kg to 1013 mg/Kg and F4 fractions (>C34 to C40) at < 10 mg/kg. Volatile hydrocarbons (boiling points in the range of < C10) were lacking in the shipped field-contaminated soil samples.

Viable hydrocarbon-degraders (9.5×10^3 – 1.1×10^4 CFU/g) and heterotrophic bacteria (1.2×10^4 – 5.0×10^4 CFU/g) were more enriched in contaminated soils than in pristine soils (2.1×10^2 – 7.0×10^3 CFU/g). The initial soil pH was pH 4.3 - 4.6. The site soils were coarse grained soils that consisted of 27% gravel, 72% sand, and 1% silt and clays and were poorly graded soil with a uniformity coefficient of $C_u = 4.2\pm0.8 < 5$.

4.2.2. Construction of temperature profile

The temperature profile was constructed from the mean hourly air temperatures for July to August for the three year period 2002 to 2005 and is shown in Fig. 4.1. A total of 4496 available hourly temperature readings were acquired from Environment Canada. The mean of all temperature measurements for this period was computed to be 5.3 °C. The daily mean of all hourly site temperatures varied between 1 and 10 °C. A temperature profile resembling the variable daily mean site temperatures or the average temperature (ambient air: 5.5 °C) was set in a cold room facility at McGill University where the experiments were conducted.

4.2.3. Pilots-scale experiments

Approximately 200 Kg soil was packed uniformly, layer-by-layer, in each pilot-scale soil tank (1m length × 0.65 m width × 0.35 m height) fabricated out of stainless steel. Compressed air (78% N_2, 21% O_2, and 1% Ar) flowed into the headspace of the pilots-scale tanks at an air flow

rate of 30 L/day to ensure adequate O_2 supply. Soil gas was sampled for O_2 and CO_2 analysis using infrared/electrochemical sensors (ATX 620, Industrial Scientific Co.) from four horizontal ports installed at various depths in the pilot-scale tanks. Activated carbon columns- fitted at the headspace exhaust outflow of each tank were extracted for detection of volatilized hydrocarbons, and only trace amounts were detected at lower than MDL indicating negligible volatilization.

Effective nutrient doses, soil pH, air flow rates, soil moisture levels, and tilling frequency were determined in previous studies [15]. Briefly, an optimal nutrient dose of a commercial fertilizer (PlantProd®) that provided a C_{TPH}: N: P molar ratio of 100: 9: 1 was used, and 2000 mg/Kg of $CaCO_3$ added to yield a soil pH of 6.5 to 7.0. Periodic tillage was performed every 10 days. An unamended and untilled landfarm, but which was supplied with air at the same flow rate was set as controls for each of the temperature modes. Triplicate soil samples were collected from each layer of surface (0-7cm), middle (7-14 cm) and bottom (14-22 cm) of the pilot-scale landfarm (soil depth: 22 cm) every 20-day interval. The soil temperatures of surface, middle and bottom layers were monitored using thermocouple temperature probes **(**Grant SQ800**,** Grant Instruments). The collected soil samples were further processed for petroleum hydrocarbon analyses, soil moisture, microbial community analyses, and viable hydrocarbon-degrading microbial population counts.

4.2.4. Total petroleum hydrocarbon analyses

The detailed analytical and quality assurance programs procedures followed for TPH analyses are described in CCME protocol for The Canada-wide standard (Tier 1 Method) [16]. Additional details are described briefly herein. An automatic Soxhlet extraction system (Gerhard Soxtherm) equipped with cooling water circulator (4 °C) was employed for soil extraction. The extraction-solvent reduction program of the extractor was developed to obtain 85% - 100% recovery of n-decane (nC10) and o-terphenyl (surrogate standard) spiked in the site soil matrix and extraction solvents (50:50 hexane: acetone). The solvent extracts were purified through silica-gel cleanup traps and then final extracts were concentrated by optimized N_2 blow-down. The filtered extracts were analyzed by GC-FID (Agilent 6890, DB-11 capillary column). TPH, F2 and F3 concentrations were quantified by a horizontal baseline method integrating resolved and unresolved peak areas after subtraction of a blank run [17].

4.2.5. Temperature-dependency of microbial respiration

The temperature coefficient (Q_{10}) is a measure of the rate of change of microbial respiration activity as a consequence of increasing the temperature by 10 °C. The Q_{10}-value was calculated according to the equation below, where R_2 and R_1 represent oxygen consumption rates measured at two different temperatures T_2, and T_1, respectively, as described in [18]. The obtained Q_{10}-values were used to evaluate temperature-dependency of microbial respiration under the variable temperature conditions.

$$Q_{10} = \left({R_1}/{R_2}\right)^{\left[{10}/{(T_1 - T_2)}\right]}$$

4.2.6. Microbial assessments

The details of microbial enumeration procedures are described in [19]. In summary, soil suspensions from 10-g composite soil samples (triplicates) were prepared for microbial enumeration of the soils collected from the three layers of landfarms. An aliquot of 0.1-mL of each soil suspension was aseptically transferred and spread onto R2A agar plates (Becton, Dickinson and Company) or mineral salt medium (MSM) agar plats amended with Arctic diesel (Shell Canada) and then plates were incubated at 5 °C.

PCR-based denaturing gradient gel electrophoresis (PCR-DGGE) analyses were conducted on the soil samples collected from the nutrient amended and control pilot-scale landfarms as described in [19]. In summary, total community DNA was extracted from 1.0 g soil samples using UltraClean Soil DNA Isolation kit (MO BIO Laboratories, Solana Beach, CA) and a 418bp bacterial 16S rDNA gene fragment was amplified for DGGE fingerprinting using primers 341F with a 5'-GC clamp (CCT ACG GGA GGC AGC AG) and 758R (CTA CCA GGG TAT CTA ATCC). For the DGGE runs (DCode universal mutation detection system, Bio-Rad), bacterial 16S rDNA were run on an 8% polyacrylamide gel with a 35%-65% denaturing gradient. The stained gels (0.6 µg ethidium bromide/ml 1×TAE for 30 minutes) was destained in 1×TAE

for 5 minutes and then viewed by UV transillumination (ChemiGenius Bioimaging system). The excised DGGE bands were eluted in 50µl Milli-Q water at 4 °C overnight and then the reamplified DNA (primers without GC clamp) for sequencing analyses was sent to Plate-forme d'analyses biomoléculaires, Université Laval, Quebec.

4.3. Results and Discussion

4.3.1. Biodegradation of TPH and non-volatile hydrocarbon fractions

This study indicated that the biodegradation patterns of petroleum hydrocarbons were significantly different between the two temperature conditions of VAR- and CST- modes, as shown in Fig. 4.2 (two-way ANOVA plus Bonferroni's post hoc tests, *p*-value < 0.05). Petroleum biodegradation was more substantially enhanced for both F2 and F3 hydrocarbon fractions in the nutrient amended soils subjected to the VAR mode that more closely approximated the variable temperature conditions during summer seasons, compared to the CST mode (Fig. 4.2a and 4.2b). As shown in Fig. 4.2b, the initial concentrations of non-volatile hydrocarbons (F3) were very similar between the soil tanks employed in the two temperature modes. Once amended with nutrients, moisture, $CaCO_3$, and tilled, F3 biodegradation was more substantially enhanced in VAR mode by more than a factor of two. The total removals of F3 in VAR- and CST- mode are 53% and 21%, respectively. On Day 40 and 60, the F3 concentrations between VAR and CST were significantly different at *p*-value of < 0.01.

The enhancement in biodegradation of higher molecular weight hydrocarbon fractions in VAR was linked to the significant decrease of UCM envelopes of the TPH chromatograms (Fig 4.3). UCM fractions largely appear in TPH chromatograms of highly weathered petroleum-contaminated soils and oil residues [20, 21]. These UCM fractions are comprised of structurally complex hydrocarbon compounds such as branched, cyclic, unsubstituted alkyl chains fractions in petroleum-contaminated soils [22]. In this study, the UCM reductions between the two temperature modes were compared only in the soil aggregate sizes with diameters ranging between 2 to 4.75 mm because the UCM fractions was significantly different in the other soil particle size fractions in the Day 0 soil samples [15]. As shown in Fig. 4.3, the various resolved peaks at which the retention time included from semi- to non-volatile hydrocarbon ranges were

notably reduced in both low temperature modes. However, the UCM fractions after the 60-day period were significantly reduced only in the amended tank operated in the VAR mode. Approximately 47% of the initial UCM areas was reduced in VAR mode (two-tailed t-test for before and after biodegradation, *p*-values = 0.0016 < 0.05) whereas the UCM reduction in CST mode was only 19%.

In this study, the TPH and hydrocarbon fraction analyses (F2, F3) integrated all extractable hydrocarbon compounds in the range of semi- (< C16) to non-volatile higher molecular weight hydrocarbons (> C16) and this ensured that biodegradation of a wide spectrum of compounds, not just of a specific target compound. Furthermore, regulatory standards and guidelines for soil quality criteria are often stated on the basis of TPH or carbon numbers, and thus the quantitative data on hydrocarbon reduction presented here allows us to determine if the extent of biodegradation was relevant to field operations.

The first-order TPH, F2, and F3 biodegradation rate constants determined by the VAR- and CST-mode experiments were compared to the first-order rate constants determined by the on-site pilot-scale experiment. An on-site pilot-scale landfarming experiment was conducted in the same Resolution Island site [14]. The nutrient amendment for the field trial landfarms was made in the first summer season and then the landfarms were for consecutive second and third summer seasons. At the site, the landfarm soil temperatures were significantly changed by air temperatures occasionally fluctuating as high as 20 °C during the treatment period [13].

Compared with the results of CST mode, the biodegradation rate constants determined under the variable site temperature conditions were in better agreement with the field-determined rate constants. As shown in Table 4.1, the laboratory TPH biodegradation rate constants of 0.018 day^{-1} in VAR mode was within the range of the field-determined rate constants of 0.017 to 0.026 day^{-1}. The laboratory F2 and F3 rate constants of 0.024 day^{-1} and 0.016 day^{-1}, respectively, in the VAR mode compared well with those rate constants.

In the field, the final TPH concentration in the nutrient amended landfarms was 200-500 mg/Kg. The final TPH concentration determined by the VAR-mode experiment was 459 ±122 mg/Kg after the 60-day period, which was generally in good agreement with the range of the final TPH level at the site. In on-site landfarming, majority of TPH reduction occurred only during the first summer season. After the first treatment season, the further TPH reductions were very negligible during the second summer season. In the VAR-mode experiment, the effective

end point of TPH biodegradation was due to reduced bioavailability in soil aggregates with diameters raging from 0.6-2 mm [15].

4.3.2. Biodegradation patterns of semi-volatile and non-volatile hydrocarbons

In VAR mode, both F2 and F3 were concurrently degraded. In contrast, preferential biodegradation pattern for lower molecular weight hydrocarbon (F2) occurred in the CST mode (Table 4.1). The initial relative abundance between F2 and F3 concentrations were near unity at the initial time (Day 0) in the contaminated soils used in both temperature modes. The preference ratio, defined as the ratio between the reduction in F2 and F3 hydrocarbon fractions from the initial values, (Δ [F2] $_{\text{Day i}}$ / Δ [F3] $_{\text{Day i}}$), indicated whether biodegradation for the two hydrocarbon fractions occurred preferentially or concurrently over time in the two different temperature modes.

In VAR, the preference ratio was 1.7 on Day 20 and then decreased to 0.7 by Day 60. The mean of the ratios for the entire treatment period was 1.0. The overall biodegradation pattern in VAR thus tended to be approximately concurrent for the two different hydrocarbon fractions. On the other hand, in CST the preference ratio increased from 1.6 on Day 20 to 2.6 by Day 40, indicating a significant preferential biodegradation of F2 over F3 during the first 40 days. The ratio then decreased to 1.1 by Day 60, and given that there was still abundant amount of F2 hydrocarbons remaining, the result indicates that F3 biodegradation rate increased by Day 60.

The preferential biodegradation observed in this study is thus different from the previous reports of the extensive biodegradation of lower molecular weight hydrocarbons (< C16) and linear alkanes and no significant biodegradation of non-volatile hydrocarbons and UCM fractions in laboratory studies conducted at temperatures of 5 °C or field studies conducted under variable site temperatures of 0-20 °C [23-25]. Garrett and co-workers [26] however indicated a different pattern of preferential biodegradation for which straight chain alkanes and simpler aromatic were degraded first, followed by branched alkanes and alkylated aromatics hydrocarbons in experiments conducted at 6 and 20 °C. This sequential pattern in the preferential biodegradation is more similar to the biodegradation pattern observed in CST- mode. These previous indications of preferential biodegradation resulted from the biodegradation experiments

performed by freshly spiking oil to (un) contaminated soils or by dispersing oil in liquid cultures [23-26].

An increasing body of literature has reported the identification of cold-adapted hydrocarbon-degrading bacteria from the field-contaminated cold regions soils, that are capable of utilizing a broad range of hydrocarbons ranging from C10 to C32 compounds, PAHs, and branched hydrocarbons (i.e. iso-alkanes) at low temperatures [12, 27]. It was found that greater portion of bacterial populations in the field-contaminated soils collected from an Alaskan contaminated site exhibited the catabolic genes related to complex, aromatic hydrocarbons (e.g. *xylE*) than simple straight-chain alkanes (e.g. *alkB*) presumably due to the selective pressures of residual hydrocarbons in which heavier hydrocarbons remained for long periods of time in the field [28]. In this study, the indigenous microbial community in the field-aged contaminated site soils possessed a variety of catabolic genes such as *alkB, ndoB, phmAc* and *xylE* encoding degradation enzymes for alkane-, naphthalene-, phenanthrene- and catechol-2,3- dioxygenase, respectively, reflecting a significant expression of biodegradation activity for a broad ranges of hydrocarbons [19]. This was the basis for significant degradation of both F2 and F3 hydrocarbons.

4.3.3. Microbial growth patterns and activity acceleration

The variable, cold site temperatures suggested more favorable conditions for the growth and efficient yielding of cold adapted hydrocarbon-degrading bacteria in nutrient-amended field-contaminated soils compared to the constant average temperature incubation. As demonstrated in Figure 4.4b, the growth pattern of viable hydrocarbon-degraders and the resulting population size were significantly different between the two temperature modes. In VAR mode, the number of hydrocarbon-degraders more rapidly increased, resulting in an increase of over two orders of magnitude of the viable population size immediately after the nutrient amendment. This data on microbial populations in the VAR mode has been previously reported elsewhere [19]. The increased population sizes of 1.2×10^7 to 6.8×10^6 CFU/g were stable up to Day 60. In the CST mode, the slow growth of the hydrocarbon-degrading populations occurred, resulting in the statistically significantly lower population size of 8.9×10^6 CFU/g. The higher population of viable hydrocarbon-degraders resulted in the rapid and greater extents of petroleum hydrocarbon

biodegradation observed in the variable site temperature condition. Psychrotolerant hydrocarbon-degraders (e.g. *Rhodococcus* sp. strain Q15; *Sphingomonas spp; Pseudomonas spp*.) isolated field-contaminated cold region soils grow over wide temperature ranges and tend to reproduce rapidly with increasing incubation temperatures near 15-20 °C but not over 30 °C [29-31]. In general, the optimal growth temperature range of psychrotrophs parallels the ambient summer site temperature range and they are tolerant to temperature fluctuations [32].

In this study, significant acceleration in microbial respiration in the nutrient amended soils subjected to the variable site temperatures were detected with increasing temperatures from 1 to 10 °C. Figure 4.5a illustrates change in O_2 gas concentrations (every 8-hour measurement for five days) in the treated and untreated landfarms when soil temperatures uniformly vary between 1 and 10 °C at 1.74 °C/day. The O_2 soil pore gas measurements were performed during the stationary phase and thus there were no significant changes in viable population size of heterotrophs and hydrocarbon-degrading populations between 1 and 10 °C for the 5-day period. Figure 4.5b presents the real-time monitored soil temperatures in response to the quarter temperature cycle for the 5 days.

Between 1 and 4.7 °C, the rate of O_2 consumption was constant and then started to accelerate at 4.7 °C (T_a: soil temperature for microbial respiration acceleration in Fig. 4.5a. Microbial respiration was thus accelerated at T_a of 4.7 °C. The calculated temperature coefficient,

The Q_{10} was 2.2 between 1.3 and 9.2 °C, representing a significant temperature-dependency of microbial activity in this temperature range. In this pilot-scale study, the temperature-dependency of microbial activity in nutrient-amended landfarms was repeatedly exhibited throughout the course of the variable site temperature regimes. Figure 4.6 illustrates the CO_2 production ratios in VAR vs. CST soils. In the VAR mode, CO_2 production increased with increasing measured soil temperatures, and *vice versa*. At points A and A' and D and D', the CO_2 production ratios ranged from 1.6 ±0.02 to 2.3 ±0.85, indicating the higher microbial (respiration) activity under the warmer temperature regimes (6-10 °C) of the temperature cycle, compared to the constant temperature of 6 °C. When the soil temperatures between the two modes were equal to each other at 6 °C (at point B, C, and E), the relative CO_2 was close to unity. At 2.7 ±0.24 °C the CO_2 production ratio decreased below unity. The pilot-scale landfarming experiment suggested that the psychrotolerant hydrocarbon-degrading communities established in the nutrient amended

contaminated soils are metabolically active in a synchronous manner with temperature variations.

4.3.4. Microbial community shift and substrate-dependency

The 16s rDNA DGGE analyses indicated a shift in bacterial community due to the nutrient amendment in both VAR- and CST- mode. As shown in Figure 4.4a, the representative dendrogram constructed by Dice similarity coefficients (0.5% optimization and 0.5% tolerance) showed the significant differences between the nutrient amended landfarm in the CST mode and the untreated landfarm (or Day 0 soils before the nutrient amendment). A new band emerged on Day 20 after the nutrient amendment and then no notable further changes in the DGGE bands were observed on Day 40 and Day 60. The shift in microbial community structure is attributable to the nutrient amendments. In VAR mode, a very similar shift in microbial community structure occurred in 20 days after the same nutrient amendment; as observed in DGGE bands presented in [19].

The sequence analyses of the newly emerged DGGE bands after the nutrient treatment were closely matched to genus *Alkanindiges* (99% *Alkanindiges illinoisensis*, GenBank) in both temperature modes. The substrate ranges of *Alkanindiges sp.* are long-chain linear (≥ C16) and branched aliphatic hydrocarbons such as hexadecane, heptadecane, pristane and squalane [33-35]. This emergence of *Alkanindiges*-related populations capable of degradation of higher molecular weight hydrocarbons in both VAR- and CST mode experiments was in agreement with the observed biodegradation of non-volatile hydrocarbon fractions (> C16) and UCM reductions in both temperature conditions. However, in CST mode the slow growth of viable hydrocarbon-degrading populations as shown in Figure 4.4b significantly limited the rate of biodegradation of non-volatile hydrocarbon fractions (F3). On a basis of pairwise comparisons of the DGGE band intensity (GelCompar II), the band intensities in the CST nutrient-amended soils (Fig. 4.4a) were increased gradually (shown in supporting document for the bend intensities). The bend intensities in DGGE profiles are significantly correlated to dominant microbial population sizes with the level of 10^5- 10^8 CFU/g [36] and thus the DGGE bands indicated the emergence and/or growth of hydrocarbon-degrading populations. However, the indication of the shift in microbial community by DGGE analyses and the emergence of hydrocarbon-degraders alone did not

extensively explain the influence of site temperature variation on petroleum hydrocarbon biodegradation.

The Respiratory Quotient (RQ), defined as the molar ratios of produced CO_2 and consumed O_2, is an alternative indicative of substrate-dependency of microbial activity [37]. RQ-values provided additional information on whether microbial communities are utilizing target substrates under aerobic (RQ < 1) or anaerobic (RQ > 1) condition [38]. For straight-chain alkanes, PAHs (i.e. naphthalene) and humic acids, theoretical RQ-values driven from stoichiometric relationship are 0.67. 0.83 and 0.88, respectively [39]. Empirical RQ-values for petroleum-related contaminated soils (e.g. jet fuel and diesel cases) have typically ranged from 0.4 to 0.82 for aerobic petroleum hydrocarbon biodegradation [39-41]. The overall RQ-value determined in the VAR- mode experiment was 0.85 ±0.07 which was higher than the RQ-value of 0.62 ±0.07 for the CST-mode. The RQ-value for the CST mode was very close to the theoretical RQ-values of straight-chain alkanes, which corresponded to the significant degradation of the resolved hydrocarbon fractions (n-alkanes) rather than higher molecular weight hydrocarbons (e.g. UCM fractions). The RQ-value results additionally indicated that enhanced biodegradation of the established hydrocarbon-degrading communities due to the nutrient amendment were more efficiently express biodegradation activity for broader range of hydrocarbon fractions under variable site temperature conditions.

4.4. Conclusions

The present study suggests that variable site temperatures representative of site temperature changes between 1 and 10 °C over two months significantly influenced the rates and extent of biodegradation of petroleum hydrocarbons using the pilot-scale landfarming experiment for the field-aged, contaminated sub-arctic soils. Compared with the results of a constant average incubation temperature of 6 °C, more rapid biodegradation of both semi- and non-volatile hydrocarbons occurred by a factor of two and biodegradation activity was extended to UCM fractions under the variable temperature condition. Preferential biodegradation of semi-volatile hydrocarbons over non-volatile hydrocarbons was significant in the constant average temperature mode, but not under the variable temperature regime.

The enhanced biodegradation under the variable temperature conditions is correlated to accelerated microbial activity and rapid growth of indigenous hydrocarbon-degrading microbial populations. The expression of biodegradation activity of established microbial community due to nutrient amendments was significantly influenced by temperature. The first-order biodegradation rate constants determined by the variable temperature-mode were in better agreement with those determined by an on-site experiment at the same site. These findings provide a potential insight of the role of temperature variability on determination of the rate and extents of petroleum hydrocarbon biodegradation in cold climates.

Table 4.1. Removal efficiency[a], preference ratio and first order rate constants[b] for TPH, F2 and F3 biodegradation between the cyclic and constant temperature modes (mean ± SE).

		VAR mode	CST mode
Removal efficiency[a]	TPH	55%	19%
	F2 (>C10-C16)	63%	22-36%
	F3 (>C16-C34)	53%	21%
	Σ UCM	47%	19%
Preference ratio: Δ[F2] /Δ[F3]	Day 20	1.7	1.6
	Day 40	0.9	2.6
	Day 60	0.7	1.1
	Mean (Day 20-60)	1 ± (0.1)	2 (±0.3)
Rate constants (day^{-1})[b]	K_{TPH}	0.018 (±0.003)	0.008 (±0.001)
	R^2	0.86	0.93
	K_{F2}	0.024 (±0.005)	0.012 (± 0.005)
	R^2	0.81	0.99
	K_{F3}	0.016 (±0.002)	0.004 (±0.0007)
	R^2	0.93	0.80

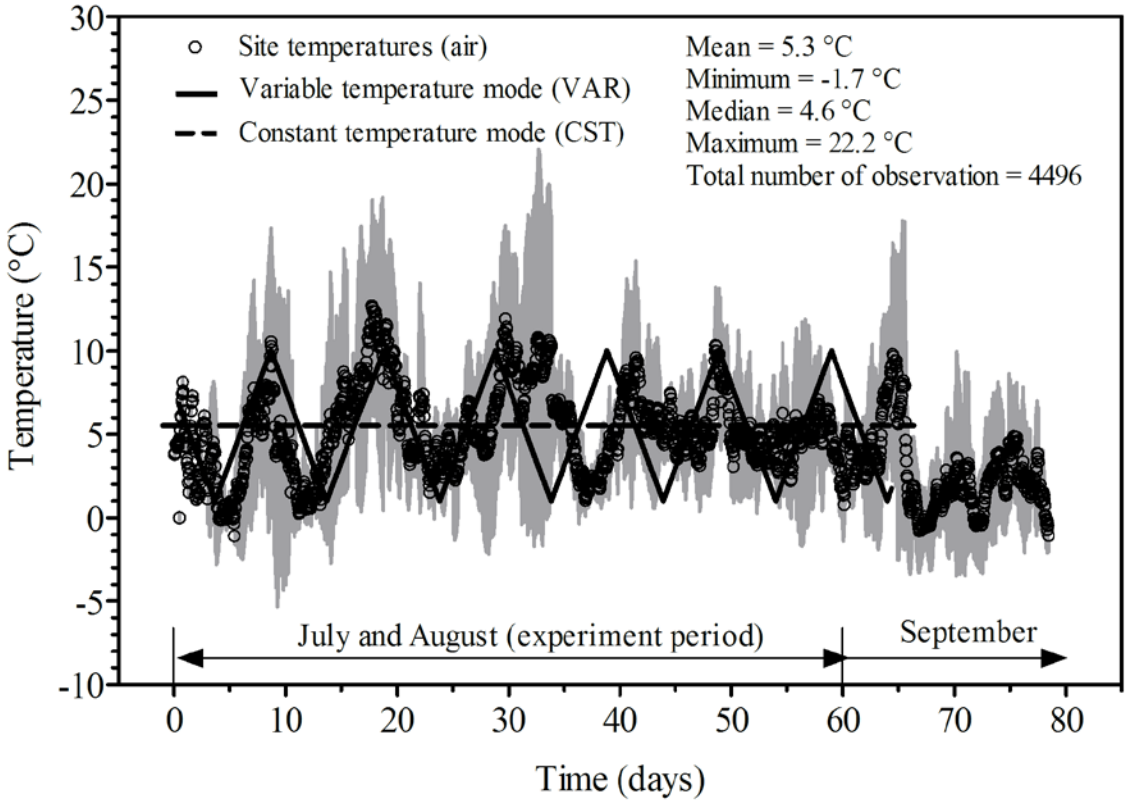

Figure 4.1. Site temperature profile for the VAR- and CST- mode pilot-scale experiment. Hourly temperature data for 2002-2005 (shown as grey data points) was obtained from Environment Canada. Average temperatures for each day over the 3-year period are shown with open circles.

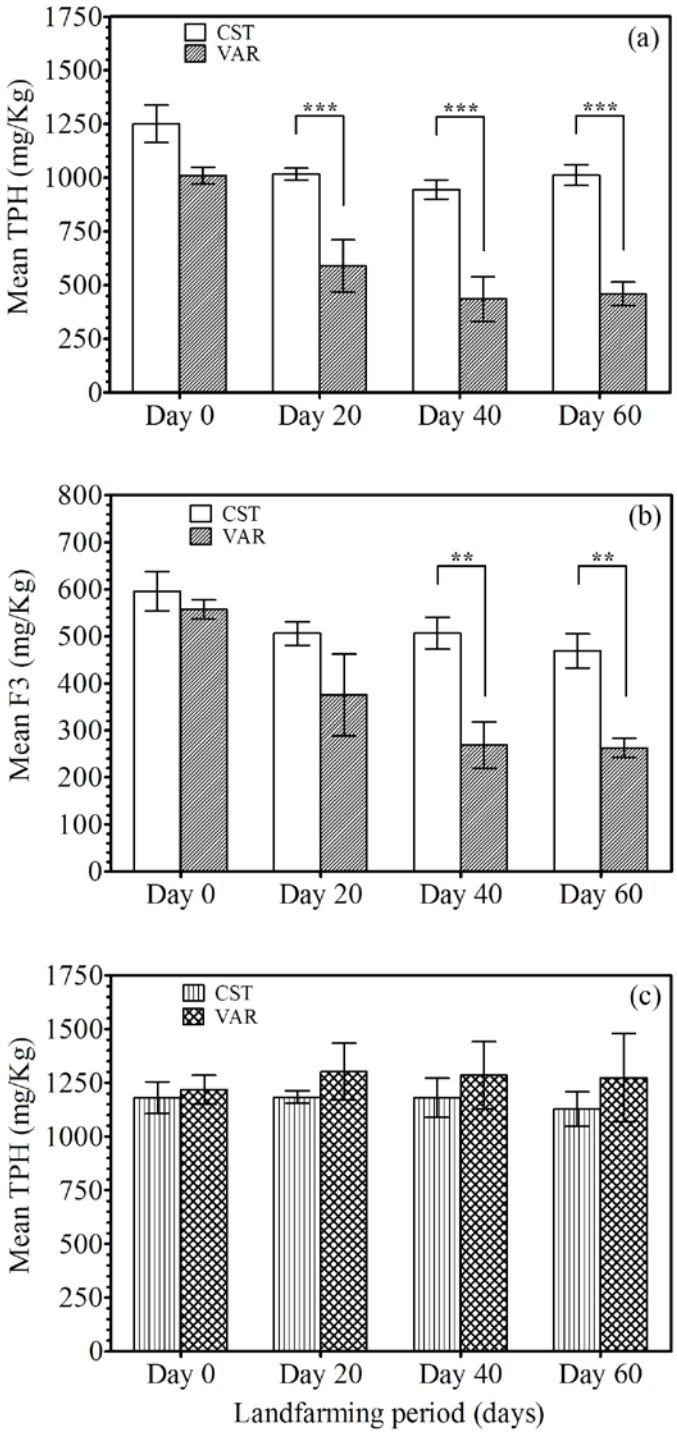

Figure 4.2. Comparisons of biodegradation of (a) TPH; (b) non-volatile hydrocarbon fractions (F3:>C16-C34) in the nutrient amended (treated) pilot-scale tanks; and (c) TPH in untreated (control) tanks between variable site temperature- (VAR) and constant average temperature- mode (CST). Error bars indicate one standard deviation from the mean; ***: $p < 0.001$ and **: $p < 0.01$ (obtained by two-way ANOVA plus Bonferroni's post test for multiple comparisons).

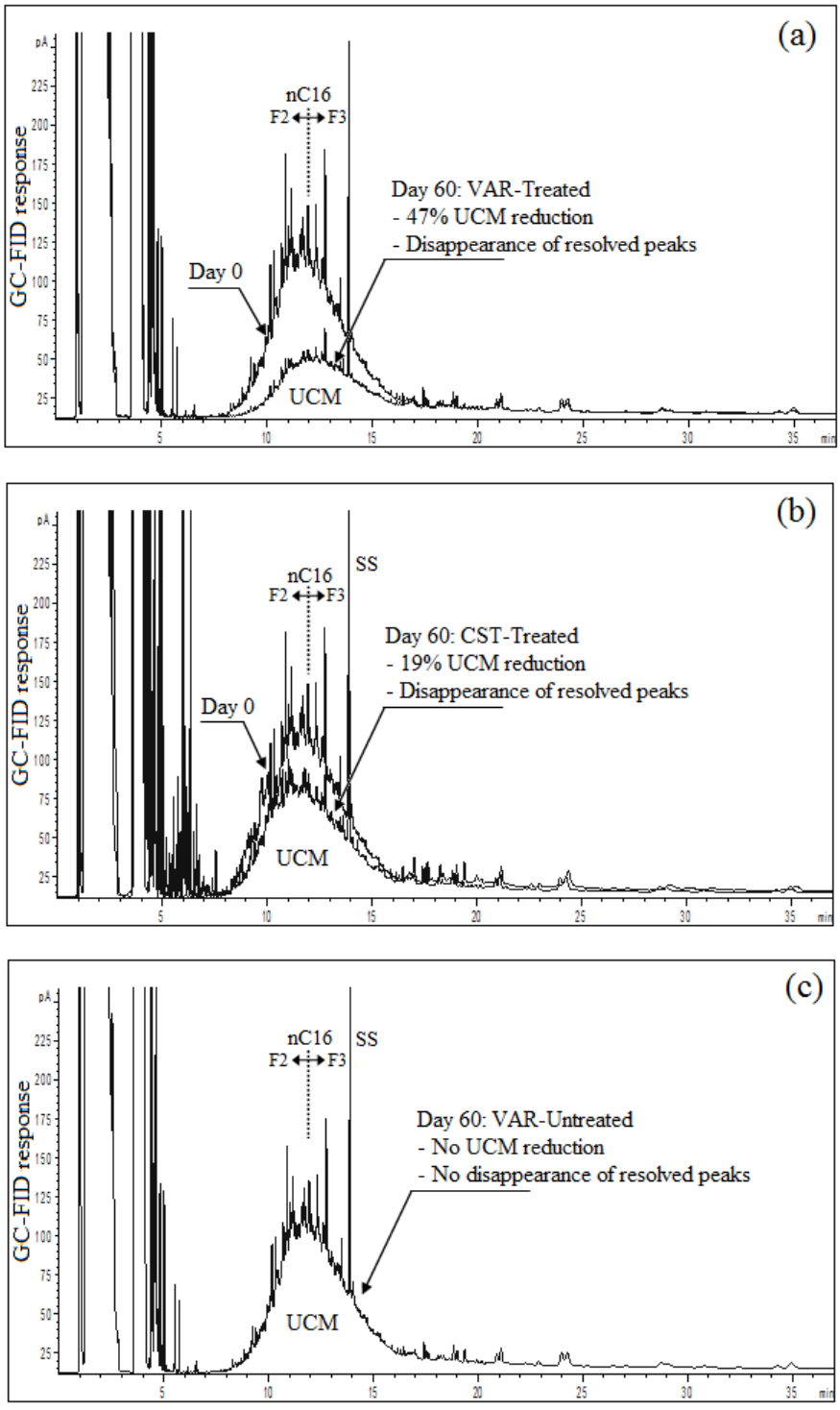

Figure 4.3. Representative TPH chromatograms of (a) VAR-Treated; (b) CST-Treated; and (c) VAR-Untreated. Note: UCM: Unresolved Complex Mixture, SS: surrogate standard

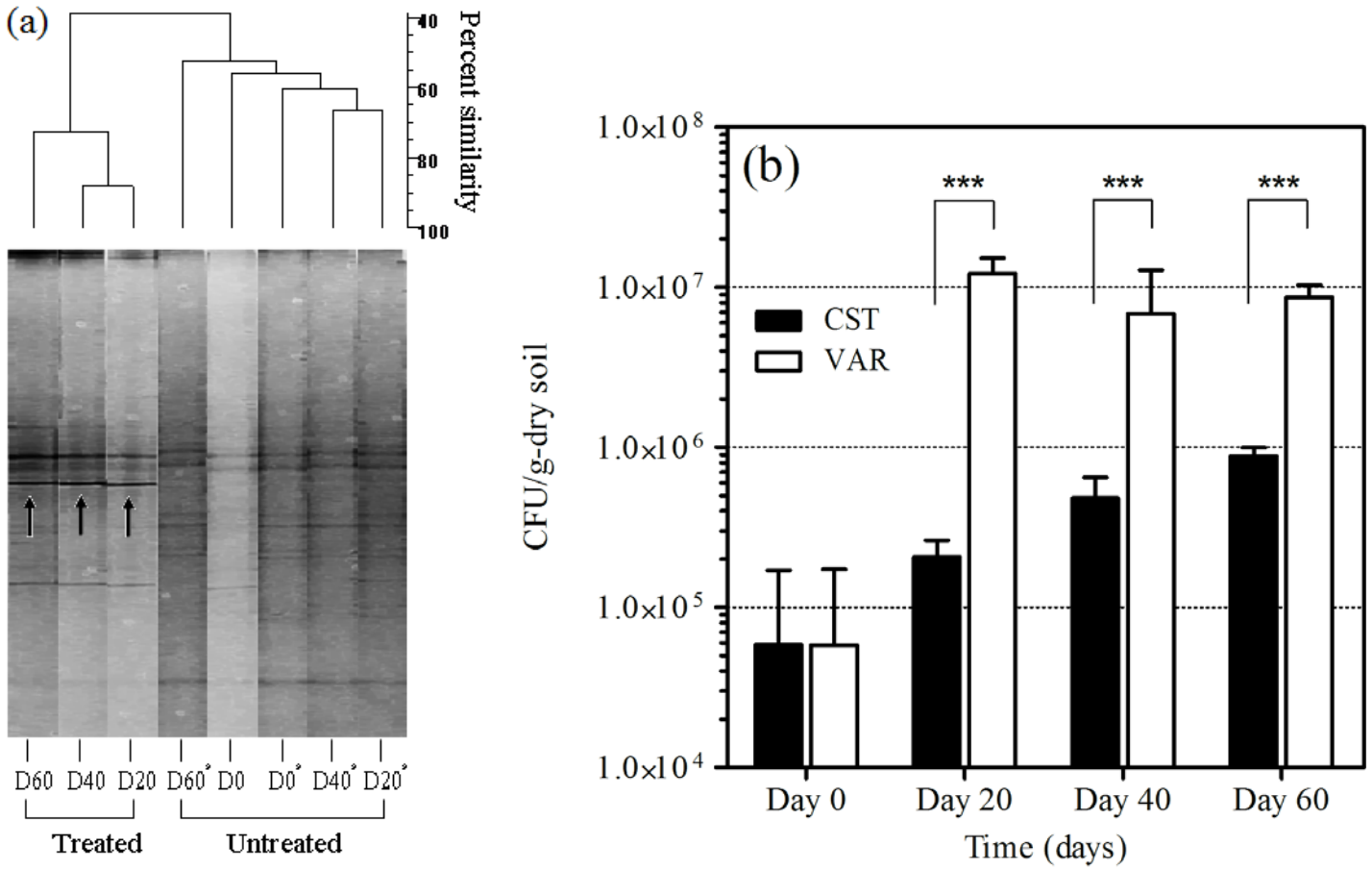

Figure 4.4. (a) Representative cluster analysis of DGGE band patterns on Day 20-60 in the both nutrient amended and untreated landfarm in the CST mode; newly emerged bands indicated by arrows on the DGGE profile are *Alkanindiges*-related populations (99% similarity) that are long-chain hydrocarbon degrading bacteria. (b) comparison of viable hydrocarbon-degrading microbial populations between VAR- and CST- mode. Data for microbial populations in the VAR mode (blank bar) was previously reported elsewhere [19]. Error bars indicate one standard deviation from the mean; ***: $p < 0.001$ (obtained by two-way ANOVA plus Bonferroni's post test for multiple comparisons). Note: no significant changes of viable hydrocarbon degraders on Day 20-60 in the both untreated landfarms (controls) were observed

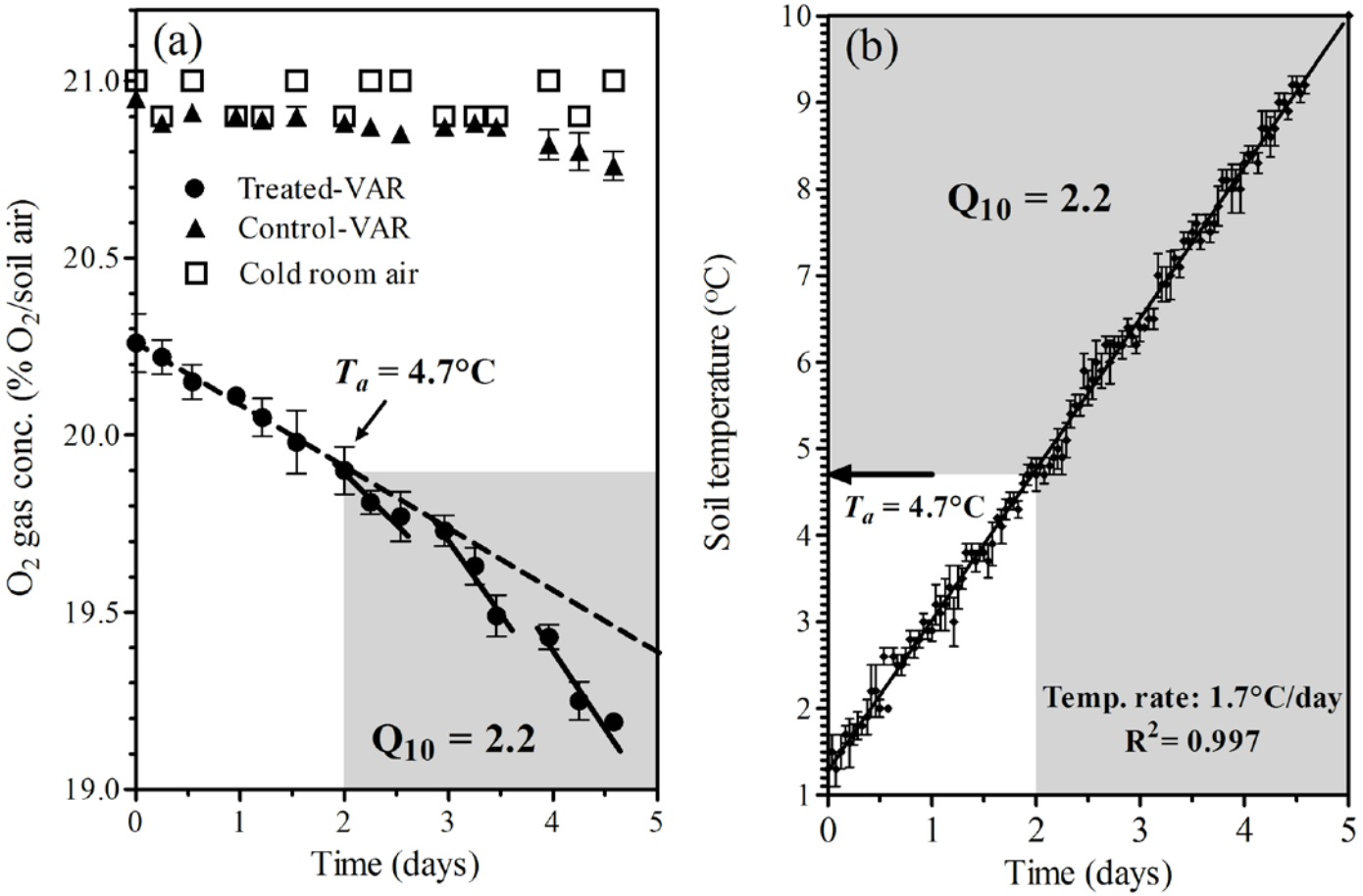

Figure 4.5. Results of 8-hour interval measurements of O_2 concentrations in the surface, middle and bottom layers of nutrient amended and untreated landfarms in VAR-mode. (a) Changes in O_2 pore gas concentrations and (b) the corresponding soil temperatures (mean soil temperature of surface, middle, and bottom layers). Onset of acceleration in microbial respiration activity at T_a of 4.7 °C in VAR-mode. Q_{10} refers to a temperature coefficient between 1 and 10 °C. The determined Q_{10} of 2.2 >1.0 implies that the rate of microbial respiration is significantly

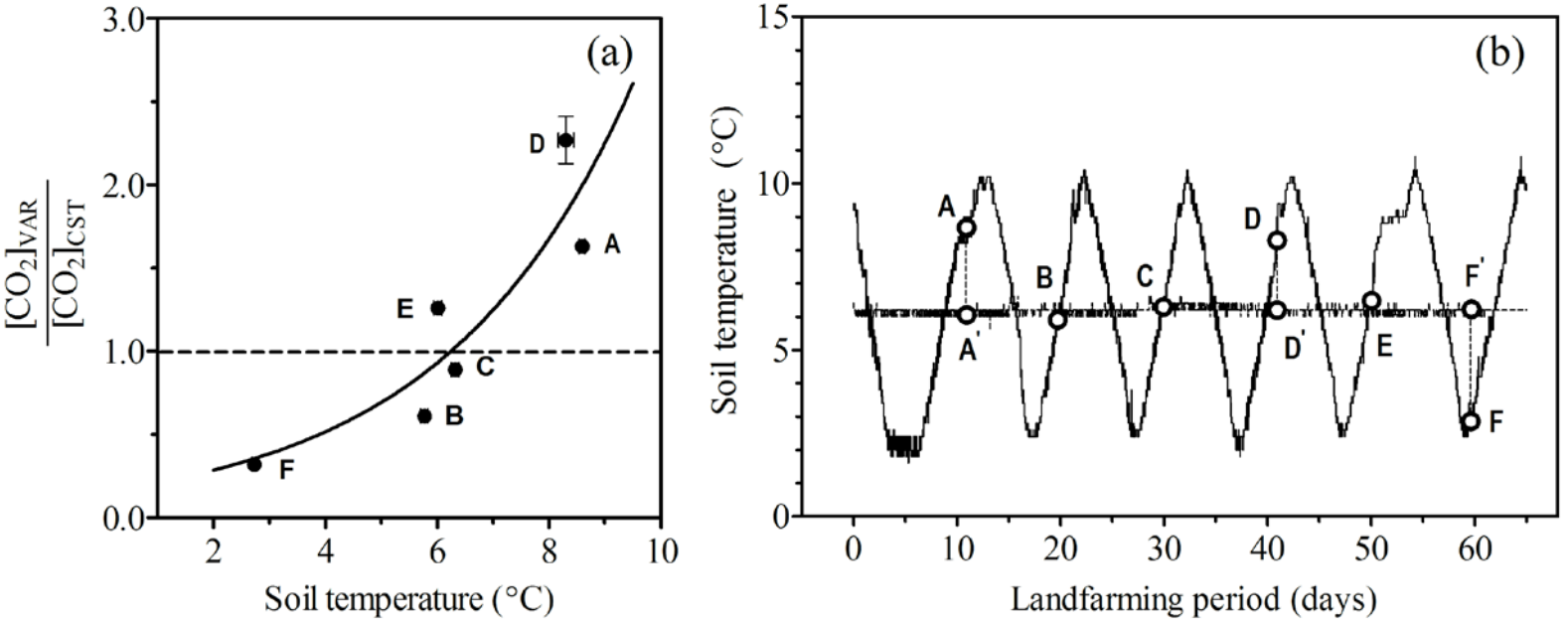

Figure 4.6. Relative comparison of CO_2 production ratios between VAR- and CST- modes.

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Connecting text: Chapter 4 and 5

In Chapter 4, pilot-scale landfarming experiments were performed in summer temperature regimes. In Chapter 5, the extent of petroleum hydrocarbon biodegradation during various temperature regimes relevant to the post summer and pre-summer seasons, including the seasonal freeze-thaw period was evaluated. Field treatment systems are left undisturbed between consecutive summer seasons. In Chapter 5, biodegradation of different petroleum hydrocarbon fractions and associated microbial activity and communities were assessed during this period.

Chapter 5

Hydrocarbon biodegradation under sub-zero seasonal freeze-thaw and post thaw soil temperature regimes in contaminated soils from a sub-Arctic site

Wonjae Chang[1], Sara Klemm[2], Lyle Whyte[2], Subhasis Ghoshal[1]

[1]Department of Civil Engineering, McGill University, Montreal, Canada
[2]Department of Natural Resources, McGill University, Montreal, Canada

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5.1. Introduction

A significant number of petroleum-contaminated sites have been identified in cold, polar and sub-polar regions as a result of fuel storage and transportation, as well as military and industrial activities [1]. Cold temperatures and the remote locations of these sites pose challenges to site remediation. The biostimulation of cold-adapted indigenous psychrotolerant and psychrophilic hydrocarbon-degrading microbial populations by supply of nutrients (e.g., N, P), moisture, and aeration is considered to be a key factor for effective bioremediation [2]. On site, *ex situ* bioremediation has been found to be feasible and has been demonstrated through field as well laboratory treatability studies [3-5] in Arctic and sub-Arctic sites. It is generally believed that the potential for bioremediation exists only during the 2- to 3-month summer season when daily average temperatures at northern sites are generally in the range of approximately 5 -15 °C, The short summer season over which bioremediation is implemented and monitored is often insufficient time for meeting remediation targets, especially where there contamination is extensive and/or high petroleum hydrocarbon concentrations are present. Treatment systems are thus generally often left dormant after summer when freezing temperatures set in and revived in the following summer [6, 7].

Significant metabolic and respiration activity of psychrophilic soil bacteria has been detected near and below sub-zero temperatures and these microorganisms likely remain active in unfrozen liquid water that is present in the soil matrix over a range of sub-zero temperatures [8, 9]. Psychrophilic bacteria are able to remain active at cold temperatures through a number of adaptations such as changes in the membrane lipid composition to maintain fluidity, expression of cold active enzymes, cold-shock proteins and extra polymeric substances for cell membrane protection [10]. The activity and viability of soil bacteria at near zero and sub-zero temperatures suggests the possibility of hydrocarbon biodegradation potential under such temperature regimes and only a few studies have evaluated microbial growth, activity and hydrocarbon degradation under such conditions [11]. Microcosm experiments have shown aliphatic hydrocarbon biodegradation during rapid, diurnal to bi-weekly freeze-thaw cycles ranging from -5 to +5 °C, as well as during incubation at constant near zero or sub-zero temperatures [12, 13]. Such rapid freeze-thaw cycles however cannot account for the effects of long term, seasonal temperature changes on microbial activity. Although rapid freeze-thaw cycles do occur at cold regions sites,

temperature changes in surface soils and surface layers are attenuated and temperatures generally decrease in winter and increase in spring in an approximately steady monotonous manner [14-16]. Furthermore, the rate of freeze-thaw plays a key role in determining the amount of pore ice, liquid pore water and the salt concentrations in liquid pore water [17], as well as on microbial survivability [18]. The hydrocarbon biodegradation activity and the microbial population dynamics or community shifts during the seasonal transition periods preceding and following summer where freezing and thawing of the surface soil layers occurs and during the winter months has not been studied extensively.

The objective of this study was to assess the extent of petroleum hydrocarbon biodegradation and associated microbial activity and its relationship to changes in temperature and unfrozen liquid water content in historically diesel-contaminated, biostimulated soils subjected to typical temperature regimes of the post- and pre-summer months at a contaminated site in Resolution Island (RI), Nunavut, Canada (61°30'N 65°00'W). A description of the site from where the soils were obtained is provided elsewhere [3]. Biodegradation experiments were carried out in pilot-scale tanks that simulated landfarm operations, and were placed in a cold room which was programmed to operate at temperatures that were representative of the mean daily temperatures of September and October (representing the freezing phase) and May and June (for the thawing phase) at in the RI site. During mid-June to July, temperature patterns change and vary between constant temperature periods and periods of rapid warming. Biodegradation activity during these temperature regimes were also evaluated following the thaw phase. A control tank which was not biostimulated, but maintained at the same temperature regimen was maintained to determine baseline petroleum hydrocarbon profiles and microbial activity. The pilot-scale tanks were monitored by periodically analyzing soils samples for semi-volatile and non-volatile hydrocarbon, microbial populations using culture-based and molecular methods, soil temperature and moisture content, and monitoring of O_2 and CO_2 concentrations in soil gases.

5.2. Experimental Section

5.2.1. Site soils

Significant volumes of aged, petroleum-contaminated soils as well as uncontaminated soils were excavated at the RI site and shipped frozen in sealed metal containers. The soils are coarse-grained sand, consisting of 27% gravel, 72% sand, and 1% silt & clays. Mineralogically the soil composition was 62% quartz, 14% plagioclase feldspar, 10% potassium feldspar and 3% kaolinite as determined in X-ray diffraction studies. Several key catabolic genes involved in encoding for enzymes for hydrocarbon degradation, such as *alkB*, *xylE*, and *ndoB*, was detected in the contaminated soils shipped from the RI site, and was indicative of the presence of hydrocarbon degrading microorganism [19].

5.2.2. Biodegradation experiments

Biodegradation experiments were conducted in stainless steel tanks operated to simulate landfarming operations at the pilot-scale. The tanks were 1.0 m long, 0.65 m wide and 0.35 m deep and contained soil to a depth of 22 cm. For soil gas extraction during the biodegradation experiment, perforated stainless steel horizontal gas collection tubes were placed at soil depths of 17.5, 12.5, 7.5 and 2.5 cm and connected to gas sampling ports fitted with valves. O_2-CO_2 soil gas concentrations were measured by an infrared gas monitor that is able to measure gas concentrations at subzero temperatures (ATX 620 multi-gas monitor, Industrial Scientific Co.). Compressed air (78% N_2, 21% O_2, 1% Ar, and trace CO_2) was consistently supplied into the headspace of the pilot-scale tanks at the slow air-flow rate of 30 L air/day. The supplied air was exhausted through the activated carbon and moisture traps (Na_2SO_4) that were fitted with each pilot-scale tank. The soil was mixed with a commercial fertilizer (20% N: 20% P_2O_5: 20% K_2O, 20:20:20 Plant Prod®) which was presterilized and applied at a dose that yielded a molar-based C_{TPH}: N: P ratio of 100: 9: 1. The site soil was acidic (soil pH 4.5) and was thus amended with 2000 mg $CaCO_3$/Kg dry soil to raise soil pH and buffer it to between 6.5 to 7.0 [20]. The gravimetric soil water content (GWC) of 12% after addition of various amendments was within

60% of the maximum water holding field capacity. The control tank was not amended with nutrient, $CaCO_3$ or moisture. Each pilot-scale tank contained about 200 Kg wet site soils, and the tanks were placed in a large cold room and subjected to the controlled temperature profile as described in Fig. 5.1. Local temperature data of the RI site were acquired from National Climate Data and Information Archive of Environment Canada in which the mean daily temperatures for the selected months were available between 1969 and 2006.

Soil temperatures and volumetric water contents (VWC) were monitored by using thermocouples and frequency domain reflectometry sensors (Decagon EC-TE sensor-FDR) and these have been validated for unfrozen water measurement in frozen soils down to -15 °C in other studies [21]. The sensors were installed at the three replicated locations of middle layers of the pilot-scale tank. The moisture sensors measure the unbound pore water rather than the total moisture content in the soils. In this study, the moisture probes were specifically calibrated using the site soils and the mean VWC obtained from the calibration for the unamended soils was 0.125 ±0.003 m^3/m^3which is calculated to be equivalent to 7.8% GWC. The measured GWC of air-dried site soils was 4.2 ±0.64%, which was regarded as bound water (i.e. hygroscopic water). Thus the measurements of the moisture sensor are in agreement with the total measured GWC of 12%.

5.2.3. Soil analyses

5.2.3.1. Petroleum hydrocarbons

An analytical method for extractable total petroleum hydrocarbons (TPH) was based on CWS PHC – Tier 1 Method which sets out procedures for analysis of the TPH extracts into 4 different fractions (F1, F2, F3 and F4) where fraction F1 represents compounds with boiling points lower than C6 (hexane), F2 represents compounds with boiling points between C10 (decane) and C16 (hexadecane), F3 represents compounds with boiling points between C16 and C34 alkanes and F4 represents compounds with boiling points above C34 alkanes [22]. In the pilot-scale soil tank, five replicate soil samples were collected in each surface, middle, and bottom layers of the soil tank on each soil sampling day with 20 days interval. The soil samples were blended with activated anhydrous sodium sulphate (Fisher Scientific) and TPH extraction

was carried out in an automatic Soxhlet extractor (Gerhard Soxtherm) with 140 mL of 50:50 hexane: acetone. Details of the extraction, post processing and GC analyses methods are presented in the Supporting Information section.

5.2.3.2. ^{14}C-hexadecane mineralization assays

Each microcosm was prepared with 10 g wet soil (triplicate) retrieved from each layer of the pilot-scale tank on unfrozen, semi-frozen, frozen and thawing phase. The retrieved samples were spiked with 50,000 disintegrations per minute (dpm) ^{14}C-hexadecane dissolved non-radiolabeled hexadecane to 100 ppm (wt. hexadecane/wt. soil) and sealed. Triplicate sterile microcosms (control) were prepared by autoclaving at 121 °C for 45 minutes at 103 kPa. All the microcosms were incubated at 5 °C. Any CO_2 evolved (including $^{14}CO_2$) in the recovery trap (1M KOH + 10% v/v ethylene glycol) which was periodically sampled for 10 weeks and analyzed for $^{14}CO_2$ recovery using a LS6500 scintillation counter (Beckman Coulter,).

5.2.3.3. Culture dependent- and -independent analyses

The detailed protocols of culture dependent and- independent analyses were conducted as described in [19]. For enumeration of viable heterotrophic and hydrocarbon-degrading microbial populations, 1.0 g of soil collected from the pilot-scale soil tank was vortexed for 30 seconds in a glass tube containing 2.5 g sterile glass beads and 9.0 ml mineral salts medium (MSM). Triplicate R2A agar plates (Becton, Dickson and Company, Sparks, MD) were made by spreading 100 µl aliquot of serial dilutions with 0.1% Na_2PO_7 and then incubated at 5 °C. Similarly, MSM agar plates were made with 150 µl aliquot of arctic-grade diesel (Shell Canada) onto 2.5cm^2 piece of filter paper on the inner glass Petri dish lid.

The procedure of total community DNA extraction and PCR amplification were described previously [23]. For quantitative detection of 16S rDNA gene copy numbers and *alkB* genes, real-time PCR was performed with the iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Briefly, plasmid standards were made by cloning the PCR-amplified target region into a pGEM-T Easy vector (Promega Corporation, Madison, WI). Bacterial 16S rDNA and *alkB* genes inserts were derived from *Rhodococcus* sp. strain Q15.

Denaturing gradient gel electrophoresis (DGGE) analyses were performed using DCode universal mutation detection system (Bio-Rad, Hercules, CA), bacterial 16S rDNA were run on an 8% polyacrylamide gel with a 35%-65% denaturing gradient. The stained gels (0.6 µg ethidium bromide/ml 1×TAE for 30 minutes) was destained in 1×TAE for 5 minutes and then viewed by UV transillumination (ChemiGenius Bioimaging system, Cambridge, UK). The excised DGGE bands were eluted in 50µl Milli-Q water at 4 °C overnight and then the reamplified DNA (primers without GC clamp) for sequencing analyses was sent to Plate-forme d'analyses biomoléculaires, Université Laval, Quebec.

5.3. Results and Discussion

5.3.1. Soil freeze thaw temperatures and unfrozen water contents

The freezing curve representing temperature changes, and the water content profile for the petroleum hydrocarbon-contaminated site soils in the pilot-scale tank are shown in Figure 5.2. A typical soil freezing curve was obtained, showing a temporary temperature increase up to the freezing temperature, T_f, of -0.2 °C, after reaching T_{sc}, the supercooled temperature of – 0.5 °C, which is associated with the release of latent heat during formation of ice [24] . The soil water content was unchanged at 0.12 m^3/m^3 (GWC of 11.9 ±0.46%) until T_{sc} was reached but decreased steadily until reaching a water content less than 0.01 m^3/m^3 at a temperature of – 2.1° C (labelled, T_{ef} or effective end point of free water) at approximately Day 40 of the freezing cycle. Between Day 21 and 40 significant and measurable amounts of liquid pore water co-existed with the ice and thus the soil was designated to be in a 'semi-frozen' state. The presence of unfrozen water results from the freezing point depression associated with increasing ion concentrations in residual liquid water once ice formation sets in, and with decreases in partial molar free energy arising from the presence of dissolved hydrocarbons in water, surface wetting by water and the surface curvature of soil particles and/or free hydrocarbon phases in the soil matrix [25]. As discussed in the following sections, hydrocarbon biodegradation and microbial activity was detected in the semi-frozen soils. Between Days 41 and 60, trace levels of unfrozen pore water were detected and soils were thus designated to be effectively 'frozen' during this period.

The soil thawing curve and soil water content diagram is presented in Fig. 5.3a. The temperature and volumetric water content profiles mirrored the patterns observed during the freezing phase, although the frozen and semi-frozen states were of shorter duration due to the applied thawing rate of 0.16 °C/day being higher than the freezing rate of -0.12 °C/day. The unfrozen water content increased significantly above -0.5 °C (T_m) and at +0.4 °C, the water content recovered to 0.127 ±0.004 $m^3/^3$ (=7.97% in GWC), which is similar to the water content at the beginning of the freezing phase.

5.3.2. Hydrocarbon biodegradation and microbial respiration activity in the freezing phase

The concentration of F2 (>C10-C16) hydrocarbon fraction decreased by approximately 13% from 579±62 mg/Kg to 503±48 mg/Kg in the nutrient-amended (treated) contaminated soils during the freezing phase as shown in Table 5.1. This decrease is statistically significant (one-way ANOVA with Dunnett's post test at $p < 0.05$) and the decrease in hydrocarbon concentrations occurred primarily occurring between Day 20 and 40, a period where temperatures ranged from 0 to -2 °C and soils were semi frozen with volumetric water contents decreased steadily from 0.1 to 0.003 m^3/m^3. No statistically significant reduction in F3 (>C16-C34) fraction hydrocarbons in the treated tank occurred during the freezing period. The control tank that was maintained at the same temperature regime but was not amended with nutrients and did not have any statistically significant reduction of either F2 or F3 hydrocarbons. The lack of hydrocarbon losses in the control which was maintained at the same temperature regime, and the significantly higher O_2 consumption and CO_2 production (Figure 5S.1) in the nutrient amended tank compared to the control, suggests that the hydrocarbon losses in the nutrient amended tank are due to biodegradation. Biodegradation of field-aged hydrocarbons at 0 °C and/or under sub-zero conditions has been reported by very few studies, and the extent of biodegradation observed in those studies is small, as observed in our experiments. In microcosm experiments with contaminated soils from an Arctic site, Erikson et al. [12], observed biodegradation rates of 0.95 mg/Kg/day of C11 to C15 alkanes at 0 °C but biodegradation was not observed at -5 °C. Rike et al. [14] reported *in situ* O_2 consumption and CO_2 production attributable to hydrocarbon

biodegradation at a polar petroleum hydrocarbon-contaminated site over a period where the soil temperatures decreased from near zero to -2 °C between the months of October and November and estimated a biodegradation rate of 3 mg/Kg/day.

The hydrocarbon degradation potential of the soil microorganisms in the nutrient-amended tank increased with time in the freezing phase, as seen from the ^{14}C-hexadecane mineralization activity in soil samples obtained at different time points from the pilot-scale tanks (Figure 5.4). It should be noted that the mineralization activity was assessed by incubation at 5 °C, and thus the different mineralization activity exhibited by the soil samples obtained at different days was not influenced by temperature. Hydrocarbon biodegradation did not occur between Day 40 and 60 as shown in Table 5.1 (Figure 5.2). A very significant change in soil respiration activity in the nutrient amended tank is observed at around Day 40, and this coincides with the lack of availability of unfrozen pore water at temperatures below -2 °C as shown in Figure 5.2. The biodegradation activity below -2 °C thus appears to be limited by the low unfrozen water levels. The hexadecane mineralization capacity at 5 °C of the frozen soil sample was comparable to the soil samples obtained in the semi-frozen state indicating the sustained presence of significant populations capable of hydrocarbon degradation. .

5.3.3. Characterization of microbial populations during the freezing phase

Plate counts of viable heterotrophs on R2A agar extracted from the soils in the nutrient amended tank indicated a significant increase in microbial populations from approximately 1.0×10^5 to 7.0×10^7 CFU/g soil (two-way ANOVA, $p < 0.05$) between Day 20 and Day 40 when the soils were in a semi-frozen state, as shown in Figure 5.5a. A smaller increase in the heterotroph population was also observed in the control tank during this semi-frozen period. There was a tenfold decline in the viable heterotroph populations during the frozen state between Day 40 and 60. The enhanced heterotrophic populations at Day 40 generally reflect the same trend of enhanced hydrocarbon biodegradation activity and mineralization activity observed in Table 5.1 and Figure 5.4. Culture-independent analyses of 16S rDNA copy numbers from qPCR studies confirmed that maximum population sizes occurred at the end of the semi-frozen period and there was no further increase in the frozen state (Fig. 5.5b).

PCR-DGGE analyses of soils sampled from the nutrient amended tanks demonstrated the emergence of microbial communities during the freezing phase as shown in Figure 6. In the semi-frozen state (Day 20 to 40), the two DGGE bands (FZ1: *Actinomycetales* and FZ2: *Rhodanobacter)* emerged and the intensity of the other of bands was also notably enhanced. Sequencing of the FZ1-band indicated a match with microbial populations closely related to *Corynebacterineae* family (similarity 91%). A strain from the *Corynebacterineae* family has been previously identified in petroleum-contaminated site in the Antarctica and has been shown to be capable of hydrocarbon degradation [26, 27]. In contrast, DGGE analysis of soil samples from the control tank showed a stable community structure, suggesting that the emergence hydrocarbon degrading populations by Day 20 was primarily due to biostimulation resulting from the nutrient amendments.

5.3.4. Hydrocarbon biodegradation and microbial activity in the thawing phase

Between Day 0 and Day 20 of the thawing phase when temperatures ranged from – 4.9 °C to -1.2 °C and soil was effectively frozen, hydrocarbon biodegradation or microbial respiration activity was not observed as shown in Table 5.2 and Figure 5.3b. However, there was microbial community shifts and PCR DGGE gels showed that *Actinomycetales* that had appeared in the freezing phase, subsequently disappeared after Day 20 of the thaw phase (Fig. 5.6b).

Statistically significant degradation of F2 fraction hydrocarbons was on Day 40 in the nutrient amended tank with F2 hydrocarbons decreasing from 452±49.8 mg/Kg in Day 0 to 402±61.9 mg/Kg, and hydrocarbon concentration decreases were not observed in the control tank. Microbial respiration activity was reactivated at sub-zero temperatures (-1.2 to -0.5 °C) in the semi-frozen soil (Figures 5.3). Heterotrophic bacterial populations also increased during the thawing phase.

The F2 hydrocarbon fraction concentrations decreased by 38% during the entire thaw and post-thaw phase and approximately 30% of this decrease occurred during the post-thaw season between Day 60 and 97 in the nutrient amended tank as shown in Table 5.2. A relatively small but statistically significant decrease in F3 hydrocarbon fraction concentrations also occurred between Day 60 and 97. Approximately 24% decrease in F2 fraction hydrocarbons also occurred in the control tank, and accounting for the fact that respiration activity in the tank showed

minimal increase even with the rapid increase in temperature between Day 84 and 97, these losses can be attributed to volatilization (Table 5S.2). Effective biodegradation of the F2 hydrocarbon fraction in the nutrient amended tanks is thus approximately 14%. Accounting for volatilization (losses in control tank) biodegradation accounted for up to 28% decrease in F2 hydrocarbon fractions in the temperature regimes where temperatures were at or above 4 °C, and suggests that the presence of adequate nutrients at the end of the summer treatment system may lead to significant reductions, at least for relatively lower molecular weight hydrocarbons.

During the constant-temperature phase of +4 °C, soil respiration activity increased moderately as shown in Figure 5.3 and 5S.1. Hexadecane mineralization assays confirmed that the greatest hydrocarbon biodegradation activity was expressed (Fig. 5.4). The constant-temperature period was maintained to determine if accelerated respiration rates, often described as a 'CO_2 burst' during soil thawing would occur at these temperatures [28]. However, microbial respiration activity was very significantly increased when the temperature changed rapidly from 4 to 9 °C on between Day 84 and 97. Tilling in the nutrient amended tank at the constant 4 °C phase only resulted in a modest increase, indicating O_2 availability to the microorganisms and of the soil during freezing were not limiting factors, and microbial activity was primarily sensitive to temperature. The CO_2 burst and enhanced biodegradation of hydrocarbons during the thaw season has been attributed to the availability of a new pool nutrients and substrates from microbes killed during the freezing phase [28], but in our study the rapid rise in temperature and the presence of amended nutrients were key factors in enhanced respiration and biodegradation activity. The biodegradation of the higher-molecular weight, F3 hydrocarbon compounds during the thawing phase could be attributed to microbial community shifts where *Pseudomonadales* (*Alkanindiges*-89% sequence similarity) was newly emerged *Alkanindiges*-related microbial populations have been frequently identified in the bioremediation site soils above 0 °C as well as involved in biodegradation of high-molecular-weight hydrocarbon fractions [19, 29] (Fig. 5.6b).

5.4. Implications and concluding remark

The pilot-scale biotreatment experiment demonstrated a significant potential of hydrocarbon biodegradation in aged petroleum-contaminated soils beyond summer seasons in cold climates when sufficient nutrients are available. During the seasonal freeze-thaw cycle, the total removals of F2 and F3 were 52% and 16%, respectively (Table 5.2). Quantifiable F2 (>C10-C16) biodegradation was detected during both freezing- and thawing phases. However, F3 (>C16) biodegradation occurred exclusively in the thawing phase.

The seasonal freezing rate did not inhibit growth and metabolic activity of indigenous hydrocarbon-degraders, resulting in the significant increase in viable population sizes and 16S rDNA in sub-zero temperature, semi-frozen soils. The microbial activity is maintained likely due to the slow rate of freezing that limits intercellular ice crystallization and cell damage, as well the availability of liquid frozen water [18, 30]. This phenomenon is relevant to field conditions at northern sites, as shown by Olsson et al. through field monitoring at an Alaska site [16] where it was found that soils started to freeze *in-situ* in September, but the soil temperatures remained near 0 °C until November and allowed for significant amounts of unfrozen pore waters.

Table 5.1. Seasonal freezing: changes in F2 and F3 conc., soil temperatures, water availability in nutrient-amended landfarm.

		F2		F3		Soil temp.	Water availability	Soil phase
		mg/kg	Sig	mg/kg	Sig.	°C	% GWC	
Day 0-Freeze		579 ±61.9	-	665 ±97.9	-	Start at 2.4	5.7	Unfrozen
Day 20		551 ±60.3	NS	660 ±63.8	NS	2.4 → 0.0	5.7→7.8	Unfrozen
Day 40		**480 ±39.8**	**S**	**620 ±59.2**	**NS**	**0.0 → -2.1**	**7.8→0.2**	**Semi-frozen**
Day 60		503 ±48.0	S	634 ±78.6	NS	-2.1 →-4.4	0.2→0.0	Frozen
% Removal	Treated	13-17	S	< 5	NS			
	Untreated	0-9	NS	< 1	NS			

Table 5.2. Seasonal thawing: changes in F2 and F3 conc., soil temperatures, water availability in nutrient-amended landfarm.

		F2		F3		Soil temp.	Water availability	Soil phase
		mg/kg	Sig	mg/kg	Sig.	°C	% GWC	
Day 0-Thaw		452 ±49.8	-	628 ±98.5	-	Start at -4.9	0.0	Frozen
Day 20		430 ±38.2	NS	632 ±56.8	NS	-4.9 → -1.2	0.0→0.3	Frozen
Day 40		**402 ±61.9**	**S**	**626 ±77.8**	**NS**	**-1.2 → 2.3**	**0.3→8.0**	**Semi-frozen**
Day 60		**395 ±39.6**	**S**	**587 ±89.1**	**NS**	**2.3 →-4.0**	**8.0**	**Unfrozen**
Day 97(CO_2 burst)		**278 ±23.9**	**S**	**559 ±61.9**	**S**	**4.0→13.7**	**8.0 →5.0**	**Unfrozen**
% Removal	Treated	38	S	11	S			
	Untreated	24	S	< 1	NS			
Total % Removal	Treated	52	S	11-16	S			
	Untreated	24-33	S	<1	NS			

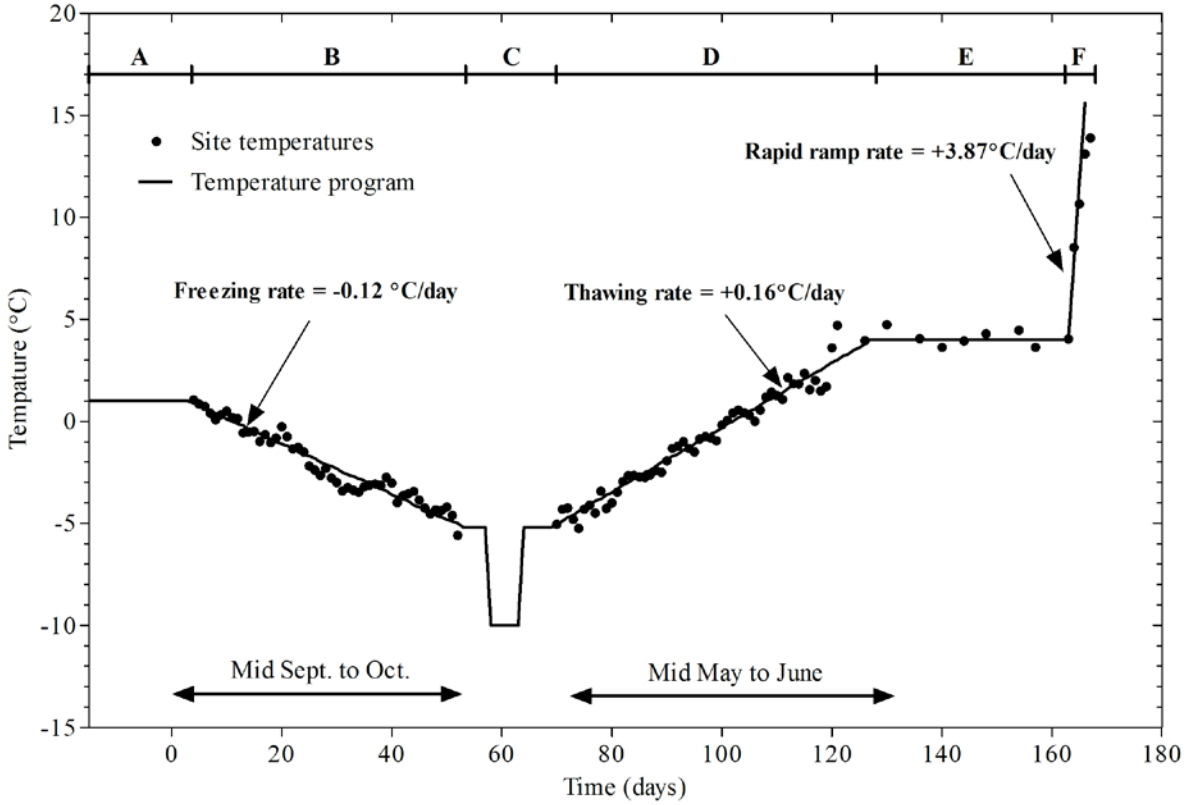

Figure 5.1 The temperature programs for the freeze-thaw experiment. The black circle dot (symbol) refers to the ambient air temperatures of the site. The mean temperatures of the site were calculated from the available data between 1962 and 2007. The daily temperature data were obtained from National Climate Data and Information Archive of Environment Canada (www. climate.weatheroffice.ec.gc.ca). The bold line is the constructed temperature program. A: soil treatment period at constant 1°C, B: freezing phase from +1 °C to -5 °C at -0.12 °C/day, approximating middle September to October of the site, C: transitional frozen period at -5 °C and -10 °C, D: thawing phase from -5 °C to 4 °C at 0.16 °C/day, approximating middle May to June, F: constant temperature-mode at +4 °C (constant average temperature of end of June and early July in 1962-2007), G: specific rapid rate increasing from 4 °C to 14.8 °C at +3.87 °C/day.

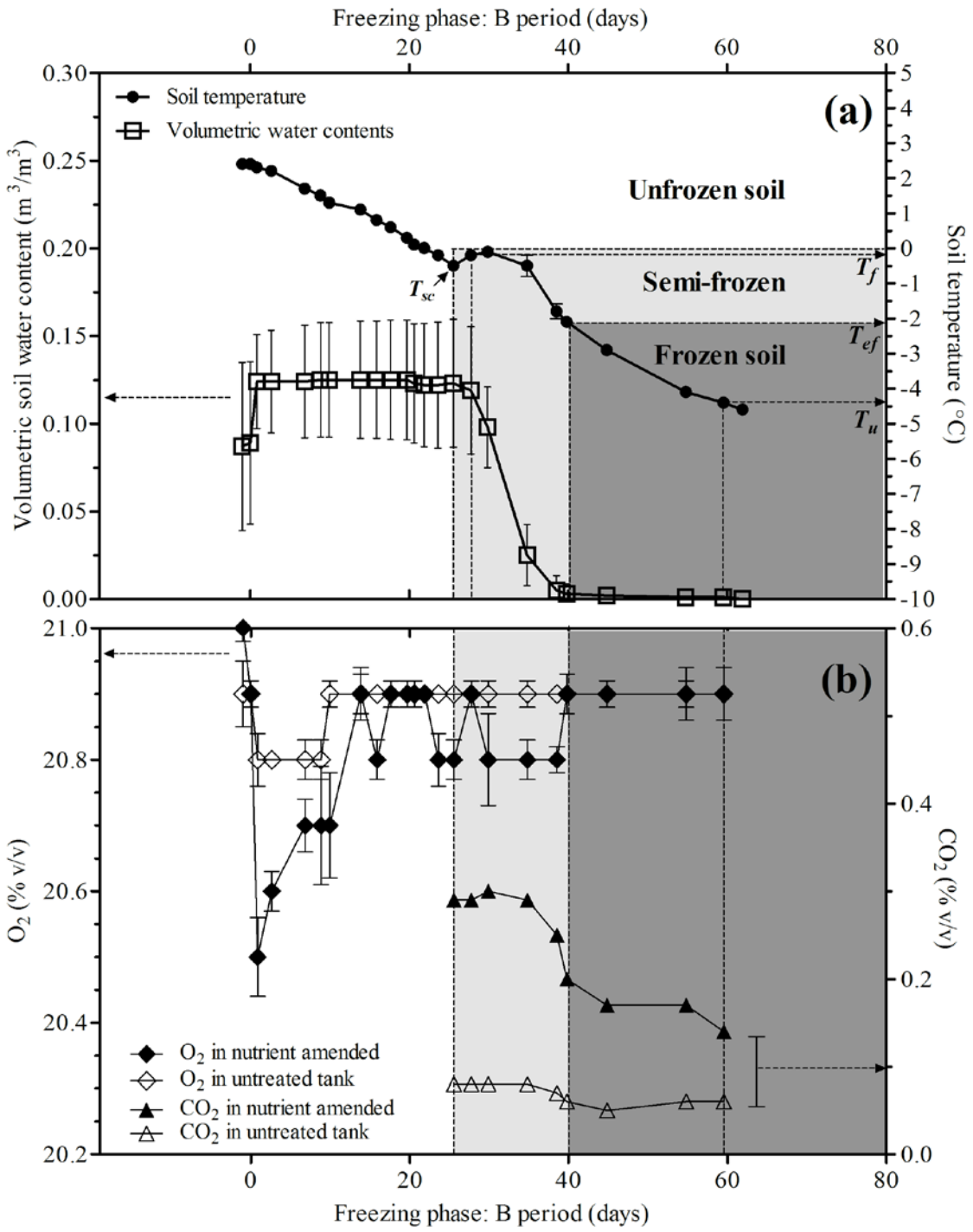

Figure 5.2. (a) Freezing curve-soil water content diagram, and (b) corresponding O_2-CO_2 soil gas concentration in the freezing phase (B period shown in Fig. 5.1). T_{sc}: supercooling temperature = -0.5°C on Day 26, T_f: freezing-point depression = -0.2°C on Day 28. $T_{ef.}$ temperature of the end of free water freezing = -2.1°C, and T_u: the lowest temperature for unfrozen water detection. The error bars refer to standard deviation of mean soil temperatures and volumetric soil water contents.

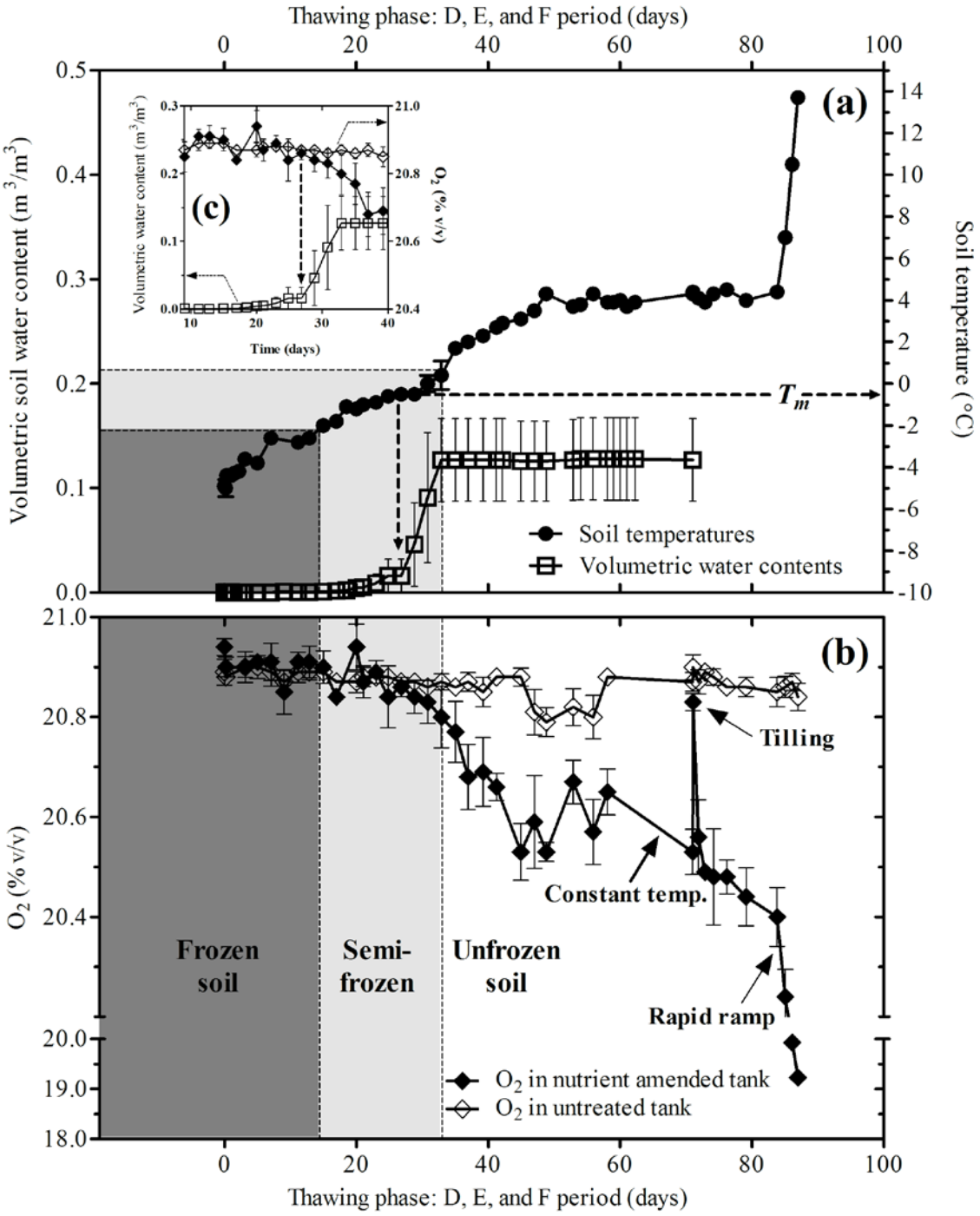

Figure 5.3. (a) Thawing curve-soil water contents, (b) corresponding O_2 soil gas concentrations in the thawing phase, and (c) onset of O_2 consumption correlated to water availability at subzero temperature.

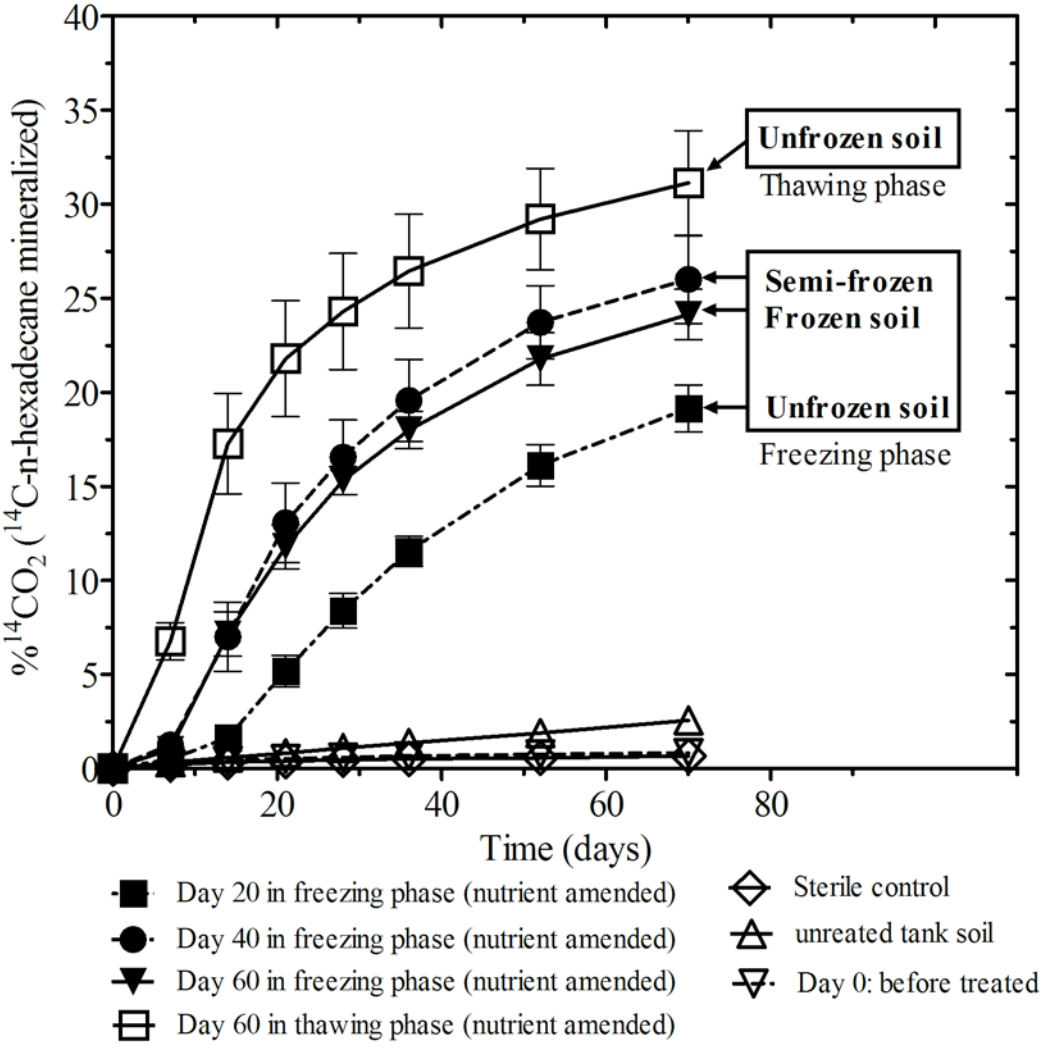

Figure 5.4. Results of ^{14}C-hexadecane mineralization assays of soil samples collected from the seasonal freezing- and thawing- phase.

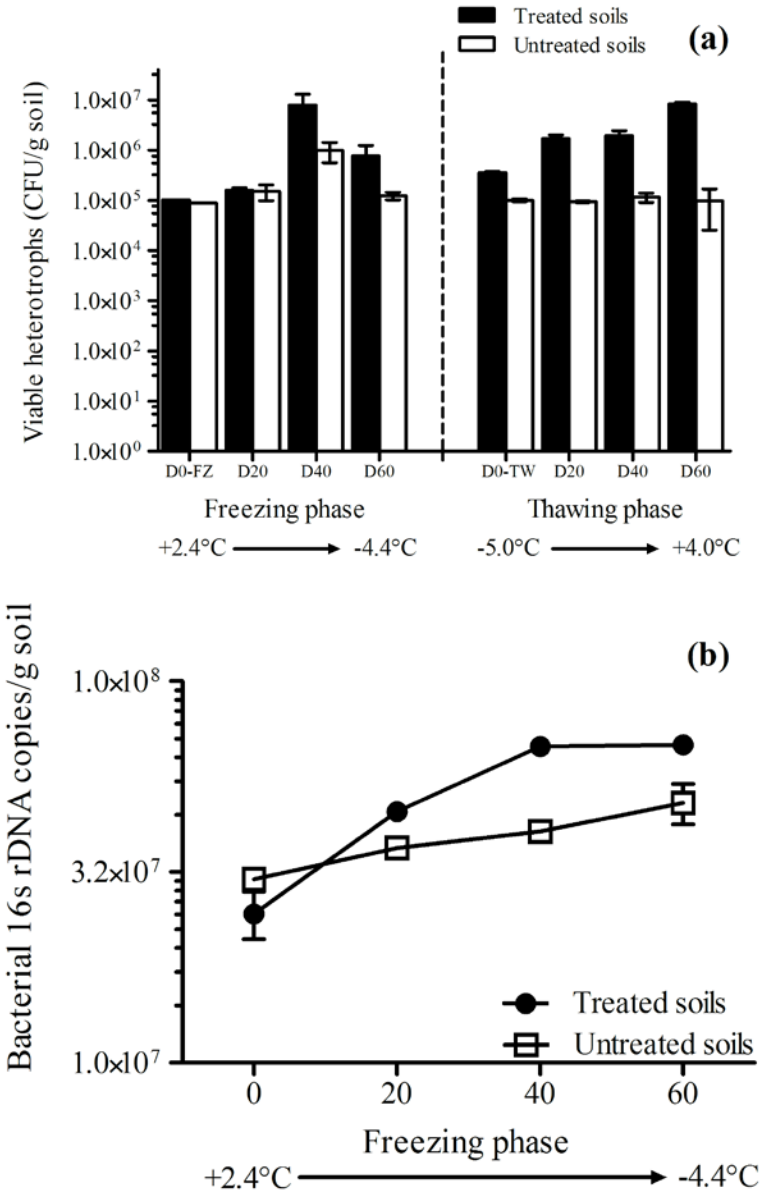

Figure 5.5. (a) Viable R2A plate counts for heterotrophs in soils samples collected from the freezing phase and (b) changes in bacterial 16s rDNA copies/g during freezing.

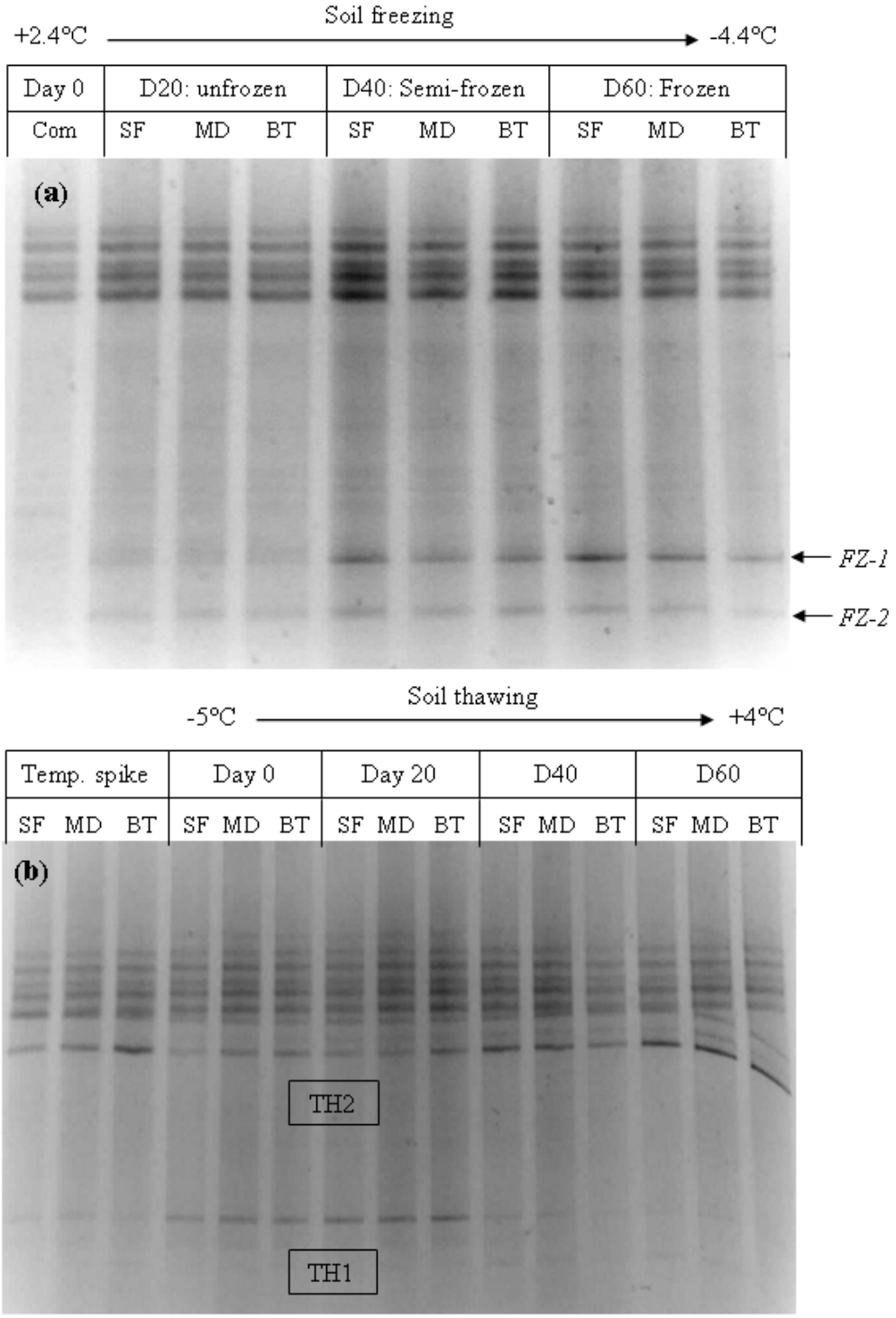

Figure 5.6. Shift in bacterial community structures assessed by the PCR-DGGE analyses in the freezing (a) and thawing phase (b). FZ1: *Corynebacterineae* (96% similarity) FZ2: *Rhodanobacter* (92% similarity) TH1 (=FZ1): *Corynebacterineae* (94% similarity) TH2: *Alkanindiges* (89% similarity).

Supplementary data

Table 5S.1. Seasonal freezing: changes in F2 and F3 conc., soil temperatures, water availability in the untreated landfarm.

		F2		F3		Soil temp.	Soil water	Soil phase
		mg/kg	Sig.	mg/kg	Sig.	°C	% GWC	
Day 0-Freeze		624 ±51.7	-	697 ±47.2	-	2.4	7.8	Unfrozen
Day 20		652 ±79.9	NS	710 ±74.0	NS	0.0	7.8	Unfrozen
Day 40		571 ±22.6	NS	710 ±58.3	NS	-2.1	0.2	Semi-frozen
Day 60		566 ±35.4	NS	691 ±53.4	NS	-4.4	0.0	Frozen
% Removal	Treated	13-17	S	< 5	NS			
	Untreated	0-9	NS	< 1	NS			

Table 5S.2. Seasonal thawing: changes in F2 and F3 conc., soil temperatures, water availability in the untreated landfarm.

		F2		F3		Soil temp.	Soil water	Soil phase
		mg/kg	Sig	mg/kg	Sig.	°C	% GWC	
Day 0-Thaw		519 ±59.0	-	658 ±104.8	-	-4.9	0.0	Frozen
Day 20		528 ±72.7	NS	633 ±76.50	NS	-1.2	0.3	Frozen
Day 40		555 ±54.0	NS	666 ±105.6	NS	2.3	8.0	Semi + unfrozen
Day 60		519 ±36.0	NS	616 ±51.40	NS	4.0	8.0	Unfrozen
Day 97(CO_2 burst)		392 ±51.8	S	652 ±54.50	NS	13.7	5.0	Unfrozen
% Removal	Treated	38	S	11	S			
	Untreated	24	S	< 1	NS			
% Total Removal	Treated	52	S	11-16	S			
	Untreated	24-33	S	<1	NS			

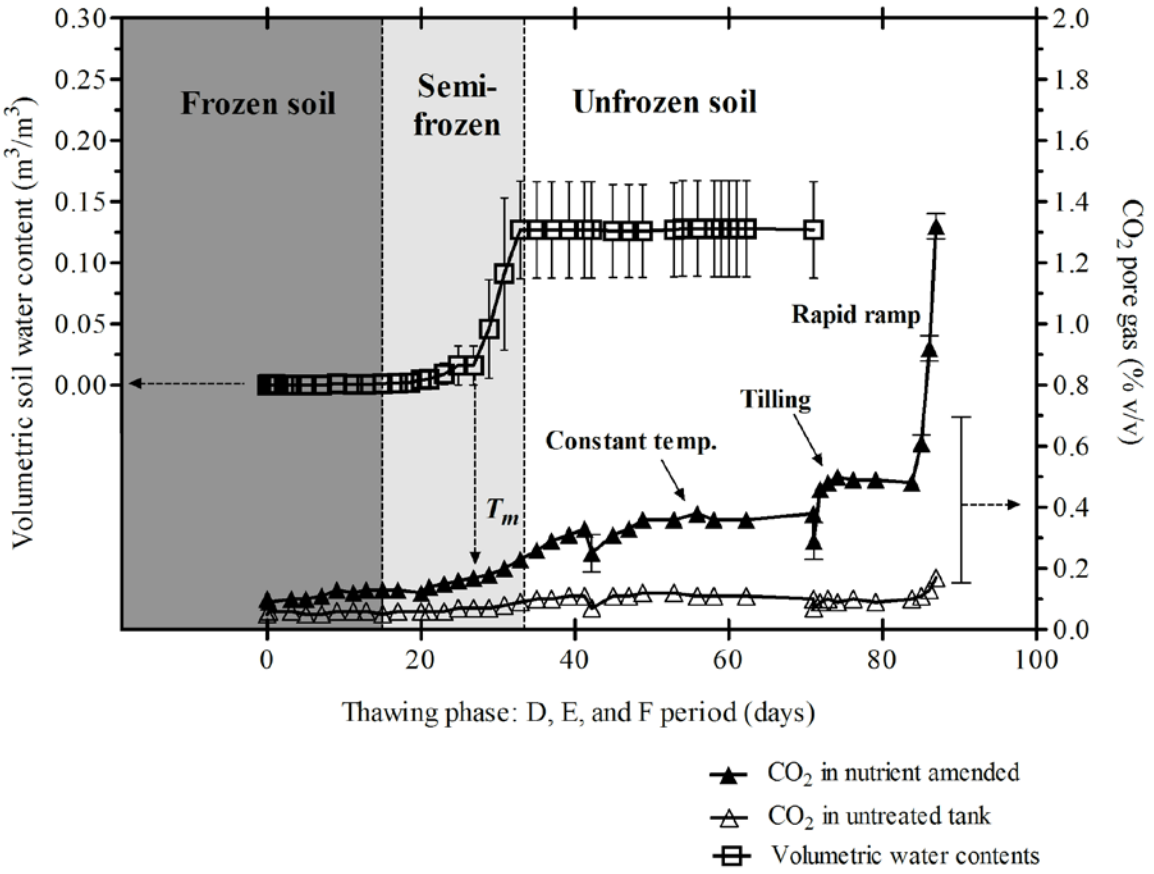

Figure 5S.1. Changes in CO_2 pore gas concentrations correlated to changes in soil water contents in thawing phase (D, E, and F period shown in Fig. 5.1). The highlighted portion with gray refers to subzero temperature-regime.

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Chapter 6

Intellectual contributions

Indigenous cold-adapted hydrocarbon-degrading microbial populations in site contaminated soils exposed to natural, variable cold climate temperatures ranging from -2 °C to +10 °C over a long period of time reflecting seasonal durations, are able to survive and degrade hydrocarbons to various extents depending on the temperature and unfrozen water content in the soil. Laboratory incubation at constant average temperatures, which are often considered representative of the variable temperature conditions, may conceal true patterns of activity of the indigenous microbial community. The microbial community present in the soils employed in this study, appeared to be sensitive to optimal temperature conditions. For example, experiments reported in this thesis showed that the indigenous microbial population were more efficient in degrading petroleum hydrocarbons over a temperature cycle ranging from 1 to 10 °C, in contrast to a fixed temperature of 6 °C. It is thus necessary that assumption and selection of laboratory temperature conditions be carefully considered to provide a better assessment of rate and extents of petroleum hydrocarbon biodegradation. Periodic temperature changes may be a rate-controlling factor in *in situ* petroleum hydrocarbon biodegradation particularly for surface and excavated soils where temperatures fluctuate in a pattern similar to the air temperature. Constant temperatures may be more representative in deeper subsurface conditions where air temperatures do not directly influence temperatures over the short term.

Petroleum hydrocarbon distribution can significantly vary with soil aggregates sizes at cold regions sites and this may result in different endpoints of petroleum hydrocarbon biodegradation. Investigation of initial petroleum hydrocarbon contamination levels in coarse, medium and fine particle or aggregate sizes provides a strategy of bioremediation to gauge the effective endpoint of biodegradation activity associated with bioavailability. This is particularly important in the decision-making process for identifying technologically feasible and cost-effective end points for bioremediation.

The experiments reported in this study showed that indigenous microbial communities strategically adapt to subzero freezing and thawing temperatures. Freezing-tolerant hydrocarbon-

degrading bacteria can grow in response to soil freezing and freezing-tolerant bacterial communities may cope well with low water availability and high osmotic stress induced during slow soil freezing. The pilot-scale investigation revealed that petroleum hydrocarbon biodegradation can be extended to below 0 °C and rapid temperature increases during seasonal transitional periods. The prerequisite for the extents of hydrocarbon biodegradation under seasonal freeze-thaw condition are that nutrients (N and P) should be not depleted in the end of summer treatment seasons and thus extra nutrient amendment may be required at the favourable N and P levels for the TPH concentrations present in the soil in the end of summer seasons.

The key findings and contributions of this study and possible future directions for this research are summarized below.

1. **This study presents the first demonstration of concurrent biodegradation patterns of both the non-volatile, higher molecular weight hydrocarbon fraction (>C16-C34) and lower molecular weight hydrocarbons fraction (>C10-C16) during pilot-scale biodegradation experiments conducted at site low temperatures.**

The rates and extent of biodegradation of the non-volatile, higher molecular weight hydrocarbon fraction was significant and comparable to the rates and extent of biodegradation observed for the lower molecular weight fraction. Biodegradation of the higher molecular weight hydrocarbon fraction occurred even in the absence of biodegradation activity of the lower molecular weight fraction. The biodegradation pattern was explained by changes in alkane ratios as a function of TPH concentrations. Comparison of the alkane ratios of C14, C16, and C18 alkanes showed that the relative abundance of C16 and C18 alkanes decreased more rapidly than that of C14 alkanes as TPH levels decreased when TPH levels were below 1000 mg/kg. The recorded observations of changes in the composition of petroleum hydrocarbon during biodegradation differ from the more commonly observed sequential degradation of lower molecular weight hydrocarbons followed by more persistent, higher molecular weight hydrocarbons.

2. **This study is the first to demonstrate that soil aggregate sizes controls the endpoint of biotreatment of petroleum hydrocarbon-contaminated soils from a northern site.**

The majority of residual TPH mass remained in soil particle and aggregate sizes ranging from 0.6 mm to 2 mm, rather than in larger or finer size fractions. Furthermore, biodegradation was not limited by nutrients, pH or the population size of hydrocarbon degraders. The soil organic matter content in the coarse and medium size fractions were observed to be similar, and thus are not likely responsible for the higher residual hydrocarbons in the medium sized fraction. Both the coarse and medium size fractions were predominantly comprised of quartz and carbonate minerals, and the clay (kaolinite) fraction was only slightly higher in the medium fraction, totalling 3% as compared to 2% in the coarse fraction. This finding suggests that the soil micropore structure may have a significant influence on biodegradation rates, as some of the oil phase may not be accessible for direct contact with microorganisms.

3. **This study demonstrates for the first time that variable cold site temperatures influence the rates and extent of biodegradation of petroleum hydrocarbons compared to those rates and extent obtained under conventional constant laboratory incubation. Despite the low temperature regimes (1-10 °C), petroleum hydrocarbon biodegradation in contaminated sub-Arctic soils was significantly enhanced due to an observed higher rate of microbial activity. Furthermore, fast microbial growth of indigenous psychrotrophic hydrocarbon-degrading populations was documented, helping these populations effectively adapt to the variable cold temperatures similar to natural cold temperature fluctuation encountered at arctic sites.**

Biodegradation of petroleum hydrocarbons was notably enhanced during the well-controlled site temperatures varying between 1 °C and 10 °C, a representative summer temperature profile of the Resolution Island site. In particular, non-volatile higher molecular weight hydrocarbons (F3: >C16) and UCM fractions were substantially biodegraded. Compared with the results of conventional constant temperature incubation at 5.5 °C (soil temperature: 6 °C), the biodegradation rate of the F3 hydrocarbon fraction observed in this study was three times higher at the variable temperatures; moreover, the total removal efficiencies of the different petroleum hydrocarbon fractions (F2, F3, UCM and TPH) were over two times greater. These

findings give important insight into the accurate assessment of on-site petroleum hydrocarbon potential and the development of efficient strategies for the implementation of bioremediation for short treatment seasons at cold sites. The shift in indigenous microbial community structure due to nutrient amendments was a short-term response observed in both temperature modes. However, the intensity of biodegradation activity of the established microbial communities was highly impacted by temperature variations. Indigenous bioactivity was efficiently enhanced at the warmer temperature regimes during the periodic temperature changes.

The use of conventional constant low temperature incubation conditions may conceal the true degradation potential of indigenous cold-adapted microbial communities in field-contaminated arctic soils that have been exposed to long-term natural cold temperature fluctuations.

4. **This study represents the first pilot-scale biodegradation experiment that quantitatively demonstrates a feasibility of petroleum hydrocarbon biodegradation in field-contaminated soils during the seasonal freeze-thaw temperature regimes.**

This pilot-scale experiment showed that 52% of the initial F2 in the range of >C10-C16, and >11 to 16% of higher molecular weight hydrocarbon fractions (>C16) were removed during the course of seasonal freezing and subsequent thawing temperature regimes representative of the RI site. Changes in pore water content at subzero temperatures where pore ice and unfrozen water coexist in contaminated soils, regulated the extent of microbial activity. Moreover, microbial growth was not inhibited during in the period in the nutrient amended contaminated soils. This implies a significant potential for petroleum hydrocarbon biodegradation during the long lasting (several months) subzero temperature regimes where some free pore water exists with ice. This represents the prolonged semi-frozen period. A rapid rate of temperature increase, which is typically seen before the summer season, triggered a burst of microbial activity and significantly enhanced biodegradation of petroleum hydrocarbons. Microbial community compositions were altered in response to soil freezing as well as thawing, and appeared to be linked to TPH biodegradation.