# Bioremediation of Petroleum Hydrocarbons: Multi-scale Investigation of Effects of Pore Size and Role of Diurnal Temperature Changes

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*"Success is not the position where you are standing, but which direction you are going."*

*Oliver Wendell Holmes*

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## ABSTRACT

Indigenous, cold-adapted, hydrocarbon-degrading bacteria have been frequently detected in soils from cold region sites suggesting bioremediation, as a cost-effective remediation technology is potentially viable for cleanup of contaminated sites in cold regions. Previous studies on bioremediation of petroleum hydrocarbons in cold regions have been conducted with coarse textured soils. However, fine-grained or clayey soils are commonly found in several cold regions, and there are limited studies of petroleum hydrocarbon bioremediation performance in such soils. The overall objective of this research was to investigate the factors controlling biodegradation of aged, clayey petroleum hydrocarbon contaminated soils from a sub-arctic site, under relevant temperature regimes.

The biodegradation rates and extents of petroleum hydrocarbons was investigated for a clayey site contaminated soil in four pilot scale biopiles maintained at 15˚C, the average temperature of the site in summer. Non-volatile petroleum hydrocarbons (C16-C32) were biodegraded 24 to 38% under continuous aeration and with amendments of 17.5% or 23.5% moisture and also 17.5% moisture with 95 mg-N/ kg soil (TPH:N = 100:5). In contrast, statistically significant biodegradation of the non-volatile hydrocarbon fraction was not observed in a biopile tank containing soils amended with 23.5% moisture content and 1340 mg-N/ kg soil (TOC:N= 100:5). The first order biodegradation rate constants for the non-volatile petroleum hydrocarbon fraction was $0.11 \pm 0.04$ $day^{-1}$ in soil slurry systems amended with 95 mg-N/ kg soil, and was significantly higher than $0.009 \pm 0.003$ $day^{-1}$ obtained from the biopile systems with the same nitrogen amendment. However, the biodegradation extent of about 46% in soil slurry systems was unchanged between 30 and 60 days, and was comparable to an extent of 50% observed in the biopile systems over 385 days. While a significant shift occurred in soil

microbial community over 60 days in the biopile tank amended with 23.5% moisture only, there was no change in the microbial community of soil samples amended with a similar level of moisture and 1340 mg-N/ kg soil, indicating that high amounts of nitrogen amendment inhibited bioremediation.

A few recent studies have investigated the effect of seasonal temperature variations and variations over a few days typically observed at cold regions sites, on the rates of biodegradation of petroleum hydrocarbons. This study investigates the effect of diurnal temperature variations on the biodegradation of petroleum hydrocarbon contaminated soil typical for the site from northern Canada from which contaminated soils were obtained. The extents of biodegradation of semi-volatile (C10-C16) and non-volatile petroleum hydrocarbons (C16- C32), after 70 days, in systems incubated at temperatures varying between 5 ˚C to 15 ˚C daily (representative of daily high and low temperatures) were similar to systems incubated at constant temperature of 15 ˚C, and were significantly higher than systems incubated at 5 ˚C. The soil microbial community of systems with daily changes in temperatures between 5 ˚C to 15 ˚C was similar to those incubated at 15 ˚C, but significantly different than those incubated at 5 ˚C, based on both 16S rRNA and *alkB* gene analyses. The results suggest that the highest temperature (15 ˚C) to which the soil was frequently exposed to, determined the microbial community profile in the site soil, and periods of low temperatures did not reduce the overall biodegradation efficiency or the microbial community compared to that maintained solely at 15 ˚C.

Soil aggregates have numerous pores smaller than a few microns which may limit the rates and extents of biodegradation. Insoluble fractions of petroleum hydrocarbons in the oil phase which may be utilized by bacteria by direct uptake may not be accessible to bacteria when present in pores smaller than bacteria. Experiments were conducted to investigate if hexadecane,

a biodegradable but effectively insoluble hydrocarbon phase, was accessible to, and degraded by hydrocarbon degrading bacteria when the hexadecane-water interface was separated from the microbial culture by membranes with specific pore sizes. In these systems, bacteria were unable to degrade hexadecane when the membrane pore size was 0.4 μm or 3 μm, but was able to degrade hexadecane when separated by pore sizes of 12 μm. To investigate the implications of these results to aggregates from clayey and sandy soils, the *in-situ* aggregate micro-structure of representative soil aggregates from petroleum contaminated clayey soil and petroleum contaminated sandy soil were compared by micro-scale computed tomography (micro CT) imaging. The "bioaccessible porosity" defined as fraction of aggregate volume corresponding to pores larger than 4 μm was comparable in case of clayey (26-27%) and sandy (24%) aggregates.

## RÉSUMÉ

Des bactéries indigènes, adaptées au froid, dégradant les hydrocarbures ont été fréquemment détectées dans les sites de région froide. Par conséquent, la bioremédiation, une technologie avec un bon rapport coût-efficacité, est potentiellement viable pour la réhabilitation des sites contaminés en régions froides. Des études antérieures sur la bioremédiation des hydrocarbures pétroliers en région froide ont été complétées avec des sols à grain grossier. Cependant, les sols retrouvés en région froide sont souvent des sols à grain fin ou argileux et les études sur la performance de la bioremédiation des hydrocarbures pétroliers dans ces conditions sont limitées. L'objectif principal de cette recherche était d'étudier les facteurs contrôlant la biodégradation de la contamination par hydrocarbures pétroliers vieillis de sols argileux provenant d'un site subarctique sous des régimes de températures pertinents.

Les taux et l'étendue de la biodégradation des hydrocarbures pétroliers ont été examinés dans quatre biopiles à l'échelle pilote contenant des sols argileux et contaminés, maintenues à une température de 15 ˚C, la température moyenne du site durant l'été. Les hydrocarbures non-volatiles (C16-C32) ont été biodégradé jusqu'à 24 à 38% sous aération continue et avec un amendement en humidité de 17,5% ou 23,5 % et 17,5% d'humidité avec 95 mg-N/ kg de sol (HPT:N = 100:5). À l'inverse, la biodégradation de la fraction d'hydrocarbure non-volatile n'a pas été statistiquement significative dans la biopile contenant des sols amendés avec une humidité de 23,5% et 1340 mg-N/ kg de sol (COT:N= 100:5). Les coefficients de vitesse de la biodégradation au premier ordre de la fraction d'hydrocarbure de pétrole non-volatile étaient de $0,11 \pm 0,04$ jours$^{-1}$ ($r^2 = 0,9$) dans les systèmes de suspension de sol amendés avec 95 mg-N / kg de sol, et étaient significativement plus élevée à $0,009 \pm 0,003$ jours$^{-1}$ ($r^2 = 0,69$) obtenues à partir des systèmes de biopiles avec la même modification en azote. Toutefois, l'étendue de la

biodégradation de 46% dans les systèmes de boue incluant sol/eau a demeurée inchangée entre 30 et 60 jours, et était comparable à une étendue de 50% qui a été observée dans les systèmes de biopiles pour une période de 385 jours. Bien qu'une modification importante est survenue dans la population de microorganismes présentes dans le biopile amendé avec une humidité de 23,5% seulement sur une période de 70 jours, il n'y avait aucun changement observé dans les échantillons de sols amendé avec une humidité similaire et 1340 mg-N/kg de sol indiquant que des amendements en taux élevés d'azote nuisent à la bioremédiation.

Des études récentes ont investiguées l'effet saisonnier de la variation de température et l'effet d'une variation de plusieurs jours typiquement observées sur les sites en région froide sur les taux de biodégradation d'hydrocarbures pétroliers. Cette étude examine les effets diurnaux des variations de température sur la biodégradation des sols contaminés en hydrocarbures pétroliers typique pour un site du nord de Canada d'où proviennent ces sols contaminés. Après une période de 70 jours, l'étendue de biodégradation des hydrocarbures pétroliers semi-volatiles (C10-C16) et non-volatiles (C16- C32) dans les systèmes incubés à des températures variant entre 5 ˚C et 15 ˚C quotidiennement (représentant les hautes et basses températures quotidiennes) était comparable à ceux incubé à une température constante de 15 ˚C, mais significativement meilleure que ceux incubés à 5 ˚C. De même que les communautés microbiennes des sols avec des changements de température entre 5 ˚C et 15 ˚C quotidiens étaient similaires à ceux incubés à 15 ˚C, mais différaient significativement de ceux à 5 ˚C d'après les analyses d'ARN ribosomaux 16S et le gène *alkB*. Les résultats suggèrent que c'est la plus haute température (15 ˚C) qui détermine le profil de la communauté de microorganismes dans les sols et que les périodes de basses températures n'ont pas réduit l'efficacité de la biodégradation en gros ou de la

communauté microbienne lorsqu'ils sont comparés aux systèmes maintenus à une température constante de 15 ˚C.

Les agrégats du sol ont de nombreux pores qui sont inférieurs à quelques microns qui pourraient limiter les taux et étendues de la biodégradation. Les fractions insolubles des hydrocarbures pétroliers présentent dans la phase huileuse peuvent être utilisée par les bactéries par absorption directe; cependant ceci n'est peut être pas le cas lorsque les pores sont plus petits que les bactéries. Des expériences ont été effectuées pour étudier si l'hexadécane, une substance biodégradable mais effectivement insoluble, était accèssible à et dégradé par des bactéries capables de dégrader les hydrocarbures dans des situations où l'interface hexadécane-eau était séparé de la culture microbienne par des membranes de pores à grandeur spécifique. Dans ces systèmes, les bactéries n'étaient pas capable de dégrader hexadécane en présence de pores de 0.4 μm et 3 μm, mais étaient capables de le dégrader lorsque le pore avait un grandeur de 12 μm. Afin d'investiguer les implications de ces résultats sur les agrégats de sol argileux et sableux, la microstructure de l'agrégat *in-situ* d'un agrégat de sol représentatif d'un sol argileux et d'un sol sableux contaminé en hydrocarbures a été comparée par imagerie de tomographie à micro-échelle (micro CT) par ordinateur.

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## PREFACE

In accordance with the "*Guidelines for Thesis Preparation*", this thesis is presented in a manuscript-based format. A general introduction precedes the results section which includes chapters 2-4. In the final chapter, summary and conclusions are presented. The results presented in these chapters are submitted to the journals. In all the publications, the author of the manuscript is the primary author of this thesis. Below is a detailed description of the efforts of each contributing author:

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# Chapter 1

# Introduction

## 1.1. INTRODUCTION

Petroleum hydrocarbons are one of the most common pollutants in the environment. Spills during oil exploration, production, transportation or storage commonly occur. There are numerous hydrocarbon contaminated sites in cold regions. In Canada, alone, more than 10,000 petroleum hydrocarbon contaminated sites have been identified, which many of them are located in arctic and sub-arctic regions [1]. The number of contaminated sites in cold regions may increase, given that it has been estimated more than 20% of world's oil resources are contained in Arctic fields [2]. Moreover, petroleum products such as diesel fuels are the main energy source for heating in remote northern areas. Crude oil contamination originating from oil exploration activities, oil pipeline ruptures and diesel fuel spills are the most commonly reported hydrocarbon pollutants on land in cold regions [3]. Bioremediation is recognized as a cost-effective strategy to remediate hydrocarbon contaminated sites. The possibility of applying this technology in cold climates has generated wide interest in recent years [4-6].

Biopile and landfarming technologies are the popular bioremediation options for hydrocarbon contaminated soils in cold climate regions [7-9], mainly due to the lower cost and relatively shorter remediation time which are of significant concerns in these regions [3]. There are studies on bioremediation of petroleum hydrocarbons contaminated soils form the sites in cold regions, however, most of these studies have been conducted with coarse textured sandy soils [5, 7, 9-14], and to the best of our knowledge there is no reported study on bioremediation of clayey hydrocarbon contaminated soil from these regions. Soil characteristics can significantly affect the bioavailability of pollutants, nutrients as well as oxygen to microorganisms. Aggregate micro-structure and in particular pore size plays an important role on restricting the bioavailability of trapped None Aqueous Phase Liquids (NAPL) in micro-pores.

When soil bacteria are excluded from intra-aggregate pores due to their sizes, the bioavailability of poorly soluble NAPL components will be severely limited. Although there are studies suggesting that organic carbon sources are protected in soil micro-pores [15, 16], there is no reported systematic study in the literature addressing the effect of pore size on bioavailability of NAPL, which has significant implications for bioremediation of different soils with different pore size distributions.

In addition to soil texture, temperature regime can also affect the biological activity and mass transfer processes which in turn govern the substrate and nutrient bioavailability. Significant number of studies have addressed the effect of temperature on soil biological activity in terms of minimum temperature feasible for bacterial metabolic activity [17-20], or the optimum temperature for maximum activity [12, 21]. Although a few studies have addressed the effect of long term seasonal temperature fluctuations [22], or short term freeze-thaw cycles [23, 24] on the rate and extent of biodegradation of petroleum hydrocarbons, the effect of diurnal temperature variations has not been addressed before. Soil temperature fluctuations of similar magnitude of daily air temperature fluctuations likely happens at soil surface, or during ex-situ remediation by biopiles, which involves aeration of the soil pile, or by landfarming, which involves treatment of excavated soils in layers spread out at the ground surface.

## 1.2. BIOSTIMULATION IN CONTAMINATED SOILS

A significant number of biostimulation studies have reported the enhancing effect of adding nutrients mainly in forms of inorganic nitrogen and phosphorus salts [4, 5, 9, 13, 22, 25, 26]. Higher microbial growth rate is believed to be the main advantageous of nitrogen amendments. Other than increased growth rate, some studies have reported a decrease in the lag phase of

microbial community upon adding nitrogen sources. However, there are also studies in the literature reporting the lack of enhancement [27-29] or inhibiting effects of nutrient amendments [25, 30, 31]. In a comparative study of different bioremediation approaches for a diesel contaminated soil, Bento et al. found that natural attenuation was more effective than biostimulation or bioaugmentation. Those authors suggested that the lack of effect of nutrient amendment in case of biostimulation treatments is due to limited bioavailability of added nutrients [32]. Braddock et al. studied the effect of nutrient addition in a sandy soil contaminated with JP-5 from an Arctic site and found that while nutrient amendment at 300 mg-N/kg soil negatively impacted the biodegradation of hexadecane and phytane during 6 weeks of experiment, significant biodegradation occurred in the mesocosms amended with lower nutrient at 100 mg N/kg soil, or the systems that were not amended with nutrients. In their study, the viable population of heterotrophic and hydrocarbon degrading bacteria were similar for nutrient amended system compared to un-amended system. They suggested that microbial activity was negatively impacted by increased soil water osmotic potential as a result of increased soluble salt concentration [10].

In another study of bioremediation of clayey creosote contaminated soil, Sabate et al. reported that aeration and adjusting the moisture level at 40% of soil WHC had enhancing effect on bioremediation of a creosote contaminated soil, whereas amendment with potassium nitrate and potassium phosphate (TOC:N:P of 300:10,1) showed inhibiting effect on rate and extent of biodegradation [33]. In a companion study, Vinas et al. found that the degradation pattern of 4-ring PAH compounds such as chrysene and benzo(α)anthracene was negatively altered by application of nutrients. On the other hand the degradation pattern of 3-ring PAH compounds was not significantly affected. Studying the microbial community by denaturing gradient gel

electrophoresis analysis (DGGE) showed that different soil microbial community composition in moisture amended and nutrient amended systems. While, initially *α-Proteobacteria* phylum was dominant in community, at later stages of biodegradation the γ-*Proteobacteria*, *α-Proteobacteria* and *Cytophaga-Flexibacter-Bacteroides* phyla were dominant in aerated and moisture adjusted systems, and *γ -Proteobacteria*, *β-Proteobacteria* and *α-Proteobacteria* groups were dominant phyla in nutrient amended systems [31]. Ruberto et al. also reported that application of 1800 mg-N/kg soil as sodium nitrate had inhibiting effect on degradation of petroleum hydrocarbons and at the same time resulting in a shift of dominant microbial populations favouring *Acinetobacter* versus *Psudomonas* genera [30].

Soil moisture content is essential for microbial activity in soil. Increasing soil moisture content enhances the nutrient availability by increasing the connectivity of water-filled pores structures on one hand, and increasing the mobility of microorganisms on the other hand, which could lead to higher $CO_2$ production rate [34]. At the same time, increasing the moisture level moves the gas-water interface toward larger pores in the soil, decreases air-liquid interfacial area and consequently results in anaerobic conditions in smaller water filled pores. The limited oxygen availability in small, water-filled pores limits the biodegradation of substrates trapped in these pores [16]. It is known that by increasing contact time contaminants diffuses into micro pores of the soil [35]. On the other hand, diffusion in water is assumed to be $10^4$ times slower than air phase [36]. Harper et al. suggested that increasing the moisture level beyond a critical level has a limiting effect on mass transfer rate [37]. Inhibiting effect of high moisture level on biodegradation was reported by Børresen et al. who showed that by increasing the moisture content from 10% to 20% the mineralization rate of hexadecane decreased from about 50 to about 12 mg/kg/d [25].

Due to high pore volume, large surface area and large portion of micro-pores associated with clay fraction of the soil, bioremediation in clayey soils faces the problem of limited bioavailability. Eyvazi et al. evaluated the biodegradation of spiked gasoline in seven different soils and found that biodegradation rate was inversely correlated to the clay content of the soil [38]. Geerdink et al. reported that desorption of organic compounds from clay particles was 1000 times slower than desorption from organic matter [39]. Aichberger et al. found that the affinity for sorption on the fine fractions of soil was increased with increasing hydrophobicity of the target pollutants [40]. This suggests that the presence of clay particles would have a more significant effect on biodegradation of higher molecular weight- more hydrophobic fraction of petroleum hydrocarbons. This is in line with findings of Nocentini et al. who studied the biodegradation of kerosene, diesel fuel and a lubricating mineral oil in sandy and clayey soils, and showed that while biodegradation rate and extent of kerosene in different soils was comparable, in case of mineral oil, biodegradation rate in sandy soil was significantly higher than clayey soil [41].

## 1.3. TEMPERATURE EFFECTS ON HYDROCARBON BIODEGRADATION

It is generally known that decreasing the temperature depresses biodegradation rates. Based on the Arrhenius equation, for the common range of activation energies in biological reactions, a 10˚C decrease in temperature results in two to three times lower reaction rates [42]. Limited biological activity at lower temperatures such as sub-zero temperatures can be attributable to several factors, such as decrease in cell membrane fluidity and lower accessibility to unfrozen water. On the other hand, bacteria have shown a complex range of adaptation mechanisms which enable them to be active at sub-zero temperatures. For example metabolic activity of permafrost bacteria has been reported at temperatures as low as -20 ˚C [19].

Based on the optimum growth temperature, researchers tend to classify the cold-adapted bacteria into two groups: psychrophiles and psychrotolerants. psychrophiles have the fastest growth rate at 15 ˚C and cannot grow at temperatures higher than 20 ˚C. While psychrotolerants have the fastest growth at temperatures higher than 20 ˚C but can grow over a wide range of temperatures. The presence of significant number of cold-adapted hydrocarbon degrading microorganisms in soils of Arctic and sub-Arctic, Antarctic and Alpine regions has been reported before [24, 43, 44]. This suggests bioremediation as a potential remediation technique for hydrocarbon-contaminated sites in cold regions.

The ability of the psychrotrophic *Rhodococcus Q15* to degrade different normal alkane compounds with variable chain lengths at 0˚C and 5˚C was shown by Whyte et al. However, the rate of mineralization was significantly dependent on molecular weight of hydrocarbon [45]. Margesin et al. reported the prevalence of seven genotypes involved in the degradation of aliphatic or aromatic hydrocarbons in soil samples taken from Alpine regions. They also reported the enrichment of gram-negative bacteria in hydrocarbon contaminated soils compared to pristine soil samples [43]. Mohn et al. reported substantial mineralization (about 65%) of radiolabeled dodecane in soil samples collected from several Canadian Arctic sites at 7 ˚C. They also showed that increasing the temperature affected the biodegradation rate constant and also microbial activity lag phase. Three times higher biodegradation rate was observed when the temperature was increased from 7 ˚C to 15 ˚C. On the other hand additional increase from 22˚C to 30˚C showed a marginal effect on biodegradation rate [5].

In another study by Coulon et al., the effect of temperature and nutrient amendment on biodegradation of hydrocarbons was studied in four kg mesocosms containing freshly contaminated sub-Antarctic soil samples. They reported significantly higher biodegradation of

diesel and crude oil at 10 ˚C and 20 ˚C compared to 4 ˚C. The extent of total petroleum hydrocarbon (TPH) degradation at 10 ˚C was slightly higher than 20˚C [21].

Most of the cold climate bioremediation studies have been conducted at constant temperature with freshly contaminated soils. Chang et al. compared the rate and extent of biodegradation of petroleum hydrocarbons at variable site temperature (10 days cycles of temperature change from 1-10 ˚C) with those at constant temperature of 6 ˚C in an aged contaminated sandy soil from Resolution Island, NU, Canada. They reported significantly higher biodegradation extent in the nutrient amended systems with variable temperature (1-10 ˚C) compared to the nutrient amended systems at constant temperature (6˚C) over 60 days period of pilot scale landfarming experiments.

The first order biodegradation rate constants of the systems with variable temperature were found to be in better agreement with the rate constant obtained from field studies at the same site [46]. In another study, they evaluated the effect of seasonal freeze-thaw temperature regime with temperature slowly decreasing from 2 ˚C to -5 ˚C in 60 days followed by another 60 day period of thawing from -5˚C to 4˚C, on petroleum hydrocarbon biodegradation. They reported 32% reduction in F2 fraction, during 120 days duration of experiment [22]. A few studies have evaluated the effect of rapid freeze-thaw cycles on contaminant bioavailability [47] or biodegradation [23, 24]. Eriksson et al. studied the effect of freeze-thaw cycles (alternate 24 hr incubation at (-5 ˚C and 7 ˚C) on biodegradation of an arctic diesel from Ellesmere Island, Nunavut, Canada. They found that while concentration of normal alkanes decreased by about 50% over 50 days in the systems incubated at 7 ˚C and -5 ˚C to 7 ˚C, no biodegradation was detected at -5 ˚C. In terms of respiration activity, $CO_2$ production rates were about 330, 260 and 50 mg/ kg soil at those temperatures [24].

Borresen et al. also studied the effect of different freeze-thaw cycles (-5 ˚C to 5 ˚C) on biodegradation of hexadecane, and found that after about 80 days, the extent of biodegradation was comparable in nutrient-amended systems with 16 days freeze-thaw cycles (-5 ˚C to 5 ˚C) to the nutrient amended systems which were constantly incubated at 5˚C. Interestingly, in un-amended systems the biodegradation extent was higher in freeze-thaw treated systems compared to un-amended systems at constant temperature of 5 ˚C. The authors suggested that this could be explained by enhanced availability of nutrients to microorganism as a results of freeze-thaw cycles [23]. Overall, there seems to be consensus that microorganisms have the ability to adapt to cold climates, including freezing temperatures. But there is very little knowledge on how temperature fluctuations at low temperature ranges on a diurnal basis, affects the bioremediation effectiveness.

## 1.4. BIOAVAILABILTY AND SOIL TEXTURE

The aging of the contaminants in soil matrices is a serious challenge to effectiveness of any remedial action on contaminated soils. The very slow release of hydrocarbons partitioned into the soil organic matter or into soil micro-pores due to long-term contact of the hydrocarbons with soil, referred to as ‘aging’, reduces substrate bioavailability to microorganisms. Several mechanisms have been proposed to explain the aging process. It has been suggested that sorption to rubbery phase of natural organic matter of soil followed by diffusion into glassy phase or diffusion to soil micro-pores are responsible for limited bioavailability (reversible sorption) or sequestration (irreversible sorption) of contaminants in soil [48]. To be degraded, hydrocarbon molecules present in smaller pores where microorganisms are size excluded, need to be dissolved and transported by diffusion to the sites where microorganisms are present [48]. Scow et al. showed that the biodegradation rate of target pollutants were significantly decreased in soil

aggregates because of slow diffusion of contaminants to microorganisms [49]. The effects of aggregate characteristics such as aggregate size on biodegradation have been widely investigated in this field [50-54]. For example, Nocentini et al. showed that limited oxygen diffusion to the core of aggregates, restricted aerobic hydrocarbon biodegradation activity in the core of the soil aggregate [55].

While it is generally believed that due to capillary pressure NAPL resides in large pores of natural porous media which are mostly considered to be a water-wet domain, it has been well documented by scientists in the oil recovery field that presence of kaolinite in the oil reservoirs increases the percent of trapped oil and so decreases the oil recovery efficiency [56]. It has been suggested that in contrary to other mineral components of porous media such as illite, the wettability properties of kaolinite is altered by presence of oil [56]. Association of oil with kaolinite in inter-angular spaces of sandstones was shown by cryogenic scanning electron microscopy [57]. Changes in wettability of kaolinite occurs through a two-step process, which involves initial destabilization of water-mineral and oil-water interfaces, and then molecular contact of polar components of oil with mineral domain soil. Subsequent bonding of polar compounds to mineral surfaces, changes the wettability properties of kaolinite [58]. An oil wet kaolinite domain, facilitates the entry of NAPL to small pores of soil aggregates. With confocal laser scanning microscopy and cryogenic-scanning electron microscopy of a creosote contaminated clayey aggregate, it was well demonstrated that PAHs are mostly resided in intra-aggregate micro pores smaller than 5 μm. In contrary in case of sandy soils, NAPL were mostly covered the surface of aggregates [59].

Aggregate pore size, has a significant effect on bioremediation or bioavailability of contaminants [15, 35, 40, 52, 53, 59-61]. Pore size controls where water reside inside the

aggregate [34], and very importantly, it can control the direct access of bacteria to entrapped pollutants (substrates) in the pores. This limiting role of pores is more crucial in the case of poorly soluble components of NAPL such as long chain (>C16) aliphatic hydrocarbons or 4-5 ring polycyclic aromatic hydrocarbon compounds compared to hydrocarbons with lower molecular weights. It has been suggested that the bioavailability of organic compounds would be decreased in presence of pore sizes in the range of 7 nm-10 μm [35], as these pore sizes would restrict bacteria or limit their colonization. The attachment of bacteria to interface of oil-water is suggested to be the limiting factor in biodegradation of NAPL components. In order to direct uptake or pseudo-solubilisation of hydrocarbons by bio-surfactants, bacteria needs to be attached to oil-water interface [62]. Direct uptake and pseudo-solubilisation are alternate uptake mechanisms of less soluble or in the other word less bioavailable compounds by bacteria in the large pores [63-66]. The limited concentration gradient for diffusive transport of entrapped, less soluble compounds to the exterior of the aggregates is expected to limit their bioavailability and biodegradation.

Noordman et al. studied the effect of surfactant on the mass transfer and biodegradation rate of hexadecane in matrices with different pore sizes. The authors conducted bioremediation experiments in the shake-flask slurry bioreactors with aggregates of known pore size distributions and found that biodegradation rates were lower in case of matrices with pore sizes of 6 nm compared to matrices with 300 nm or more pore sizes. They found that under intense mixing conditions contaminant mass transfer to microorganisms was limited only in small 6 nm pore sizes. They also reported that adding surfactant had stimulating effect on both degradation and mass transfer of hexadecane. Stimulated degradation was attributed to induced emulsification or enhanced uptake of substrate by cells in presence of surfactant. Enhanced mass

transfer was observed in column studies and was found to be correlated to specific area of matrices rather than pore size [67]. Nam and Alexander also studied the effect of pore size and pore surface properties on biodegradation of phenanthrene. They reported 60-67% mineralization in non-porous glass beads and silica beads with pores sizes of 2.5, 6 and 15 nm. In case of hydrophobic polystyrene beads different biodegradation extents was observed depending on bead pore size. While 44% mineralization was observed for non-porous beads, only 2.5-6.6% mineralization was detected for bead pore sizes of 5 – 400 nm.

X-ray computed tomography (CT) has been used for more than two decades in the environmental field for purposes such as studying the transport of NAPL phase in subsurface environment [68] or characterization of porous medium [69]. Recent advanced scanning instruments such as micro-CT with resolutions as low as 1 μm have provided some new opportunities for more in depth characterization of porous medium micro-structure [70]. Nunan et al. used micro scale computed tomography (micro-CT) to study the microbial habitat in soil pore networks at resolutions as small as 4 μm for two different soil managements [71].

To obtain the desired information from acquired images by CT scanner, a series of image analysis steps are usually employed, among them the critical step is believed to be the image threshodling. By thresholding each pixel in the image is assigned as either background (void) or object (soil). In case of natural porous media and in particular a clayey aggregate this is complicated process for several reasons; such as limited attainable resolution which is still larger than some soil constituents, noise in scanning and reconstruction and finally the nature of environmental porous media which is composed of constituents with a range of densities.

The thresholding methods could be classified as global and local methods. In global methods, a single threshold value is applied to whole image, while in local methods; different values are considered as threshold values for different regions of the image. Another classification is based on if the spatial information of the image is incorporated in threshholding process. As an example of global method without considering the spatial information is Otsu method which is based on minimization of standard deviation of range of grey scale values of object and background [72]. The method introduced by O'Gorman which is based on maximizing the connectivity in image regions is an example of global methods which considers the spatial information of the image [73].

Two-Sigma method with low pass filter is a local thresholding method based on the sigma probability of the Gaussian distribution and involves smoothing the image by averaging only those neighboring pixels which lie within fixed intensity ranges of the central pixel. The technique is based on the fact that most image noises are Gaussian in nature. The two sigma probability is defined as the probability of a random variable being within two standard deviations of its mean [71]. The indicator kriging (IK) is another method introduced by Oh and Lindquist, which is essentially a local method which includes the spatial information in terms of covariance function for the image [74]. Based on comparisons with ground truth image, Wang et al. found that IK method produced the most similar results to ground truth image [75]. Overall, CT scanning followed by proper image analysis procedure can provide information on distribution, length, connectivity as well as neck diameter of pores inside the aggregate, which in turn can be used to interpret and predict the efficiency of bioremediation for a specific soil texture.

## 1.5. RESEARCH OBJECTIVES

The overall objective of this study was to investigate the limiting factors in bioremediation of aged, clayey petroleum hydrocarbon contaminated soils from a contaminated site in a sub-Arctic region in Northwest Territories (NWT).

The following are the specific objectives of this study.

1. Assess the effects of nutrient amendments and moisture levels on bioremediation of the hydrocarbon-contaminated soils at the average summer temperature of the site, in pilot scale biopiles.
2. Assess the effect of diurnal temperature variation from 5˚C to 15˚C, that represent site temperature profiles, on the extent of petroleum hydrocarbon biodegradation and the microbial community in microcosms containing the site soils.
3. To study the role of pore size on biodegradation of a model high molecular weight hydrocarbon and assess the implications of pore sizes in soil aggregates on the bioavailability of high molecular weight petroleum hydrocarbons.

## 1.6. THESIS ORGANIZATION

In Chapter 2, we evaluate the feasibility of bioremediation of an aged petroleum hydrocarbon contaminated soil from a sub-Arctic site in Northwestern Territories (NWT). The experiments were carried out in pilot-scale biopiles, and the effects of different doses of nutrients, as well as moisture amendment are studied in long term experiments. To further determine the bioremediation endpoint, the experiments were carried out for about 385 days. Given the simplicity and cost of operation, biopiles are one of the poplar techniques in soil remediation, particularly in case of remote sites in cold regions. The temperature was kept at 15 °C during the

experiments, which is the average summer temperature of the site. The concentration of different fractions of petroleum hydrocarbon in soil samples, the levels of $CO_2$ and $O_2$ in soil air phase as indicators of soil biological activity, and finally soil microbial community were monitored during the course of experiments. The TRFLP technique was used for fingerprinting of changes in soil microbial community. Also, to investigate the role of nutrient bioavailability, microcosm scale slurry phase experiments were carried out with the same contaminated soil. Given, the intense mixing during slurry experiments as well as partial disruption of soil aggregates it was assumed the nutrient bioavailability will be increased in the case of slurry phase experiments compared to solid phase biopile experiments.

Temperature has a significant effect on biodegradation of hydrocarbon contamination in soil. In Chapter 3 we evaluate the effect of three temperature regimes of constant incubation at 5 °C, constant incubation at 15 °C and daily fluctuation between 5 °C and 15 °C on the extent and kinetic of biodegradation of semi-volatile (>C10-C16) and non-volatile (>C16-C32) fractions of petroleum hydrocarbons in microcosm scale experiments in 70 days. Daily fluctuation between 5 ˚C and 15 ˚C resembles the ambient air temperature of the site in August. In case of surface soil or *ex-situ* treatments such as landfarming and biopiles, soil temperature can experience the same daily variations as air temperature. Also, the effect of temperature regimes on soil microbial community at day 70 was further studied using pyrosequencing, an advanced method which can provide detailed information about the structure of microbial community. The results of community analysis are presented for both 16S rRNA as well as *alkB* gene harboring bacteria. This enables us to compare the temperature effect in entire microbial community as well as community specialized of alkane biodegraders. To also evaluate the effect of temperature on soil bacterial biodiversity, biodiversity indices are compared among different temperature regimes.

Lastly, the effect of temperature on soil biological activity in terms of $CO_2$ respiration was studied.

In Chapter 4, the effect of soil pore sizes on biodegradation of high molecular weight petroleum hydrocarbons is discussed. Firstly, the role of pore size on biodegradation and accessibility of n-hexadecane by *Dietzia maris* strain which was isolated from NWT site and proved significant capacity to biodegrade alkanes was studied. The experiments are carried out in specially designed bioreactors with fixed oil-water interfacial areas fitted with membranes with specific pore sizes. Four different systems as without membrane and with membranes with 12 μm pore diameter, 3 μm pore diameter and 0.4 μm pore diameter were considered. Further, pore size distribution of representative aggregates from two different contaminated soils with different characteristics (NWT (fine textured soil), RI (coarse textured soil)), are determined by micro-CT scanning and image analysis. Micro-CT scanning enabled us to non-destructively delineate the three dimensional aggregate micro-structure at very low resolutions relevant to soil bacterial habitat. Image analysis was performed using modified Indicator Kriging thresholding method (IK) followed by an in-home developed procedure to preserve the whole aggregate body for statistical analysis. To further highlight the environmental implications of findings from pore experiments and CT-scanning, the bioremediation end point and biodegradation rate constants of semi-volatile and non-volatile fraction of petroleum hydrocarbons for NWT soil and RI soil are compared.

Chapter 5 describes a summary of the thesis and the general conclusions of this doctoral research.

## 1.7. CONTRIBUTIONS

The specific contributions of this work are listed below:

1. **Feasibility of biopile remediation of a clayey petroleum hydrocarbon contaminated soil from a sub-arctic site:** Although there are reported studies on bioremediation of petroleum contamination in cold regions; they have been mainly conducted in coarse textured soils and focused on the effect of nutrient amendment. We demonstrated the effectiveness of bioremediation of a clayey petroleum contaminated soil at typical temperature of the summer at the site in pilot scale biopiles. Although at significantly lower biodegradation rates than slurry bioreactors, the bioremediation endpoint was attained in biopiles was similar to that soils bioremediated in slurry (mixed) bioreactors where soil aggregates were less aggregated. This suggests that irreversible binding between hydrocarbons and clay or entrapment of NAPL in micro-pores of soil primary aggregates regulates the bioremediation endpoint. The significant capacity of soil indigenous microbial community for biodegradation of petroleum hydrocarbons under aerobic conditions and without nutrient amendment was characterized. It was further shown that application of high doses of nutrients negatively impacts the biodegradation rate and extent and also inhibits any change in soil microbial community.

2. **Effects of diurnal temperature variation on biodegradation of petroleum hydrocarbons in soil and on soil microbial community:** Though, the effect of temperature on biodegradation of hydrocarbon contaminants has been extensively studied; the effect of diurnal temperature variations has not been addressed before. In this study, we evaluated the effect of temperature as well as diurnal temperature variations on biodegradation extent of

C10-C24 petroleum hydrocarbons in a site-contaminated soil. Moreover, the effect of temperature regimes on soil general microbial community as well as those specialized in biodegradation of alkanes (*alkB* gene harbouring community) was characterized with pyrosequencing technique, which provides high resolution sequencing data. The results reveled that in case of the diurnal variable temperature, higher temperature limit regulates the soil microbial community. Moreover, the extent of biodegradation was comparable between the systems incubated at higher limit of temperature variation and those experienced variable temperature. Our data showed the significance of soil microbial community structure in biodegradation of petroleum hydrocarbons.

3. **The effect of soil pore size on accessibility and biodegradation of insoluble NAPLs:** The effect of pore size on biodegradation of hexadecane as a model NAPL compound was investigated in specially designed bioreactors. Although there are several studies addressing the effect of pore size on biodegradation rate and extent of hydrocarbons, the specific design of bioreactors enabled us to more precisely determine the bioaccessibility of NAPL in different pore sizes. Our results showed that for the bacteria used in experiments, the NAPL are not accessible when entrapped in pores with 3 μ diameters, which is significantly larger than bacterial size. Based on findings from pore experiments we defined the term of "bioaccessible porosity" as fraction of soil aggregate which are accessible to bacteria, and employing micro-CT scanning at very high resolution we were able to map bioaccessible porosity inside representative aggregates from contaminated soils for the first time.

4. **A new image analysis method for characterization of soil intra-aggregate "bioaccessible porosity"** Different approaches has been used for analysis of X-ray scanning

images of soil aggregates, however the majority of them have ignored the importance of shape analysis in order to consider the whole soil aggregate body. We developed a method for determining the aggregate boundary based on alpha-shape of aggregates, which further enabled us to characterize the pore network of whole aggregate. Analysis with the developed method provides more representative information about aggregate porosity, fraction of open pores as well as pore network connectivity compared to other approaches which consider only a sub-sample of core of aggregates.

Some of the work presented in this thesis has been or will be submitted for publication:

Akbari A., Ghoshal S. "Pilot-Scale Bioremediation of a Petroleum Hydrocarbon-Contaminated Clayey Soil from a Sub-arctic Site". Submitted to *Journal of Hazardous Materials*

Akbari A., Ghoshal S. "Effects of Diurnal Temperature Variation on Microbial Community and Petroleum Hydrocarbon Biodegradation in Contaminated Soils from a Sub-arctic Site" Submitted to *Environmental Microbiology*

Akbari A., Ghoshal S. "The Effect of Soil Pore Size on Biodegradation of High Molecular Weight Hydrocarbons" to be submitted

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# Chapter 2

# Pilot-Scale Bioremediation of a Petroleum Hydrocarbon-Contaminated Clayey Soil from a Sub-arctic Site

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## ABSTRACT

Bioremediation is a potentially cost-effective solution for petroleum contamination in cold region sites. This study investigates the extent of biodegradation of petroleum hydrocarbons (C16–C34) in a pilot-scale biopile experiment conducted at 15˚C for periods up to 385 days, with a clayey soil, from a crude oil-impacted site in northern Canada. Although several studies on bioremediation of petroleum hydrocarbon-contaminated soils from cold region sites have been reported for coarse-textured, sandy soils, there are limited studies of bioremediation of petroleum contamination in fine-textured, clayey soils. Our results indicate that aeration and moisture addition was sufficient for achieving 47% biodegradation and endpoint of 530 mg/kg for non-volatile (C16-C34) petroleum hydrocarbons. Nutrient amendment with 95 mg-N/kg showed no significant effect on biodegradation compared to a control system without nutrient but similar moisture content. In contrast, in a biopile amended with 1340 mg-N/kg, no statistically significant biodegradation of non-volatile fraction was detected. Terminal Restriction Fragment Length Polymorphism (T-RFLP) analyses of *alkB* and 16S rRNA genes revealed that inhibition of hydrocarbon biodegradation was associated with a lack of change in microbial community composition. Overall, our data suggests that biopiles are feasible for attaining bioremediation endpoints in clayey soils. Despite the significantly lower biodegradation rate of 0.009 $day^{-1}$ in biopile tank compared to 0.11 $day^{-1}$ in slurry bioreactors for C16-C34 hydrocarbons, the biodegradation extents for this fraction were comparable in these two systems.

## 2.1. INTRODUCTION

Significant numbers of oil contaminated sites have been identified in sub-Arctic regions, and contamination at these sites is linked to oil exploration and production, storage, transportation activity as well as past, improper disposal practices [1]. Bioremediation has been proposed as a cost-effective technique for remediation of hydrocarbon contaminated soils in cold regions [2]. Biostimulation can be successfully implemented in the summer at remote sites in subarctic regions [3]. Several studies have shown that cold-adapted, hydrocarbon-degrading, indigenous microbial populations are present in cold region sites and can be biostimulated through aeration, moisture adjustment and nutrient amendment in landfarm or biopile systems [4-7]. These microorganisms have genes for hydrocarbon degradation such as *alkB*, *alkM*, *alkB1*, *alkB2*, *xylE*, *ndoB, nidA* [6], and employ several strategies for cold adaptation such as alteration of the lipid content of the cell membranes, and expression of anti-freeze proteins [8].

Previous treatability assessments of hydrocarbon contamination in cold regions reported in the literature have been generally conducted with sandy or gravelly soils [2, 3, 7, 9-12]. To the best of our knowledge there are no reported studies on bioremediation of clayey, aged, hydrocarbon-contaminated soils from subarctic regions. Although sandy and gravelly soils are common in cold regions, fine textured soils are also present in significant parts of the subarctic [13, 14]. Cold region soils are often contain limited nitrogen and soil organic matter, and microbial populations in the soils are sensitive to osmotic stress [9].

The high surface area and large number of nano- and micro-pores in clay fractions of soils lead to poor bioavailability of pollutants, nutrients, and oxygen, which in turn limit the extent and rate of pollutant biodegradation. In clayey soils the microbial bioavailability of aged

petroleum hydrocarbons are limited by their poor solubility, extensive partitioning into the clay mineral phase and by the slow sorption-retarded diffusion from pores in clays [15]. Thus, long term studies are needed to adequately characterize biodegradation patterns of poorly soluble high molecular weight hydrocarbons in clayey soils. The vast majority of studies reported in the literature have been conducted for shorter periods of time of up to 90 days [2, 5, 9, 10, 12]. In addition, generally studies are conducted with freshly spiked hydrocarbons [5, 11, 12, 16]. Freshly spiked contaminations are more bioavailable due to limited contact time for interaction and binding between oil and soil matrices. These factors may cause overestimation of biodegradation rates.

Laboratory studies aimed at assessing biodegradation rates of hydrocarbons in soil are generally performed in small scale microcosms [5, 9-12, 16]. It has been shown that biodegradation rates derived from microcosm experiments are usually higher than the rates observed in the field [17]. However, larger-scale mesocosm studies provide estimates of biodegradation rates representative of those obtained in field experiments and operations [2, 18]. This study evaluates the feasibility of bioremediation of a clayey soil with aged hydrocarbon-contamination from a subarctic site in laboratory pilot-scale biopiles. The goal of the study was to assess the rates and extents of biodegradation of aged petroleum hydrocarbons in the clayey soils under selected biostimulation conditions. The specific objectives of this study were to assess (i) the effects of selected nitrogen and moisture doses on the rates and extents of biodegradation of petroleum hydrocarbon fractions; and (ii) to compare the rates and extents of biodegradation between unsaturated, aggregated clayey-soil in biopiles with those from well mixed soil slurry suspension microcosms of the same soil.

Pilot-scale biopiles each containing 300 kg of contaminated soils were operated for durations between 130 to 385 days to characterize long-term biodegradation patterns given that biodegradation rates were slow. The experiments were carried out at average summer temperature of 15 ˚C, in a temperature-controlled system. The contributions of volatilization and biodegradation to losses of petroleum hydrocarbons of different molecular weight fractions in the biopiles were quantified. T-RFLP analysis was performed to investigate the shifts in the indigenous microbial communities in systems with significantly different biodegradation extents resulting from different biostimulation treatments. Continuously mixed soil slurry microcosms studies were conducted to compare the biodegradation rates and extents for the aged hydrocarbons in clayey soils under conditions where soil was disaggregated, relative to those in the biopiles. Thus the study elucidates factors that control bioavailability and biodegradation of petroleum hydrocarbons in historically contaminated clayey soils from a subarctic region.

## 2.2. MATERIALS AND METHODS

### 2.2.1. Site Soil

The soil was shipped frozen from a contaminated site in the Northwest Territories, Canada, and was stored at -4˚C. The physico-chemical properties of the soil are summarized in Table 2.S1 in Supplementary Material section. The soil was classified as a clay loam with about (34 % clay, 33% slit and 33% sand) according to USDA classification and belongs to the Cryosolic Order according to Food and Agriculture Organization (FAO) classification system. The total organic matter of the soil was 2.3% by wt. Inorganic nitrogen was not detected in soil, but organic nitrogen content was 1400 mg/kg soil. Total phosphorus and inorganic phosphorus were 600 mg/kg soil and 420 mg/kg soil, respectively. There was an abundant population of hydrocarbon

degrading bacteria (about $10^6$ CFU/g soil) as determined by plating soil extract on Bushnell Haas plates exposed to diesel vapors.

### 2.2.2. Pilot Scale Biopile Experiments

Stainless steel tanks with the dimensions of 1m × 0.7m × 0.35m (L × W × H) were used as pilot-scale laboratory biopiles. Perforated tubes in the bottom of the tanks were used to inject air into soil layer above. Tanks were sealed, except for an air exit port at the top, which was fitted with an activated carbon trap to remove volatilized hydrocarbons from the effluent air stream. Based on 2000-2010 weather data the daily average temperature of the site in summer (June-August) was in the range of 13.9 ˚C to 16.4 ˚C, and thus the biolpile tanks were maintained at 15 ˚C inside a cold room. Tanks were sterilized with 70% ethanol solution prior to the experiments.

Approximately 300 kg of the soil was transferred to each tank to maintain minimum headspace. The soil was transferred in small amounts and was spread in several layers over the whole surface area of the tanks to ensure homogeneity. Then, calculated amounts of either sterilized water in case of control tanks or sterilized nutrient solution in case of nutrient amended tanks were carefully sprayed over each soil layer. The layers were then mixed by small shovels to ensure adequate mixing of the amendments. Moisture-free compressed air (78% $N_2$, 21% $O_2$, and 1% Ar) was injected into the soil at the rate of 2 mL/s. The air flow rate was chosen to ensure that the soil gas $O_2$ concentration was always higher than 80% of the atmospheric concentration and that biodegradation was not limited by the aeration rate. For microbiological and chemical analyses, soil samples were collected periodically using sterilized augers from five different random points in each of three layers of the soil. Samples at time zero were collected after nutrient or moisture amendment. Composite samples of each layer were prepared by mixing

the soil samples from the same layer. The samples were stored at -20˚C for further analysis. Additional details for the biopile experiments are provided in the Supplementary Material section.

As presented in Table 2.1, different amounts of nutrients and moisture were added to different biopiles each containing about 300 kg of soil. Nitrogen was applied at doses of 1340 mg-N/kg soil or 95 mg-N/kg soil, and soil moisture content was adjusted at either 23.5% (73% of water holding capacity) or 17.5% (55% of water holding capacity) in different tanks. A variety of nitrogen doses corresponding to molar ratios of C: N ratios ranging from 100:2-100:50, where C represents the total organic carbon or Total Petroleum Hydrocarbon (TPH), has been assessed in the literature [19-21]. In the HMNA tank (high moisture content of 23.5% and high nutrient dose at 1340 mg-N/ kg soil), N as ammonium nitrate and P as diammonium phosphate were added to attain a molar ratio of $C_{TOC}$: N: P in soil at 100:5:0.8. The LMNA tank (low moisture content of 17.5% and low nutrient dose at 95 mg-N/ kg soil) was amended

**Table 2.1.** Operating conditions for pilot-scale biopiles

| Biopile | Amended nutrients* (mg N or P/kg Soil) | | Moisture (wt%) |
|---|---|---|---|
| | N | P | |
| High moisture content (HMA) | - | - | 23.5 |
| High moisture content and high dose nutrient amended (HMNA) | 1340<br>[TOC:N] = 100:5 | 75<br>[TOC:P]** = 100:0.8 | 23.5 |
| Low moisture content (LMA ) | - | - | 17.5 |
| Low moisture content and low dose nutrient amended (LMNA) | 95<br>[TPH:N] = 100:5 | -<br>[TPH:P]** = 100:11 | 17.5 |

Note: * N and P added as ammonium nitrate; and diammonium phosphate; ** In calculation of C: P ratios, the background inorganic phosphorus was included.
Temperature: 15 ˚C ; Air flow: 2 mL/s

with lower doses of nitrogen at the ratio of $C_{TPH}$: N at 100:5. Given the background inorganic phosphorus concentration in the soil (Table 2.S1 in Supplementary Material section), no additional P was added to the LMNA tank, and the $C_{TPH}$: P ratio was calculated as 100:11. Two other tanks as LMA (low moisture content of 17.5% and no nutrient), and HMA (high moisture content of 23.5% and no nutrient) were maintained as controls without nutrient amendment.

### 2.2.3. Slurry-Phase Biodegradation Experiments

Slurry-phase biodegradation experiments were performed in batch microcosms comprised of 250 mL glass flasks fitted with crimp caps and Teflon laminated septa. Soil (5 g) and 35 mL of sterilized-distilled water were added to each microcosm. Three set of microcosms, nitrogen amended at 95 mg-N/kg soil with ammonium nitrate, un-amended and a killed control containing the biocide sodium azide were designed for slurry experiments. Microcosms were maintained in triplicate and incubated inside the shaker at 175 rpm and at the temperature of 15˚C.

### 2.2.4. Chemical Analyses

Nine subsamples from three different composite soil samples collected from three different depths of biopiles were analyzed for TPH. TPH extraction from soil was performed with an acetone-hexane mixture using an Automatic Soxhlet Extractor (Gerhardt, UK), and solvent extract after cleanup and concentration was analyzed using a Gas Chromatograph (GC) fitted with a flame ionization detector (Agilent 6890) and a DB1 column. The extraction and analysis procedure was according to the Canadian Wide Standard-Tier 1 Method [22]. Details of TPH extraction procedure can be found in the Supplementary Material section.

Ammonium analysis was performed according to a colorimetric method [11]. Nitrate and nitrite was measured by ion chromatography [23].

### 2.2.5. Microbial Analyses

Terminal Restriction Fragment Length Polymorphism (T-RFLP) was performed on both 16S rRNA and *alkB* genes. *alkB* genes are common markers for hydrocarbon-degrading populations [24]. Three replicate samples from Day 0 and Day 60 of HMA tank and Day 60 of HMNA tank were analysed. The detailed description of the DNA extraction procedure and T-RFLP methods are described in the Supplementary Material section.

### 2.2.6. Statistical Analyses

Differences in petroleum hydrocarbon concentration of different pilot scale biopiles were evaluated with two-way ANOVA followed by Bonferroni t-test for pairwise multiple comparisons, with time and treatments as factors. Differences among diversity indices between Day 0 and Day 60 of different treatments were evaluated by the paired t-test. All statistical analyses were done using SigmaPlot 12.0.

## 2.3. RESULTS AND DISCUSSION

The results of TPH analyses from all four biopiles are presented in Fig 2.1. Statistically significant reduction in TPH concentration (based on two-way ANOVA-Bonferroni, $p<0.001$) was observed in all biopile tanks. However, the extent of reduction over 110 days was significantly higher (35-43%) in HMA, LMA and LMNA tanks compared to 11% reduction in HMNA tank. TPH reduction in biopiles results from both volatilization and biodegradation; however, as discussed in the following section, the reduction in the F3 (C16-C34) fraction of petroleum hydrocarbons is solely attributable to biodegradation. As shown in Fig 2.1B, statistically significant reduction in F3 concentrations (24-38% in 110 days) was observed in HMA, LMA and LMNA tanks, whereas the reduction in the HMNA tank was not statistically

significant over the 110-day period. The results suggest that under high nitrogen concentrations, microbial activity in terms of biodegradation of petroleum hydrocarbons was inhibited. However $CO_2$ production (respiration) indicates that HMNA systems were still biologically active (Fig 2.S1 in Supplementary Material section). TPH and F3 biodegradation rates and extents were similar in the LMNA and LMA tanks. The LMNA tanks which showed relatively higher biodegradation extent by day 110, was maintained for longer period of times.

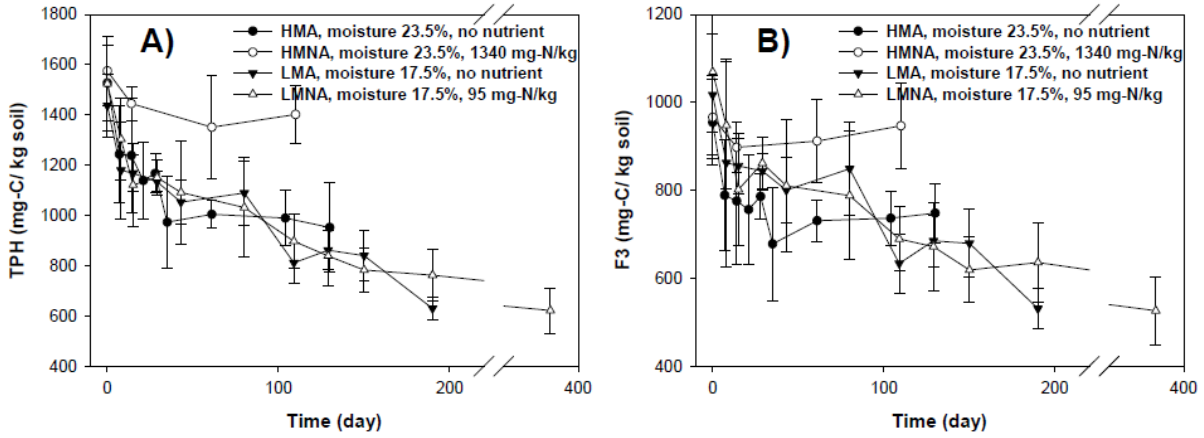


**Fig. 2.1.** Pattern of TPH reduction (A) and F3 biodegradation (B) in four pilot scale biopiles. Each data point is the average of nine different soil samples and error bars represent the standard deviation of the mean.

### 2.3.1. Hydrocarbon Losses by Volatilization and Biodegradation

Different approaches have been employed in the literature to determine the relative contributions of abiotic and biotic losses in biodegradation experiments such as using biomarker ratios [4] or including killed control systems [25]. Hopanes (m/z=191) and steranes (m/z=217) which are common conserved biomarkers of petroleum oils were not detected in our samples by GC-MS analyses. Similar lack of biomarkers have been reported for other aged petroleum oil samples [26]. Each biopile tank contained 300 kg of soil, as thus maintaining a killed control biopile was not feasible given the challenges in ensuring uniform distribution of biocides in the

soil. However, TPH analysis of activated carbon samples from the exhaust air trap of all tanks showed negligible amount of hydrocarbons in the F3 range, as shown in Fig 2.S2 (Supplementary Material section), which is a sample GC chromatogram of solvent-extracted activated carbon from the exhaust traps. Thus, F3 fraction reductions in the biopile experiments are attributed to biodegradation. Moreover, the results of F3 reduction in HMNA tank, indicating no statistically significant reduction in 110 days, can be considered as a control for volatilization extent in HMNA system which has similar moisture content. A lack of F3 volatilization have been reported by others in remediation studies of diesel contaminated soil with composting [27], and in laboratory-scale biopiles [28] with similar air flow conditions as our experiments. Also, significantly lower reduction in the F2 (C10-C16) fraction (25%) in HMNA tank in 110 days compared to the HMA tank (61%) indicates that F2 fraction loss in HMA biopile is largely due to biodegradation.

Fig. 2.2 shows the pattern of relative reduction of F2 and F3 fractions for three biopiles with significant biodegradation extent, and for a thin layer of soil continuously exposed to an air stream, representing a scenario with maximum possible volatilization. The relative reduction of F2 versus F3 fractions is significantly higher in case of the air-dried soil layer. The higher volatilization in the air dried samples is due to significantly higher surface area for mass transfer between air and soil-phase hydrocarbons in the thin layer of soil was exposed to a continuous air stream compared to the 0.7 m thick soil layer in biopiles. Also, the soil moisture content dropped to less than 2% in 24 h which would further enhance volatilization in air dried soils whereas in biopile soils the moisture content remained unchanged at approximately 17.5%, or 23.5% throughout the experiments. Fig. 2.2 clearly shows the distinct patterns of relative F2 and F3 removal in biopiles and air dried systems, suggesting different mechanisms of TPH loss in these

two different systems. The slightly higher ratio of F2 reduction over F3 reduction in HMNA reflects lower F3 reduction in this system, compared to the low moisture content soil biopiles (LMNA and LMA), as discussed in section 3.2.

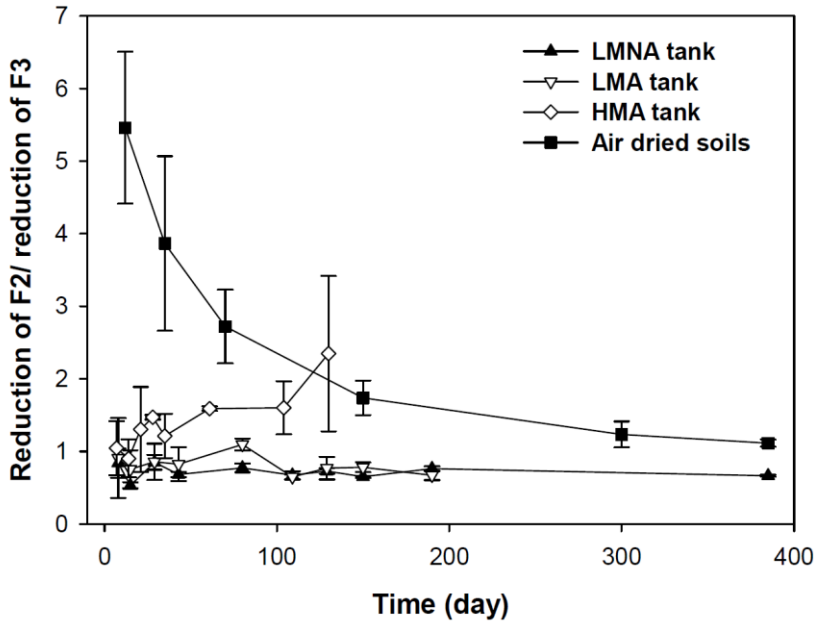


**Fig. 2.2.** Comparison of the relative reduction in F2 and F3 hydrocarbon fractions, in air dried soils and soils in LMNA, LMA and HMA biopiles. The data points represent the average of 5 and 9 different soil samples from air dried and biopile experiments, respectively. The error bars represent the standard deviation of the mean.

### 2.3.2. Biodegradation Endpoint and Rates

The TPH degradation rate in the LMNA tank which was initially 12.5 mg/kg/day, decreased to less than 1 mg/kg/day between days 190 and 385 suggesting limited bioavailability of a fraction of the hydrocarbons in the clayey soil. The residual concentration of F2 and F3 fractions after 385 days of treatment were 94.5 and 525.8 mg/kg soil.

The first order TPH degradation rate constant based on the data for the 385 days of LMNA tank was calculated as $0.010 \pm 0.003$ $day^{-1}$ ($r^2=0.79$) based on pseudo-first order kinetics

(Equation 1), which factors in the residual concentration of the target compound at the end of treatment. In equation 1, C represents the concentration at any time, and $C_{res}$ represents residual (un-degraded) hydrocarbon concentration, and $k$ is the first order rate constant. The pseudo-first order kinetics rather than the simple first order degradation kinetics was a significantly better fit to the hydrocarbon biodegradation data with significant residual levels, as reported elsewhere[16].

$$\frac{d(C-C_{res})}{dt} = k\,(C - C_{res}) \qquad (1)$$

The calculated TPH degradation rate constants were within the range already reported in the literature. For example, Nocentini et al. reported the rate constants in the range of 0.02 $day^{-1}$ for bioremediation of a freshly-contaminated clayey soil with diesel at 30$^{o}$C [16]. Zytner et al. reported first order degradation rate constants in the range of 0.008-0.01 $day^{-1}$ for bioremediation of a diesel contaminated clayey soil [29]. Significantly higher degradation rates, in case of sandy soils have been reported in literature [2, 3]. The first order biodegradation rate constant of the F3 fraction in LMNA tank considering the data over the 385 days was calculated to be 0.009 $\pm$ 0.003 $day^{-1}$ ($r^2$=0.69) which is significantly lower than the F3 hydrocarbon biodegradation rate reported by Chang et al. in case of bioremediation of a sandy soil [2].

The results of slurry experiments which involved incubation of soil slurries of the low dose nitrogen amended site soils (95 mg-N/kg soil) and killed controls are presented in Fig. 2.3. There was no significant difference between the results of un-amended and low dose nutrient amended systems. Rapid biodegradation of F3 and TPH occurred in the systems but biodegradation ceased after 30 days. The F3 modified first order biodegradation constant was determined as 0.11$\pm$ 0.04 $day^{-1}$ ($r^2$=0.9) which is significantly higher than LMNA tank (0.009 $day^{-1}$) amended with similar

nitrogen level. Higher mixing rate in slurries enhances the bioavailability and biodegradation rate compared to biopile, however, the bioremediation endpoint of slurries (F3: 578.6 mg/kg soil, TPH: 696.2 mg/kg soil) was comparable to LMNA biopile (F3: 525.8 mg/kg soil, TPH: 620.9 mg/kg soil). This could be an indicator of either strong binding between clay and the residual oil phase hydrocarbons, or sequestration of oil phase hydrocarbons in micropores of smaller primary aggregates that remained intact in the soil slurry.

High surface area and large number of micropores which are characteristics of clay aggregates reduce the accessibility and availability of hydrocarbons to microorganism, which in turn can affect the rate and extent of biodegradation. In our previous study we have proposed that the abundance of pores with neck diameters smaller than the size of bacteria have limiting effect on biodegradation extent of relatively insoluble hydrocarbons such as aliphatic hydrocarbons in the F3 fraction (>C16) [30]. Many of these components are known to be biodegraded only through direct contact or by pseudo-solubilisation as a result of biosurfactant production by microorganisms [31]. In the case of entrapment in micro-pores where bacteria are size-excluded, the hydrocarbon molecules cannot diffuse out of the pores to be available to microorganisms at any significant extent due to their extremely low solubility.

Increasing the moisture content is generally believed to enhance microbial activity by increasing microbial motility and nutrient bioavailability [32]. This was observed from the $CO_2$ production results in pilot tanks (Fig. 2.S1 in Supplementary Material section). Although $CO_2$ production rate is an indicator of total microbial activity in the soil but our results indicated that in high moisture amendment (HMA) tank the extent of biodegradation of F3 hydrocarbons was slightly but statistically significantly higher than the LMA tank at day 130 ($p$=0.07, student t-test, n=9).

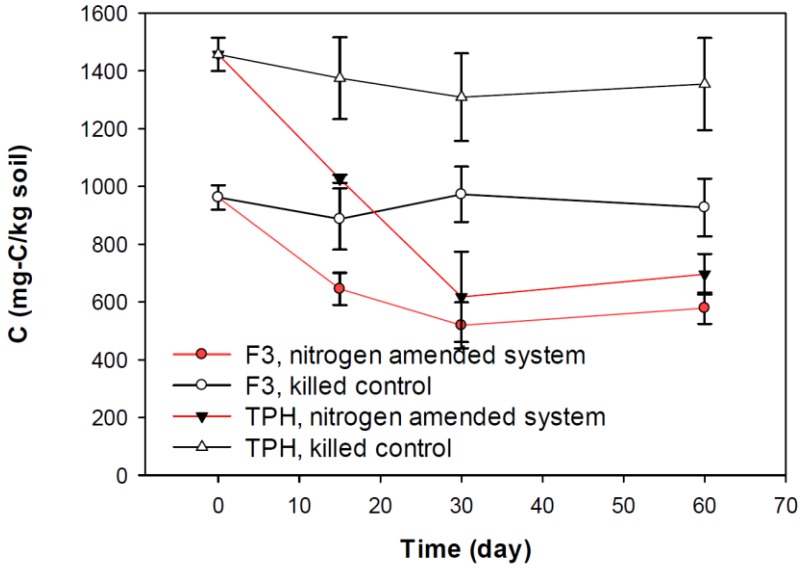


**Fig. 2.3.** TPH and F3 time profiles in slurry phase biodegradation experiments with hydrocarbon-contaminated site soils with nutrient amendment (95 mg-N/kg soil) and killed control systems. Each data point is the average of measurements from reactors maintained in triplicate and the error bars represent the standard deviation of the mean.

Moisture amendment altered the soil aggregate size distribution. The results from aggregate size characterization by sieving of the un-amended site soil and soil amended with moisture and/or high nutrient dose are shown in Fig. 2.4. Increasing moisture content to 23.5% resulted in a shift to larger aggregate sizes. The addition of nutrient at the high dose did not additionally alter aggregate sizes. Addition of moisture resulted in more than 60% w/w of soil aggregates becoming 4.75 mm or larger after one week of incubation which is attributable to plasticity of clays and aggregation as a result of moisture addition [33]. The increase in aggregate size, would lead to greater diffusion length for oxygen and soluble hydrocarbons [34], and could have contributed to lower degradation in the HMA tank than in the LMA and LMNA tanks. Furthermore, as moisture content increases, air-water interface which are hot spots for high biological activity due to availability of both water and sufficient oxygen, moves to larger pores.

Water saturation of the smaller pores, which are more abundant than larger pores, and the reduced oxygen diffusion through water compared to air is likely to reduce biodegradation activity in the smaller pores.

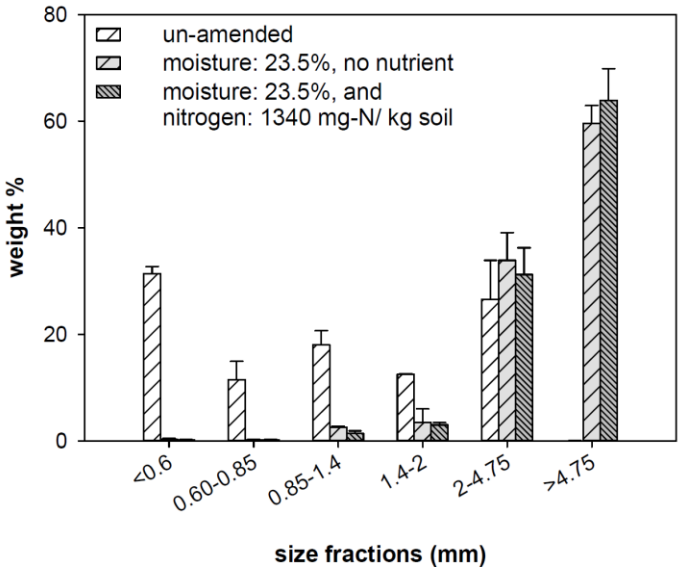


**Fig. 2.4.** The extent of aggregation in the soil as a result of moisture and high dose nutrient amendment.

### 2.3.3. Effect of Nitrogen Amendment on Biodegradation Performance

As shown in Fig. 2.1, nitrogen amendment at 95 mg-N/kg soil in LMNA tank exhibited no enhancement of biodegradation rate and extent compared to the associated un-amended control (LMA) tank. There are several studies in the literature reporting no enhancement of biodegradation of hydrocarbons with nutrient addition [35-37]. In a bioremediation study of diesel contaminated soil by Bento et al., no effect of 53 mg-N/kg amendment was observed and they suggested that the lack of effect is related to limited nutrient bioavailability [38]. However, the results of nutrient analysis for soil samples taken from LMNA tank indicated 47 mg-N/kg soil decrease in ammonium concentration and 37 mg-N/kg soil increase in nitrate concentration in the first 22 days of biopile experiments. The nitrite concentration was 2 mg-N/kg soil at day

22. The presence of nitrite, consumption of ammonium and generation of nitrate are indictors of the aerobic process of nitrification by soil autotrophic bacteria and confirm the nitrogen bioavailability to soil microbial community.

To assess if the lack of effect of nutrient addition on hydrocarbon biodegradation was attributable to limited bioavailability of hydrocarbons, another set of slurry phase biodegradation experiments were carried out where the soil was spiked with fresh oil, #2 furnace oil (Shell Canada). As shown in Fig. 2.S3 (Supplementary Material), the low dose nutrient amendment (95 mg-N/kg soil) did not enhance hydrocarbon biodegradation compared to un-amended systems, and in both systems approximately 900 mg/kg TPH representing a range of C10-C16 hydrocarbons was biodegraded in 30 days.

The results from HMNA tank (Fig. 2.1) showed that nitrogen amendment at 1340 mg-N/kg inhibited biodegradation of petroleum hydrocarbons of both F2 and F3 fractions, suggesting that microbial activity of hydrocarbon degraders was negatively impacted. Muller et al. reported impaired growth of bacteria in the presence of 750 mM ammonium is due to osmotic pressure or ionic effects rather than toxicity of ammonium [39]. It is likely that the salt concentrations (416 mM as N) added in the HMNA tank resulted in too high osmotic potential for hydrocarbon degraders to thrive. There are few reported studies in the literature indicating the inhibiting effect of nitrogen amendment on hydrocarbon degradation. Braddock et al. reported that nutrient application at 300 mg-N/kg soil inhibited the biodegradation of hexadecane [9]. However, in the same time period significant hexadecane reduction was observed in the systems amended with lower dose of nutrients, 100 mg-N/kg soil (3.3 g-N/L water), and in un-amended systems. Sabate et al. and Vinas et al. reported that application of about 1600 mg-N/kg soil in the form of potassium nitrate inhibited the biodegradation of 4-ring PAH compounds while the degradation

of 3-ring PAH compounds was not significantly affected during bioremediation in a clayey, creosote-contaminated soil. [20, 40].

To further investigate the observed markedly different biodegradation patterns in HMNA and HMA tanks which had similar moisture contents, the microbial community structure of 16S rRNA and *alkB* gene harbouring bacteria of soil samples from these two tanks were analyzed by T-RFLP. The relative abundance of different OTUs (Operational Taxonomy Unit) obtained from T-RFLP analysis of *alkB* genes are presented in Fig. 2.5A. The data clearly shows the microbial community in the soil sample from Day 0 underwent a significant shift in the HMA tank over 60 days. In contrast, there was a high degree of similarity in the community structure between Day 0 soil samples and soil samples taken at Day 60 from the HMNA tank. As shown in Fig 2.5A, the proportion of OTU of 137 is about 50% of total population at Day 0 and at Day 60 of HMNA tank. This portion is significantly decreased to about 28% in samples from Day 60 in the HMA tank. Also, in Day 60 samples from HMA tank, the OTU of 116 appeared at a relatively significant fraction of about 20%. The appearance of new OTUs in HMA tank area associated with 24% biodegradation of F3 fraction. The extent of similarity in the microbial community between the Day 0 soil samples and the Day 60 soil samples from the HMA and HMNA tanks are quantified in the non-metric multidimensional scale plot (Fig 2.6) calculated based on Bray-Curtis similarity index.

Changes or the lack thereof in the microbial community on the basis of 16S rRNA T-RFLP analyses with all three restriction enzymes (*AluI*, *BfaI* and *MseI*) was very similar to *alkB* T-RFLP results. The relative abundance of different OTUs obtained from 16S rRNA T-RFLP analysis of soil samples digested with *MseI* enzyme are presented in Fig. 2.5B. As shown in the figure, a significant shift was occurred in soil bacterial community of HMA tank in 60 days,

whereas in case of all three enzymes little or no shift was observed in soil community structure of samples from HMNA tank. This is in contrary to findings by Vinas et al. (2005) who found that inhibiting effect of high nutrient dose was associated with a shift in microbial community defined by an increase in relative abundance of *γ-Proteobacteria* and *β-Proteobacteria* phyla and a decrease in relative abundance of *α-Proteobacteria* phylum. Overall, our results indicated that as a result of applying a high dose of nutrients as in the HMNA tank, the microbial population community experienced negligible shift during the experiments and was associated with no statistically significant change in F3 hydrocarbon concentrations. On the other hand as a result of moisture addition and aeration in the HMA tank, a significant shift in microbial community occurred, which was associated with significant F3 biodegradation.

Richness and effective diversity numbers calculated based on Shannon and Simpson indices as well as evenness indices are presented in Table 2.S2 in Supplementary Material section. The calculated indices in most cases indicated a statistically significant difference between the microbial populations at Day 60 of HMA tank compared to population at Day 0 ($p<0.05$), whereas, the values for HMNA tank at Day 60 were never significantly different than indices of Day 0. In general, diversity numbers were all higher in case of HMA tank, Day 60, which is associated with significant biodegradation extent. Our results are in agreement with studies by Zucchi et al. and Dell'Anno et al. which reported increased diversity along with effective treatment of contaminated soil [41, 42]. Dell'Anno et al. reported an increase in microbial diversity during bioremediation of hydrocarbons present at relatively low levels in contaminated sediments (TPH < 1000 mg/kg). Effective bioremediation in case of fresh oil contamination (which has a large pool of normal alkanes and less diverse source of carbon than weathered and aged petroleum contamination) along with nutrient amendments is reported to lead to enrichment

of selective species and consequently reduced biodiversity indices [24, 43]. Overall, our data suggest that at relatively low contamination levels and in weathered contamination which has relatively varied TPH composition than fresh oil, a more diversified microbial community is more effective for TPH biodegradation. This is consistent with the complementary mechanism of contribution of bacterial community richness to community functionality [44].

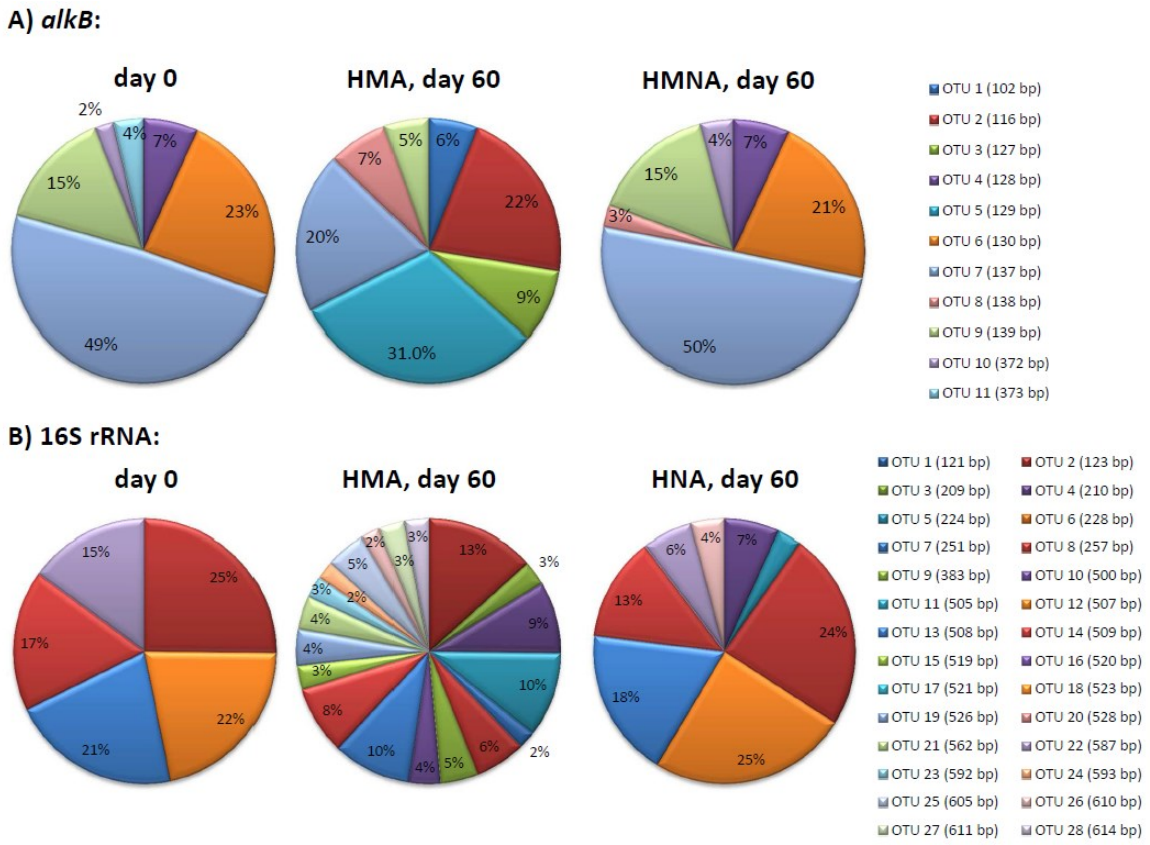


**Fig. 2.5.** Terminal restriction fragment length polymorphism (T-RFLP) analyses of *alkB* genes (A) and 16S rRNA genes (B) from soil samples of HMA pilot tank (23.5% moisture, no nutrient) and HMNA pilot tank (23.5% moisture, 1340 mg-N/kg soil).

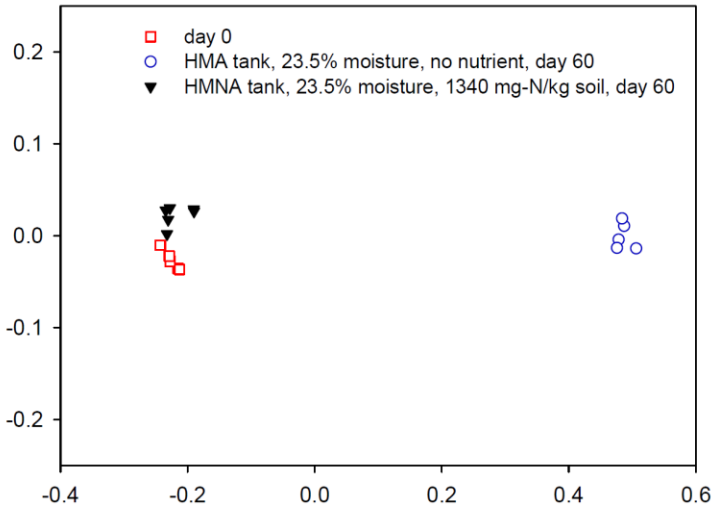


**Fig. 2.6.** Non-metric multi-dimensional scaling plot showing similarities of microbial communities based on T-RFLP of *alkB* gene, for samples of HMA pilot tank (23.5% moisture, no nutrient) and HMNA pilot tank (23.5% moisture, 1340 mg-N/kg soil).

## 2.4. CONCLUSION

As a result of biostimulation, significant biodegradation of petroleum hydrocarbons occurred in a clayey hydrocarbon contaminated soil from a subarctic site at the average summer temperature of the site. Significant biodegradation was achieved as a result of aeration and moisture amendment, or aeration and low dose nitrogen amendment. Low dose of nitrogen amendment did not show enhancement in F3 biodegradation rate and extent compared to the tank with similar moisture content and without nutrient amendment. On the other hand, biodegradation of hydrocarbons with higher molecular weights (F3) was completely stopped as a result of high dose nutrient amendment. Characterization of soil microbial community by T-RFLP revealed that soil microbial community experienced almost no change in high dose nutrient amended systems, whereas it underwent a significant shift associated with an increase in microbial diversity indices in case of high dose moisture amended systems in 60 days. About 600 mg/kg of TPH, mainly consisted of more hydrophobic and less soluble fraction of F3 was remained non-

biodegraded in the soil even after more than one year in the biopile tanks. Comparable residual concentrations were observed in slurry phase bioreactors, suggesting that the bioremediation endpoint is regulated by irreversible binding between hydrocarbons and clay or entrapment of NAPL in micro-pores of soil primary aggregates.

## 2.5. ACKNOWLEDGEMENTS

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## 2.6. SUPPLEMENTARY MATERIAL

### Biopile Operation Information

To sample the soil gas phase six perforated tubes were designed at three different depths of biopile. The air sampling tubes were attached to sampling valves which were opened at each sampling times. $CO_2$ and $O_2$ concentration in the soil air phase were determined using an ATX 620 $CO_2$-$O_2$ analyser (Industrial Scientific Co.).Nine measurements at 20 s intervals were performed at each sampling time and each port. To monitor the temperature and moisture of the soil at different depths, sensors [5TE, Decagon devices] were placed at different depths of each biopile. Readings was performed every 30 min and data was stored at data logger (Em50 Digital/Analog Data Logger, Decagon Devices).

**Table 2.S1.** Physico-chemical properties of the soil, NWT, Canada.

| | | |
|---|---|---|
| | Maximum water holding capacity | 32% |
| | pH | 7.4 |
| | Clay | 34% |
| | Silt | 33% |
| | Sand | 33% |
| Microbial Population | Total heterotrophic bacteria (CFU/g soil) | $1.35 \times 10^6$ |
| | Hydrocarbon-degrading bacteria (CFU/g soil) | $2.38 \times 10^6$ |
| Background nutrients | Inorganic nitrogen | Not Detected |
| | Total Kjeldahl nitrogen (mg/kg soil) | 1400 |
| | Inorganic phosphorous (mg/kg soil) | 410 |
| | Total phosphorous (mg/kg soil) | 600 |
| | Calcium (mg/kg soil) | 43000 |
| | Iron (mg/kg soil) | 19000 |
| | Magnesium (mg/kg soil) | 12000 |
| | Potassium (mg/kg soil) | 550 |
| | Sodium (mg/kg soil) | 73 |
| | Total organic carbon (TOC) (mg/kg soil) | 23000 |

## Chemical Analyses

For TPH analysis, soil samples (10 g) were mixed with sodium sulphate to remove the moisture and then were extracted by a 140 mL mixture of acetone-hexane in automatic Soxhlet extractor (Gerhardt, UK) for two hours at 150˚C under pressure. The extracted organic phase was passed through a column of silica gel and sodium sulphate to remove the moisture and natural organic matter of the soil. Mixture of dichloromethane (DCM) and hexane (50-50% by weight) was used

as eluent solvent in the column clean up step. Then the mixture was concentrated under nitrogen to 2 mL. Slurry samples were first centrifuged at 1000 g to separate the soil and aqueous phases and each phase was extracted with solvents. The aqueous phase was decanted and stored and the residual soil was mixed with 10 g sodium sulphate. 40 mL of DCM was added as solvent to soil and aqueous phase containing extraction tubes and mixed overnight. Extraction was repeated two more times for three and one hours with 30 mL and 20 mL of solvent. Each time the solvent was separated by centrifuge. At the end of the extraction process, the whole 70 mL solvent for each sample was concentrated under nitrogen atmosphere to reduce the solvent volume to about 20 mL. Column clean up and concentration under $N_2$ atmosphere were performed prior to GC analysis. TPH concentrations for aqueous phase samples were found to be negligible in all bioreactors. 0.5 ml of o-Therphenyl was initially added to each sample to track the extraction recovery efficiency. The recovery efficiency was always higher than 90%. α- andostrane was used as internal standard. To remove the bias in analyses, in all set of extractions one blank sample (surrogate plus sodium sulphate without soil), was analyzed. The activated carbon samples were extracted in similar way but using $CS_2$ as solvent. The prepared samples were injected to a 6890 Agilent GC with DB1 column. The oven temperature was increased from 40˚C to 280˚C at the rate of 15˚C/min. Helium with the flow rate of 10 mL/s was used as carrier gas.

The total petroleum hydrocarbon concentration was determined by integrating the area under peaks for hydrocarbon compounds with boiling points higher than n-C10. F2, and F3 fractions were determined by integration the area under the peak for compounds with boiling points in the ranges of n-C10:n-C16 and n-C16:n-C34, respectively.

**Microbial Community Analyses Methodology**

DNA extraction from soil samples was carried out using MoBio PowerSoil DNA isolation kit® according to the protocol provided by the manufacturer. PCR screening for either 16S rRNA sequence or *alkB* genes was performed in the next step. Each PCR reaction vial was consisted of 35.8 µL DNA free water, 5.0 µL buffer solution, 1.2 µL $MgCl_2$ solution, 0.5 µL dNTP 2.5 mM, and 2.5 µL the universal bacterial primer 338 as forward primer ([6~FAM]ACTCCTACGGGAGGCAGC) and 2.5 µL 1390 reverse primer [GACGGGCGGTGTGTACAA], 0.5 µL Tag polymerase and finally 2.0 µL of DNA extraction product from the soil. Three replicate of PCR reaction vials was prepared for each sample. After PCR the replicate vials were pooled again as a single sample. Thermal cycle of PCR was as follow: 3 min at 94˚C, followed by 30 cycles of 45 sec at 94˚C, 30 sec at 60˚C, 1.5 min at 72˚C, and 10 min extension at 72˚C in the end of the cycles. In addition to extracted DNA samples, positive and negative control samples were also included. The quality of extraction and PCR were further examined with running electrophoresis gel. Digestion for 16S rRNA samples was conducted with three different enzymes as *AluI*, *MseI* and *BfaI* (New England Biolabs®). At two different steps, after PCR reaction and also after digestion sample clean-up was conducted using UltraClean® PCR clean up kit (MoBio laboratories®). The *alkB* gene PCR screening was performed similarly with *alkB-1f* ([6~FAM]AAYACNGCNCAYGARCTNGGNCAYAA) and *alkB-1r* [GCRTGRTGRTCNGARTGNCGYTG] as forward and reverse primers and *HpaII* (New England Biolabs®) as digestion enzyme. The purified digests were sent to Plate-forme d'Analyses Génomiques de l'Université Laval where the samples were analyzed to determine the fragment size distributions using an ABI 3130XL genetic analyzer. Diversity and evenness

indices based on the equations described elsewhere [30] and non-metric multidimensional scale similarities were calculated based on Bray-Curtis similarity index.

**Respiration Results of Pilot Scale Biopile Experiments**

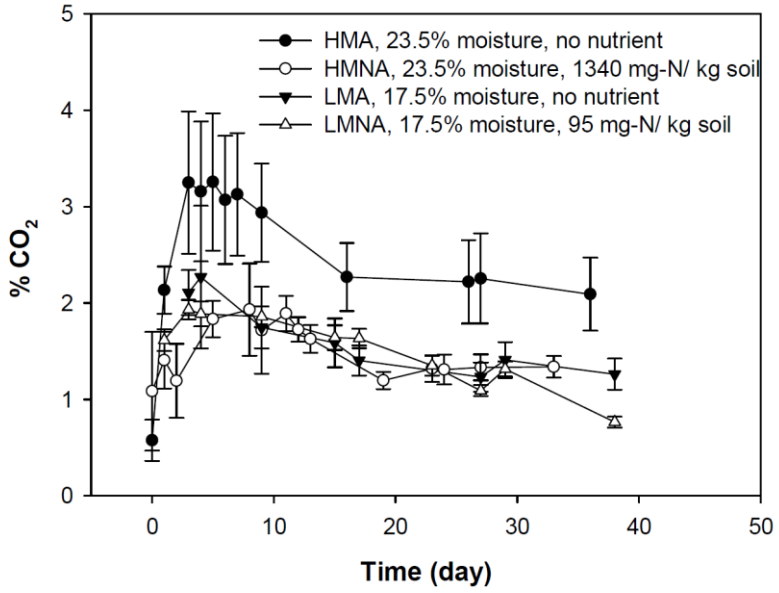


**Fig. 2.S1.** $CO_2$ concentration in soil air phase of four biopile tanks. Each data point is the average of 18 readings from 6 different sampling ports installed at different depths

**GC Chromatogram of Volatilized Fraction o TPH**

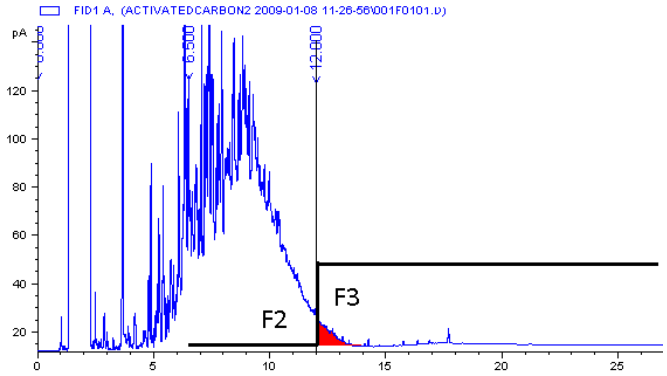


**Fig. 2.S2.** A sample GC chromatogram of TPH content extracted from activated carbon traps installed on exhausting air stream from biopile tanks showing the fraction of volatilized TPH.

**Results of Slurry Phase Biodegradation with Freshly Spiked Soil**

Contaminated soil from the site was spiked with fresh grade #2, furnace oil, Shell Canada, which is largely composed of F2 (>C10-C16) and F3 (>C16-C34) hydrocarbon fractions and thus enabled us to determine the biodegradation extent of compounds belonging to each of these two fractions. This oil was chosen as it was representative of the petroleum hydrocarbon present in the contaminated soil with similar composition. The experiments were carried out similar to non-spiked slurry experiments.

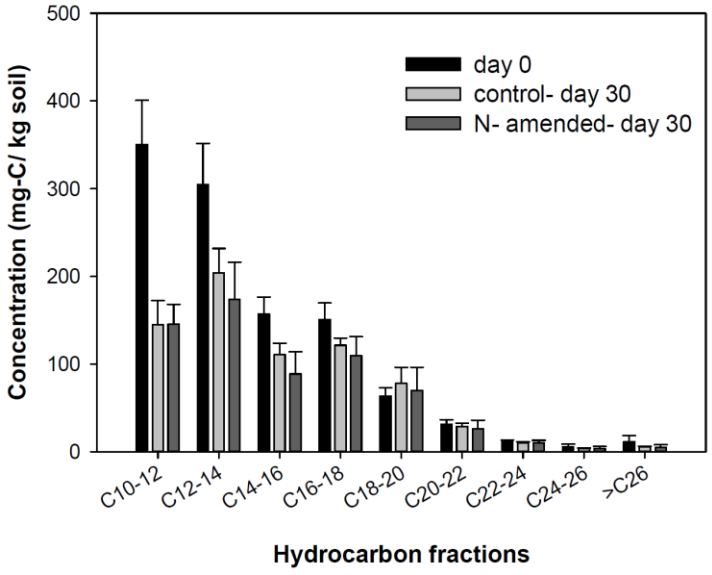


**Fig. 2.S3.** The biodegradation extent of different fraction of resolved peaks in slurry phase experiments spiked with furnace oil with control (un-amended) and nitrogen amended (95 mg-N/kg soil) systems . Each data point is the average of three different slurry bioreactors and the error bars represent the standard deviation of the mean.

**Diversity Indices in Pilot Scale Biopiles**

**Table 2.S2.** Microbial diversity indices calculated based on 16S rRNA and *alkB* T-RFLP analyses for soil samples taken at day 0 and at day 60 from HMA (23.5% moisture, no nutrient) and HMNA (23.5% moisture, 1340 mg-N/kg soil) pilot tanks. The means and standard deviation (in parentheses) of 3-5 different soil samples are presented.

| | **16S rRNA** | | | | | | | | | ***alkB*** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***MseI*** | | | ***BfaI*** | | | ***AluI*** | | | ***Hpa*** | | |
| | **day 0** | **HMA d60** | **HMNA d60** | **day 0** | **HMA d60** | **HMNA d60** | **day 0** | **HMA d60** | **HMNA d60** | **day 0** | **HMA d60** | **HMNA d60** |
| Richness | 6.33 | 16.67 | 9.00 | 6.67 | 10.33 | 6.50 | 8.00 | 11.67 | 10.50 | 6.60 | 7.00 | 6.25 |
| | (1.15) | (2.08) | (1.41) | (0.58) | (0.58) | (0.71) | (1.00) | (1.15) | (0.71) | (0.55) | (0.00) | (0.50) |
| Shannon's diversity | 5.31 | 13.73 | 6.49 | 3.58 | 8.62 | 4.70 | 5.42 | 10.00 | 7.41 | 4.20 | 5.71 | 4.06 |
| | (0.54) | (2.10) | (0.16) | (0.30) | (0.53) | (2.29) | (0.47) | (1.05) | (1.23) | (0.25) | (0.11) | (0.15) |
| Simpson's diversity | 4.80 | 11.48 | 5.33 | 2.53 | 7.57 | 3.69 | 4.15 | 8.96 | 5.74 | 3.21 | 4.96 | 3.14 |
| | (0.67) | (2.14) | (0.31) | (0.20) | (0.56) | (2.19) | (0.26) | (0.95) | (1.38) | (0.11) | (0.12) | (0.14) |
| Shannon's evenness | 0.91 | 0.93 | 0.86 | 0.67 | 0.92 | 0.73 | 0.81 | 0.94 | 0.85 | 0.76 | 0.90 | 0.77 |
| | (0.08) | (0.01) | (0.05) | (0.03) | (0.01) | (0.15) | (0.01) | (0.01) | (0.05) | (0.01) | (0.01) | (0.02) |
| Simpson's evenness | 0.78 | 0.69 | 0.60 | 0.38 | 0.73 | 0.47 | 0.52 | 0.77 | 0.54 | 0.49 | 0.71 | 0.51 |
| | (0.19) | (0.04) | (0.13) | (0.03) | (0.04 | (0.16) | (0.04) | (0.02) | (0.01) | (0.03) | (0.02) | (0.04) |

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# Chapter 3

# Effects of Diurnal Temperature Variation on Microbial Community and Petroleum Hydrocarbon Biodegradation in Contaminated Soils from a Sub-arctic Site

Ali Akbari, Subhasis Ghoshal

**Connecting text:**

In Chapter 2, pilot scale biopile experiments were performed at constant temperature of 15 ˚C, the average summer temperature of the site. To investigate the effect of diurnal temperature variation between 5 ˚C to 15 ˚C, the typical temperature regime of the site in August, the rate and extent of biodegradation of petroleum hydrocarbons as well as soil microbial community under three temperature regimes of constant incubation at 5 ˚C, constant incubation at 15 ˚C, and incubation at variable temperature regime of 5 ˚C to 15 ˚C, were compared.

The results of this research have been submitted for publication as: Akbari A., Ghoshal S. "Effect of Diurnal Temperature Variation on Microbial Community and Petroleum Hydrocarbon Biodegradation in Contaminated Soils from a Sub-arctic Site" in the *Environmental Microbiology*

## SUMMARY

Contaminated soils are subject to diurnal and seasonal temperature variations during on-site *ex-situ* bioremediation processes. .We assessed how diurnal temperature variations similar to that in summer at the site from which petroleum hydrocarbon-contaminated soil was collected, affects the soil microbial community and the extent of biodegradation of petroleum hydrocarbons compared to constant temperature regimes. Microbial community analyses for 16S rRNA and *alkB* genes, with pyrosequencing indicated that the microbial community for soils incubated under diurnal temperature variation from 5˚C to 15˚C (VART5-15) evolved similarly to that for soils incubated at constant temperature of 15˚C (CST15). In contrast, under a constant temperature of 5˚C (CST5), the community evolved significantly differently. The extent of biodegradation of C10 to C16 hydrocarbons in VART5-15 systems was comparable to those of CST15 systems at 48% and 41%, respectively, and both were significantly higher than CST5 systems at 11%. The emergence of γ-*Proteobacteria* was observed in the *alkB* gene harbouring community in VART5-15 and CST15 systems but not in CST5 systems. The *Actinobacteria* phylum was abundant at all temperature regimes. The results suggest that changes in microbial community composition as a result of diurnal temperature variations can significantly enhance petroleum hydrocarbon bioremediation performance in cold regions.

## 3.1. INTRODUCTION

Temperature affects various aspects of biological activity in soil. Temperature is an important factor regulating cellular biochemical reaction rates, viscosity, membrane fluidity and nutrient uptake [1]. For example, it has been suggested that the bacterial substrate affinity decreases with decrease in temperature as a results of lipid membrane stiffening [2]. Because different bacteria respond differently to temperature changes in the environment, temperature changes could results in a shift in microbial community structure. Different bacteria have different enzymatic activity and preferred carbon substrates, and consequently temperature change may also indirectly alter the biodegradation activity of microbial communities. On the other hand, the bioavailability of hydrocarbons in the environment is not expected to be significantly affected by typical changes in the ambient temperature because physico-chemical properties of hydrocarbons such as solubility and diffusion of hydrocarbons are not significantly altered by such temperature changes [3].

Petroleum contamination is a major source of soil and groundwater contamination in cold regions [4]. Previous studies have demonstrated the potential of cold adapted indigenous microbial community of soils from cold regions to degrade petroleum hydrocarbons [5-7]. Margesin et al. reported the prevalence of seven genotypes containing genes involved in hydrocarbon degradation (*alkB*, *alkM*, *alkB1*, *alkB2*, *xylE*, *ndoB*, *nidA*), as well as enrichment of gram negative bacteria upon soil contamination in 20 petroleum hydrocarbon contaminated and pristine soil samples from Alpine regions [6]. Whyte et al. also reported prevalence of *Rhodococcus* sp. strain Q15 genotypes in pristine and petroleum contaminated soil samples and *Pseudomonas putida* GPo1 in contaminated soil samples from Arctic and Antarctic regions [8]. The potential for biostimulation of indigenous hydrocarbon degraders in soils for bioremediation

in cold regions have been assessed. Those studies have been largely conducted at constant incubation temperatures: some studies have selected incubation temperatures representative of the average temperatures encountered at the sites [7], whereas others have used a series of constant incubation temperatures to identify the minimum feasible [9, 10] and/or the optimum temperature [11, 12] for bacterial metabolic activity. Ferguson et al. reported a continuous increase in the biodegradation rate of $^{14}$C-octadecane in petroleum contaminated Antarctic terrestrial sediments with the temperature increase from 4 ˚C to 42 ˚C [12]. On the other hand, Coulon et al. reported a slightly higher biodegradation extent of diesel and crude oil at 10 ˚C compared to 20 ˚C in artificially contaminated sub-Antarctic soils [11]. There are a few studies on the effects of seasonal long term temperature variations [13], and seasonal (Chang et al., 2011b) or short term freeze-thaw cycles [13-15] spanning a few days on biodegradation of petroleum hydrocarbons in soils. For example Chang et al. found higher biodegradation rate in case of repeated 10-day cycles of temperature changes between 1 ˚C to 10 ˚C resembling site temperature profile in months of July and August compared to constant average temperature of 6 ˚C [16]. Although, previous studies characterized the effects of temperature on soil microbial community by employing molecular techniques such as DGGE [16] or PLFA [17], the use of advanced high-throughput pyrosequencing, which provides higher resolution sequencing data, has not been used before to investigate the effects of temperature on microbial community in soils contaminated with petroleum hydrocarbons.

Diurnal temperature changes can be of significant magnitude, however, the effect of such rapid temperature variations on soil microbial community, biological activity and bioremediation efficiency has not been addressed before. Although rapid temperature changes may not influence temperatures in soils a few metres below the ground surface, surface soil or the layers of

excavated soil in *ex-situ* treatment processes such as landfarms and biopiles, are likely to experience temperature changes of similar magnitude to the ground-level air temperature changes during the day. Surface soil diurnal temperature fluctuations up to 20 ˚C have been reported in sub-Antarctic regions [11].

Temperature changes involving freeze-thaw cycles can induce a change in soil microbial community structure, diversity and functions [13, 18, 19], which is mainly attributable to released nutrients from destroyed or damaged microbial cells. However, even in case of above zero temperature changes, soil microbial community and its functions are affected by temperature. The objective of this study was to investigate how constant incubation temperatures and diurnal temperature changes from 5 ˚C to 15 ˚C over the duration of the summer season impacts indigenous soil microbial community composition and the biodegradation of petroleum hydrocarbons in a contaminated soil. Microcosm experiments were conducted with a weathered, crude oil-contaminated soil from a sub-arctic site in Northwest Territories (NWT), Canada, with three temperature regimes: constant temperature of 5 ˚C (CST5), constant temperature of 15˚C (CST15), and daily varying temperature from 5 ˚C to 15 ˚C (VART5-15) representative of the summer temperature profile of the site. After 70 days of incubation of microcosms at three temperature regimes, the soil microbial community as well as *alkB* gene harbouring community was characterized using pyrosequencing. The *alkB* gene is responsible for encoding of transmembrane alkane 1-monooxygenase AlkB protein, which is involved in initial activation of aliphatic hydrocarbon metabolism and has been used as functional biomarker for community of aliphatic hydrocarbon degraders in petroleum contaminated soils [20, 21]. Aliphatic hydrocarbons are a major component of crude oil.

The extent of biodegradation of semi-volatile (F2: >C10–C16) fraction and non-volatile (F3: >C16–C34) fraction of petroleum hydrocarbons in different microcosms were determined. The F2 and F3 fractions comprised about 99% of total petroleum hydrocarbon content of the soil. The effects of temperature changes on soil respiration, as indicator of soil general biological activity, was assessed in parallel microcosms by $CO_2$ gas measurements in the soil air phase.

## 3.2. RESULTS AND DISCUSSION

### 3.2.1. Hydrocarbon Biodegradation Extent at Different Temperature Regimes

The extent of biodegradation of total petroleum hydrocarbons in different microcosms incubated at different temperature regimes are shown in Fig. 3.1. As shown in Fig. 3.1A, there were statistically significant reductions (Two-way ANOVA - Tukey test, p value <0.05) in the concentrations of the semi-volatile hydrocarbon fraction (F2: >C10-C16) in all treatments. The extent of reduction was significantly higher for CST15 and VART5-15 at 41 and 48%, respectively, compared to an 11% reduction in the CST5 systems. Moreover, as illustrated in Fig 3.1B statistically significant reduction (Two-way ANOVA - Tukey test, p value <0.05) in concentrations of the higher molecular weight, non-volatile hydrocarbon fraction (F3: >C16-C34), was observed in VART5-15 and CST15 systems whereas the F3 fraction concentration remained virtually unchanged in 70 days duration of experiments in CST5 systems. The results indicate comparable performance of VART5-15 and CST15 systems in terms of biodegradation of petroleum hydrocarbons.

The reduction in petroleum hydrocarbon concentrations occurred largely as a result of biodegradation. Small scale soil microcosms amended with sodium azide to inhibit biological activity were set up and incubated at 15 ˚C. No $CO_2$ production was observed in these killed controls. After 70 days, no reduction in the F3 fraction concentration and less than 10%

reduction in F2 fraction hydrocarbon concentration were obtained. Thus, all of the F3 fraction reduction and the major portion of F2 reduction in the test systems are attributable to biodegradation, rather than abiotic losses such as volatilization.

There is limited information in the literature about the effects of short-term or seasonal temperature variations on biodegradation of hydrocarbons. Chang et al. evaluated the effect of typical summer site temperature patterns of 10-day cycles of temperature changes from 1 to 10 ˚C over two months, on the biodegradation of hydrocarbons in an aged contaminated sandy soil from Resolution Island, NU, Canada [16]. They reported that when soil was amended with nutrients, 63% and 53% reduction in concentrations of F2 and F3 fractions in 60 days was observed in the variable temperature mode (1 to 10 ˚C) compared to 36% and 21% reduction in F2 and F3 fractions in systems incubated at constant temperature of 6 ˚C. The higher biodegradation rates under incubation at variable temperatures was explained by the fact that biological activity, as measured by respiration, is exponentially increased with increasing temperature in the range of 5 to 10 ˚C. Eriksson et al. investigated the effect of 48 h freeze-thaw cycles on biodegradation rate of a hydrocarbon contaminated soil from a Canadian sub-arctic site and found 30% higher degradation rate in freeze-thaw treated systems (-5 ˚C to 7 ˚C) compared to the systems incubated at constant temperature of 7 ˚C [15].

The authors suggested that enhanced biodegradation under variable temperature mode was most likely attributed to the selection of certain populations of hydrocarbon degraders at the variable temperature regime. Biodegradation of spiked radiolabeled hexadecane in an Arctic soil sample with no added nutrients and subjected to freeze-thaw cycles (-5 ˚C to 5 ˚C) was enhanced in comparison to systems incubated at 5 ˚C in a study by Borresen et al. [14]. Furthermore, as the duration of each freeze-thaw cycles was decreased from 16 to 4 days during the 48-day

incubation period, higher hexadecane mineralization was observed. Overall, these studies show that biodegradation of petroleum hydrocarbons in cold regions soils are enhanced under variable temperature regimes compared to systems maintained at constant temperature equal to the upper limit or the mean of the temperature range.

In this study, the biodegradation extent in the VART5-15 system was very similar but not higher than that for the CST15. The calculated first order rate constants for VART5-15 systems was 0.0094+ 0.0004 d-1 ($r^2$=0.93) for F2 fraction, 0.0031$\pm$ 0.0003 $d^{-1}$ ($r^2$=0.62) for F3 fraction and 0.0052$\pm$ 0.0003 $d^{-1}$ ($r^2$=0.85) for TPH. The calculated rate constants are significantly lower than previously reported values in the literature (Paudyn et al., 2008; Chang et al., 2010), which is attributable to limited bioavailability of petroleum hydrocarbons in unsaturated clayey soil. Long contact time which may lead to adsorption of oil in micro-pores of clayey aggregates [22] reduces the availability of oil to soil microorganism. The limited bioavailability of hydrocarbons and presence of significant number of micro-pores in soil aggregates from this soil has been reported in our prior studies [23, 24].

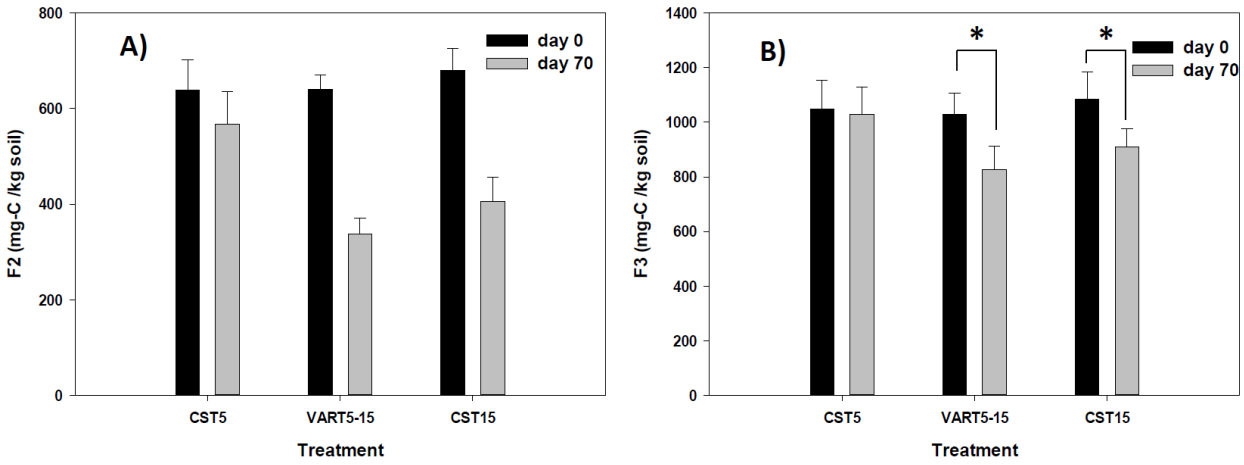


**Fig. 3.1.** The results of TPH analysis at day 0 and day 70 for different treatments of CST5 (constant incubation at 5 ˚C), VART5-15 (diurnal fluctuation between 5-15 ˚C) and CST15 (constant incubation at 15 ˚C). A) semi-volatile fraction (F2: >C10–C16) of hydrocarbons B) non-volatile fraction (F3: >C16–C34). The error bars represent the standard deviation of 9 different samples from 3 different systems. In (B) '*' indicates statistically significant difference (Two-way ANOVA - Tukey test, p value < 0.05)

### 3.2.2. Microbial Community Structure under Different Soil Treatments

The pyrosequencing results for 16S rRNA genes for soil samples taken from CST5, VART5-15, and CST15 systems are shown in Fig. 3.2A. Eight major phyla of soil microbial community (*Actinobacteria*, *Proteobacteria*, *Chloroflexi*, *Bacteroidetes*, *Acidobacteria*, *Verrucomicrobia*, *Firmicutes*, *Planctomycetes*) were detected, however, at Day 0 only the first 6 phyla contributed to more than 1% of total community population.

At Day 70, the microbial community in VART5-15 systems was more similar to community in CST15 systems and both were significantly different than the community of CST5 systems and Day 0 samples. At phyla level, the relative abundance of γ-*Proteobactera*, α–*Proteobactera*, *Verrucomicrobia*, *Bacteriodetes* and *Acidobacteria* was higher in the CST15 and VART5-15 systems compared to CST5 systems and at Day 0. However, the relative abundance of

*Actinobacteria* and *Chloroflexi* were lower in the CST15 and VART5-15 compared to the systems incubated at 5˚C (CST5) or Day 0 sample. The information in Fig. 3.2 shows the difference in shifts in communities from Day 0 for CST5 versus VART5-15 and CST15 systems over 70 days. Mair et al. has recently shown that Gram-negative hydrocarbon degrading soil populations were favourably enriched at 20 ˚C compared to 10 ˚C, whereas the Gram-positive population was not significantly affected by incubating temperature [17]. Chang et al. also studied the effect of seasonal freeze-thaw on microbial community of petroleum hydrocarbon contaminated soil with DGGE fingerprinting. They reported that a shift in the microbial community occurred as a result of change in temperature. In nutrient amended systems, during freezing two bands representing genera related to *Corynebacterineae* (member *of Actinobacteria* phylum) and *Rhodanobacter* (member *of* γ*-Proteobactera* phylum) appeared between days 40-60 of freezing periods. These genus disappeared with increasing temperature after about 60 days in thaw phase when another band related to *Alkanindiges* (member *of* γ*-Proteobactera* phylum) appeared [13].

As shown in Fig 3.2A, a significant portion (16.2% and 14.6%) of the microbial community in VART5-15 and CST15 belongs to *Verrucomicrobia* phylum and *Verrucomicrobiaceae* family compared to CST5 (2.9%) and Day 0 sample (1.4%). *Verrumicrobia* is commonly found in soil. Buckley et al. reported that *Verrumicrobia* are present in more than 90% of 16S rDNA clone libraries generated from soil microbial communities with average relative abundance of about 10% [25]. Similar to our study, the *Verrumicrobia* was found to be significantly enriched and presented as dominant phyla in microbial communities of soil samples taken from an alpine site in summer time, when the temperature changed between 4-15˚C in summer [26].

Among γ-*Proteobacteria* phylum, *Xanthomonadaceae* family and its major genus *Thermomonas* exhibited the highest increase in relative abundance in CST15 and VART5-15 compared to Day 0, whereas the relative abundance of *Xanthomonadaceae* family in CST5 was comparable to Day 0 sample (Table 3.1). The *Xanthomonadaceae* family and *Thermomonas* genus are closely related to *Pseudomonas* and *Xanthomonas* genera, which are well known hydrocarbon degraders. The presence of *Thermomonas* in hydrocarbon contaminated soil has been reported [27, 28]. Kaplan et al. reported that *Thermomonas* and *Rhodanobacter* were among dominant phylotypes during biodegradation of high molecular weight hydrocarbons in a sandy soil, and these observations are in agreement with our results. They also suggested that different bacterial phylotypes are associated with the extent of weathering or degradation of petroleum oils [28].

A significant reduction in abundance of *Actinobacteria* phylum and in particular the *Willimisia* genus was observed in CST15 and VART5-15 systems compared to Day 0, whereas the relative abundance of the *Actinobacteria* phylum and *Willimisia* genus were increased in CST5 after 70 days period of experiments. *Willimisia* is a recently identified genus and is very similar to *Gordonia*, several members of which are isolated from oil contaminated soils [29]. Some members of *Willimisia* genus have also been isolated from oil contaminated soils [30, 31]. Some members of this genus have been shown to degrade nitroaromatic compounds [32]. A significant decrease in relative abundance of *Actinobacteria* from winter to summer in soil microbial community from the alpine site was reported before [26].

In the *Chloroflexi* phylum, there was significant decrease in the relative abundance of *Anaerolinea* family in CST15, and VART5-15 systems compared to Day 0, whereas the relative abundance of this family was decreased in CST5 systems compared to Day 0. Sutton et al.

reported an increase in abundance of *Anaerolinea* in diesel contaminated soil samples taken from a contaminated site compared to background un-contaminated soil sample [33]. *Anaerolinea* are obligate anaerobes.

The results of *alkB* gene pyrosequencing in phyla level are presented in Fig 3.2B. Similar to 16S rRNA results, the community of species harboring *alkB* genes in VART5-15 systems at 70 days were closer to CST15, than CST5 systems. The *alkB* communities in all systems after 70 days were significantly different from that at Day 0. *Actinobacteria* phylum was abundant at all different temperature regimes at Day 70, and its relative abundance was higher in CST5 compared to CST15 and VART5-15 systems. The total reads of *Actinobacteria* phylum belonged to *Actinomycetales* at the order level in all three temperature regimes, the majority of which (63-73%) was further assigned to *Rhodococcus* at genus level. Also, 3 to 8% of total reads of *Actinobacteria* phylum in all three temperature regimes were assigned to the *Dietziaceae* family (*Dietzia* genus).

Species harbouring *alkB* genes from γ-*Proteobactera* phylum emerged in the CST15 and VART5-15 systems. While 67-70% of assigned reads to *Proteobactera* phylum was attributed to γ-*Proteobactera* class in case of CST15 and VART5-15, none of the reads from CST5 sample were assigned to this class. Moreover, 25-30% of reads assigned to γ-*Proteobactera*, matched γ-*Proteobactera* HdN1 at the species level in VART5-15 and CST15 systems, which is an anoxic strain capable of utilizing a range of alkanes including high molecular weight alkanes up to C30 (Zedelius et al., 2011).

The non-metric multidimensional scaling plot of similarities, calculated based on Bray-Curtis measure, between different communities at genus level for 16S rRNA results and OTUs of

*alkB* results is shown in Fig. 3.2C. This plot shows a significant difference in community between CST5 and CST15 samples as well a high similarity between VART5-15 and CST15. Higher similarity between VART5-15 and CST15 microbial community on the basis of 16S rRNA and *alkB* genes implies that effect of increasing temperature on the microbial community dynamics is more influential than effects of decreasing the temperature. Pettersson et al. reported significant shifts in the PLFA profile of the microbial community with increasing incubation temperatures, when incubated for 80 days at fixed temperatures ranging from 5 to 30$^{o}$C [34].

Similar to our observation, they reported relatively small effects on PLFA profiles when the incubation temperatures were decreased. Those authors suggested that the change in PLFA pattern is more likely linked with bacterial phenotypic plasticity with temperature (change in fatty acid composition of bacteria) rather than change in species composition. However, our results clearly demonstrate the shift in community in terms of change in species composition over time when subjected to diurnal changes in temperature.

The calculated biodiversity indices for different treatments are presented in Table 3.2. The results indicated significantly lower diversity in CST5 systems both in terms of 16S rRNA and *alkB* genes compared to CST15 and VART5-15 systems. Dell'Anno also reported higher microbial diversity in marine sediments incubated under aerobic condition at 35˚C compared to 20˚C [35]. In our study the higher biodiversity is associated with higher biodegradation extent, suggesting that a more diversified community may be more effective for biodegradation of aged petroleum hydrocarbons in the site soils, which could be explained by complementary mechanism of contribution of bacterial community richness to community functionality [36].

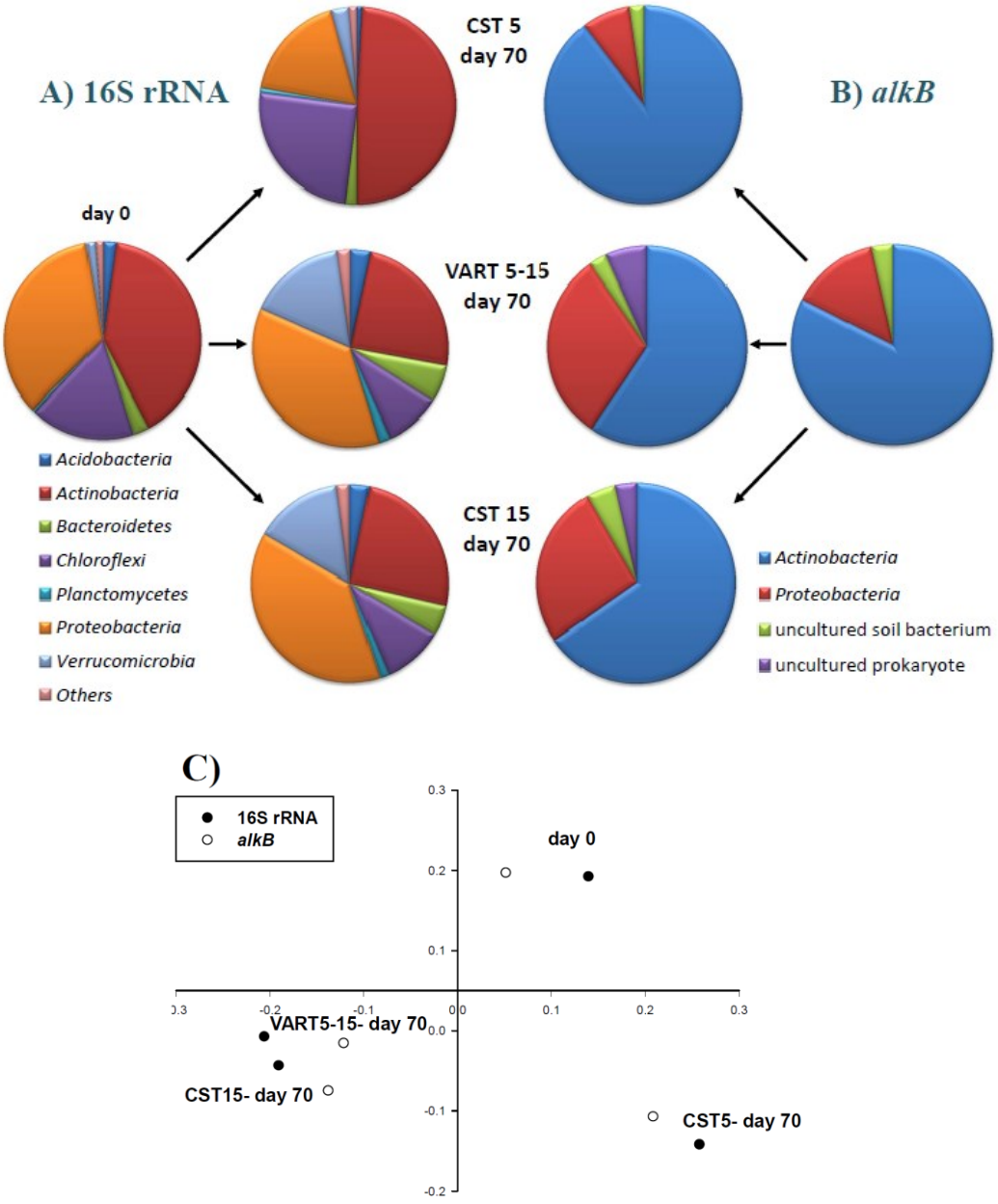


**Fig. 3.2.** Overall phylogenetic diversity of bacteria from day 0 and day 70 of CST5 (constant incubation at 5 ˚C), VART5-15 (diurnal fluctuation between 5-15 ˚C) and CST15 (constant incubation at 15 ˚C) for A) 16S rRNA gene diversity and B) *alkB* sequences. Phyla with more than 1% abundance have been presented in the graph. C) Non-metric multidimensional scaling (NMDS) plot showing the effect of different treatments on bacterial community structure based on 16S rRNA gene and *alkB* gene results

**Table 3.1.** Major bacterial families identified based on 16S rRNA results with relative abundance of more than 1% at day 0 and day 70 of different systems of CST5 (constant incubation at 5 ˚C), VART5-15 (diurnal fluctuation between 5 to 15 ˚C) and CST15 (constant incubation at 15 ˚C) along with the most commonly identified genera in each family**.**

| **Family** | **Affiliation** genera | **d0** | **CST5, d70** | **VART5-15, d70** | **CST15, d70** |
|---|---|---|---|---|---|
| ***c__Chloracidobacteria*** | ***Acidobacteria*** | ***0.9*** | ***0.4*** | ***1.9*** | ***2.5*** |
| ***Actinomycetales;Other*** | ***Actinobacteria*** | ***5.0*** | ***7.0*** | ***1.8*** | ***1.4*** |
| ***Dietziaceae*** | ***Actinobacteria*** | ***1.4*** | ***0.8*** | ***0.1*** | ***0.2*** |
| | *Dietzia* | 1.4 | 0.8 | 0.1 | 0.2 |
| ***Intrasporangiaceae*** | ***Actinobacteria*** | ***2.1*** | ***4.7*** | ***3.1*** | ***3.0*** |
| ***Microbacteriaceae*** | ***Actinobacteria*** | ***6.9*** | ***3.8*** | ***2.2*** | ***2.1*** |
| | *Agromyces* | 2.5 | 1.2 | 1.6 | 1.2 |
| | *Salinibacterium* | 2.8 | 2.4 | 0.3 | 0.7 |
| ***Micrococcaceae*** | ***Actinobacteria*** | ***0.7*** | ***1.8*** | ***1.1*** | ***0.9*** |
| | *Arthrobacter* | 0.6 | 1.8 | 1.1 | 0.9 |
| ***Mycobacteriaceae*** | ***Actinobacteria*** | ***4.1*** | ***3.1*** | ***5.1*** | ***4.8*** |
| | *Mycobacterium* | 4.1 | 3.1 | 5.1 | 4.8 |
| ***Nocardiaceae*** | ***Actinobacteria*** | ***2.7*** | ***4.1*** | ***1.4*** | ***1.3*** |
| | *Rhodococcus* | 2.6 | 4.1 | 1.4 | 1.3 |
| ***Nocardioidaceae*** | ***Actinobacteria*** | ***1.1*** | ***1.4*** | ***0.6*** | ***1.2*** |
| ***Williamsiaceae*** | ***Actinobacteria*** | ***13.5*** | ***19.5*** | ***7.0*** | ***8.7*** |
| | *Williamsia* | 13.5 | 19.5 | 7.0 | 8.7 |
| ***Chitinophagaceae*** | ***Bacteroidetes*** | ***1.3*** | ***0.6*** | ***4.6*** | ***3.9*** |
| ***Anaerolinaceae*** | ***Chloroflexi*** | ***9.4*** | ***12.5*** | ***4.2*** | ***5.3*** |
| | *Anaerolinea* | 3.8 | 10.0 | 3.2 | 3.7 |
| | *T78* | 4.4 | 1.2 | 0.6 | 0.9 |
| ***Pirellulaceae*** | ***Planctomycetes*** | ***0.1*** | ***0.3*** | ***1.1*** | ***1.0*** |
| ***Comamonadaceae*** | ***Betaproteobacteria*** | ***11.8*** | ***1.6*** | ***5.6*** | ***4.0*** |
| | *Acidovorax* | 6.0 | 0.4 | 1.9 | 0.9 |
| | *Polaromonas* | 2.1 | 0.2 | 1.3 | 1.0 |
| ***Haliangiaceae*** | ***Deltaproteobacteria*** | ***1.4*** | ***0.1*** | ***0.8*** | ***0.6*** |
| ***Alteromonadaceae*** | ***Gammaproteobacteria*** | ***3.2*** | ***0.5*** | ***6.4*** | ***6.0*** |
| | *HB2-32-21* | 3.1 | 0.5 | 6.3 | 6.0 |
| ***Ectothiorhodospiraceae*** | ***Gammaproteobacteria*** | ***1.1*** | ***1.0*** | ***0.3*** | ***0.6*** |
| ***[Gammaproteobacteria_PYR10d3]*** | ***Gammaproteobacteria*** | ***1.1*** | ***0.9*** | ***2.1*** | ***2.3*** |
| ***Xanthomonadaceae*** | ***Gammaproteobacteria*** | ***10.0*** | ***9.6*** | ***13.2*** | ***18.5*** |
| | *Thermomonas* | 7.8 | 9.1 | 11.2 | 16.4 |
| ***Verrucomicrobiaceae*** | ***Verrucomicrobia*** | ***0.9*** | ***0.8*** | ***12.2*** | ***10.4*** |

**Table 3.2.** Microbial diversity indices of samples of day 0 and day 70 of different systems of CST5 (constant incubation at 5 ˚C), VART5-15 (diurnal fluctuation between 5-15 ˚C) and CST15 (constant incubation at 15 ˚C) calculated based on 16S rRNA results at genus level and *alkB* clustered OTUs by RDP.

| | 16S rRNA | | | | *alkB* | | | |
|---|---|---|---|---|---|---|---|---|
| | day 0 | CST5,d70 | VART5-15,d70 | CST15,d70 | day 0 | CST5,d70 | VART5-15, d70 | CST15,d70 |
| **Richness** | 141 | 124 | 145 | 132 | 314 | 294 | 350 | 362 |
| **Shannon's index** | 3.73 | 3.35 | 3.74 | 3.56 | 4.48 | 4.03 | 4.54 | 4.61 |
| **Simpson's index** | 0.04 | 0.08 | 0.05 | 0.06 | 0.03 | 0.06 | 0.03 | 0.03 |
| **Shannon's true diversity** | 41.73 | 28.55 | 42.26 | 35.26 | 88.08 | 56.08 | 93.31 | 100.53 |
| **Simpson's true diversity** | 22.30 | 13.27 | 21.01 | 16.80 | 30.19 | 17.78 | 31.86 | 32.15 |
| **Shannon's evenness** | 0.75 | 0.70 | 0.75 | 0.73 | 0.78 | 0.71 | 0.77 | 0.78 |
| **Simpson's evenness** | 0.16 | 0.11 | 0.14 | 0.13 | 0.10 | 0.06 | 0.09 | 0.09 |

The cumulative $CO_2$ production in different microcosms incubated at different temperature regimes are shown in Fig. 3. Some $CO_2$ is likely produced from degradation of the background organic matter which was 2.3% w/w in addition to that from degradation of the petroleum hydrocarbons. There was significant difference in respiration activity between CST15 and CST5 systems, which is attributable to higher respiration activity and growth ($3.47\times10^7$ CFU/g for CST15 versus $2.20\times10^6$ CFU/g for CST5) at higher temperatures [37]. Comparable biodegradation of petroleum hydrocarbons was observed in the CST15 and VART5-15 systems (Figure 1), although respiration activity was higher in CST15. This suggests that the similar *alkB* community composition, rather than microbial metabolic activity levels was more directly linked to petroleum hydrocarbon degradation. The direct influence and significance of soil microbial community on petroleum hydrocarbon biodegradation has also been reported for a diesel-contaminated soil from an arctic site [38].

In this study, significantly higher relative abundance of γ- *proteobacteria* phylum was observed in the VART5-15 and CST15 systems, and indicates that members of this phylum are more responsive to higher temperatures. The enrichment or appearance of this phylum among *alkB* gene harbouring community is associated with significant biodegradation of higher molecular weight hydrocarbons. In contrast, the *Actinobacteria* phylum, the other hydrocarbon degrading phylum in the site soils, appeared to be well adapted to all temperature regimes. Moreover, similar microbial communities in CST15 and VART5-15 systems, implies that at least for the site soil in this study, the upper limit temperature of the variable temperature regime regulated the composition of the soil microbial community. Our findings have significant environmental implications for understanding the effect of diurnal or short-term temperature changes on the performance of on-site bioremediation technologies. For example, often soils are heated in cold regions during biopile remediation of hydrocarbon contaminated soils [39]. Results of this study suggest that comparable remediation efficiency could be achieved by short cycles of heating rather than the continuous heating of soil piles.

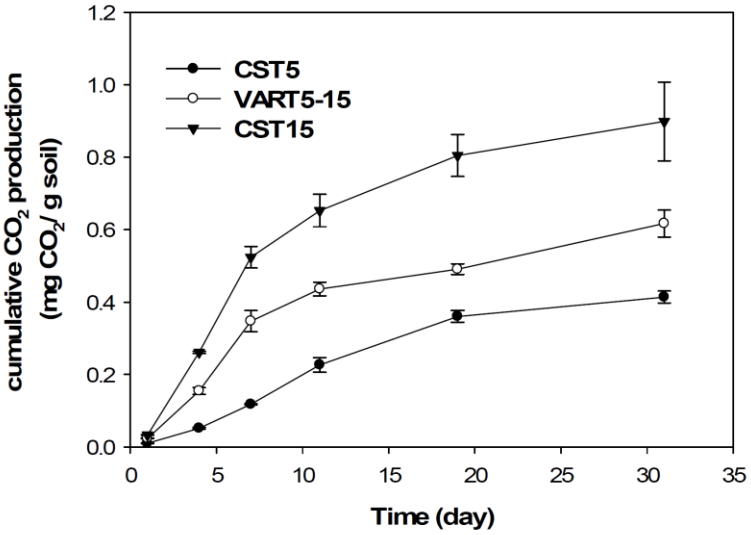


**Fig. 3.3.** The cumulative soil respiration of different systems as CST5 (constant incubation at 5 ˚C), VART5-15 (diurnal fluctuation between 5-15 ˚C) and CST15 (constant incubation at 15 ˚C). The error bars represent the standard deviation of 9 measurements from 3 different bioreactors.

## 3.3. EXPERIMENTAL PROCEDURE

### 3.3.1. TPH Biodegradation Experiments

The weathered petroleum hydrocarbon contaminated soil was shipped frozen from a subarctic site in Northwestern Territories (NWT) and was incubated in cold room at -4 ˚C prior to experiments. The clay, silt, and sand fraction of the soil were about 34%, 33% and 32% respectively, and initial TPH concentration was about 1600 mg-C/kg soil. The detailed information of soil physical and chemical properties has been reported elsewhere [23]. There were significant levels of background organic nitrogen (1400 mg-N/kg soil), inorganic and organic phosphorous (593 mg-P/kg soil as total phosphorus and 367 mg-P/kg soil as inorganic phosphorus) as well as micro nutrients (calcium: 42 g/kg soil, iron 18 g/ kg soil, magnesium 12 g/kg soil) in the soil. Significant numbers of heterotrophic bacteria ($2.93\times10^6$ CFU/g) as well as hydrocarbon degrading bacteria ($1.72\times10^6$ CFU/g) were present in soil as determined by plate counts with R2A agar plates and Bushnell Hass plates amended with diesel. In a preliminary study we have shown that the TPH biodegradation rate was not enhanced by nutrient (ammonium nitrate and diammonium phosphate as sources of nitrogen and phosphorus) amendment (data not shown).

The typical hourly averaged air temperature profile of the site is presented in Fig. 3.4, which shows diurnal temperature changes from 5 to 15 ˚C. The TPH biodegradation experiment was conducted at microcosm scale with well-sealed 1 L glass jars which were sterilized prior to experiments. Each microcosm contained 600 g of site contaminated soil. Microcosms representing each temperature regime were prepared in triplicate. Two sets of microcosms were incubated at constant temperatures of 15 ˚C and 5 ˚C inside incubators, and microcosm subjected to variable temperatures from 5 to 15 ˚C were maintained inside a cold room which was

equipped with programmable temperature controller. The temperature program involved a temperature increasing step with the rate of 1 ˚C/h from 5 ˚C to 15 ˚C, followed by incubation at 15 ˚C for two hours, and then a temperature decreasing step at the rate of 1 ˚C/h to 5 ˚C and finally, incubation at 5 ˚C for 2 hours.

The moisture content in all sets was adjusted at 19% (about 60% of soil water holding capacity) by weight and remained constant during the experiment. Samples were taken from microcosms periodically for further TPH or community analysis. At each sampling event, samples were taken using sterile techniques from different depths of soils of each microcosm, and then composited. Three replicates from each composite sample were analysed for TPH content (totally 9 samples for each set).

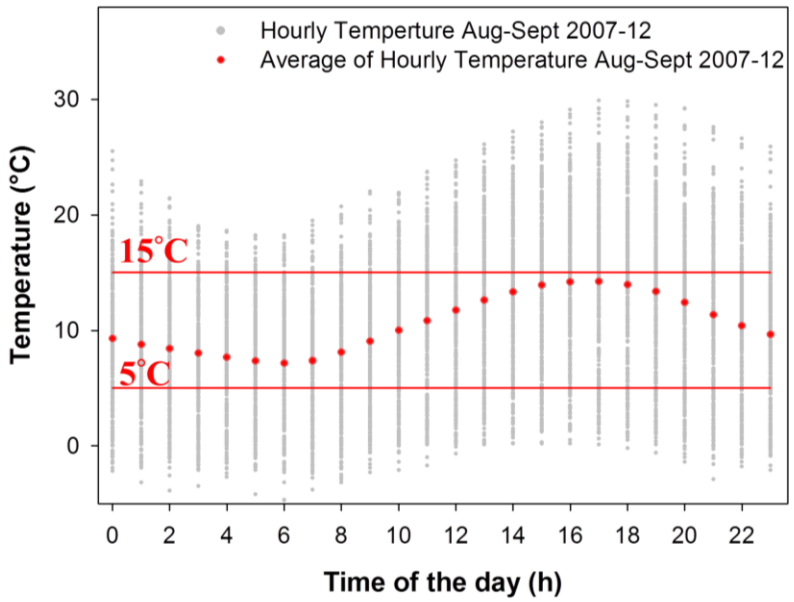


**Fig. 3.4.** The hourly temperature of the air at the site for different days of the month of August and September 2007-2012 (gray diamonds). The red line represents the average of hourly data of different days of the month. Temperature data was obtained from Environment Canada.

### 3.3.2. TPH Analysis

The TPH analysis were done according to Canadian-wide Standard method (CCME) method [40]. The procedure has been reported in detail elsewhere [23]. Briefly, soil samples were extracted with organic solvent using automatic Soxtherm (Gerhardt) at high temperature and under pressure. The extracted organic phase was later passed through a cleanup column containing silicagel and sodium sulphate to remove the water and soil natural organic matter. After concentration of the samples under $N_2$ blow down systems, the samples were injected in a GC (Agilent 6890) equipped with DB1column and FID detector and the concentration of semi-volatile fraction, (F2, >C10-C16) and non-volatile fraction (F3, >C16-C34) were determined. o-terphenyl was added as surrogate to all samples prior to analysis to assess the extraction efficiency. A clean sample was included in all extraction sets to verify any contamination from the extraction procedure.

### 3.3.3. Microbial Community Analysis

Genomic DNA was extracted from the soil samples using the commercial DNA extraction kit (PowerSoil DNA isolation kit, MO-BIO Laboratories, Carlsbad, CA). The extracted DNA was then amplified separately by PCR with both 16S rRNA and *alkB* gene primers containing unique multiples identifier (MID) tags. 16S rRNA primers were a mixture of 3 forward primers namely 5′-CCTACGGGRGGCAGCAG-3′, 5′-ACWYCTACGGRWGGCTGC-3′ and 5′-CACCTACGGGTGGCAGC-3′ targeting the hyper variable V3 region (*E.coli* position = 338) and 1 reverse primer, 5′-TACNVGGGTHTCTAATCC-3′ targeting the hyper variable region V4 (*E.coli* position = 802). These set of primer has proved accepted recovery for DNA of bacterial group with high G-C content [41]. Cycling conditions for 16S rRNA gene amplifications

involved initial 5 denaturing step at 94 ˚C followed by 30 cycles of 94 ˚C for 1 min, 30 s at 55 ˚C, 1.5 min at 72 ˚C and a final extended elongation at 72˚C for 8.5 min. *alkB* genes were amplified by degenerate primers *alkB*-1f (5′-AAYACNGCNCAYGARCTNGGNCAYAA-3′) and *alkB*-1r (5′-GCRTGRTGRTCNGARTGNCGYTG-3′) [42]. Cycling conditions for *alkB* gene was as follow: 5 min denaturing step at 95 ˚C, 35 cycles of 30 s at 95 ˚C, 30 s at 55 ˚C, and 30 s at 72 ˚C, and a final elongation step of 15 min at 72 ˚C. PCR reaction mixture for *alkB* included: 20 µl Ultrapure, DNAse free, RNAse free water (Invitogen), 80 pmol of *alkB* primer (IDT), 4 µl of 2.5 mM deoxynucleoside triphosphates (Invitrogen), 2.5 µl of 50 mM $MgCl_2$ (Invitrogen), 0.5 U of Taq DNA polymerase (Invitrogen) and 5 µl of the 10x Taq DNA polymerase buffer. To remove impurities amplicons were then purified by MO-BIO UltraClean PCR Clean-UP Kit. The concentration of amplicons were then determined with Quant-iT™ PicoGreen kit, and finally the quality of samples were examined by Bioanalyzer 2100 (Agilent Technologies). The purified samples were then submitted to McGill Genome Center for sequencing with GS FLX Titanium machine.

Analysis of sequences was performed with Qiime pipeline [43], which includes initial trimming and removing sequences with quality score less than 25 as well as sequences with 200-2000 bp length. 16S rRNA sequences were clustered at 97% similarity with UCLUST. RDP classifier was used for taxonomic assignment.

After initial trimming, *alkB* sequences were submitted to the rdp fungene pipeline for further processing including framebot, alignment and clustering. Diversity indices were calculated based on OTUs defined at 97% similarity. For taxonomic assignment, the trimmed sequences were compared to NCBI non-redundant protein sequence database using the Basic Local Alignment Search Tool (blast) for translated nucleotide queries (blastx, NCBI). The blastx was used instead

of nucleotide blast (blastn) since protein hits are better preserved and leads to higher hits [44] with blastx. The blastx results were then exported to Megan software version 3.7 [45] for taxonomic assignment with default parameters.

### 3.3.4. Respiration Experiments

Soil respiration was conducted in microcosm scale with 10 g soil in 40 mL amber vials equipped with gas tight valve and sampling tubes and incubated at the same temperature regimes as TPH biodegradation experiments. CO2 measurement was conducted at different sampling points with Micro-GC (3000 Agilent) equipped with PLOT U column and a TCD detector. After triple measurements, headspace and soil air void was purged with sterilized air. Air was sterilized by passing through filters with 0.4 μm pore sizes.

## 3.4. ACKNOWLEDGMENTS

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# Chapter 4

# The Effect of Soil Pore Size on Biodegradation of High Molecular Weight Hydrocarbons

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**Connecting text:**

In Chapter 2, pilot scale biopile experiments were performed for a clayey petroleum hydrocarbon contaminated soil over 385 days to determine the remediation endpoint. To better understand the role of micro-structure of clayey soil and particularly the role of pore size, well-controlled bioreactor experiments were conducted with different pore sizes. Moreover, the in-situ aggregate micro-structure of representative contaminated clayey and sandy soils were characterized using micro-CT scanning. The information obtained from CT scanning was used for interpretation of biodegradation experiments results, partly presented in Chapter 2.

The results of this research will be submitted for publication as: Akbari A., Ghoshal S. "The Effect of Soil Pore Size on Biodegradation of High Molecular Weight Hydrocarbons"

## ABSTRACT

Bioremediation in clayey soils is known as a challenging task mainly due to large number of micro-pores in clayey aggregates. In this study, we investigated the role of pore size on biodegradation of hexadecane as a model of effectively insoluble NAPL component by *Dietzia maris*, a model hydrocarbon degrader isolated from a clayey hydrocarbon contaminated soil, in specially designed bioreactors with fixed oil-water interfacial areas fitted with membranes with different pore sizes. The results indicated that in bioreactors fitted with membranes with pore sizes of 3 μm or 0.4 μm no biodegradation occurred, whereas, significant and comparable biodegradation was observed in bioreactors fitted with 12 μm membrane and bioreactors without membrane. We further examined the implications of findings from pore experiments in terms of bioremediation effectiveness in the contaminated site by characterizing the clayey aggregate micro-structure and in particular "bioaccessible porosity" of representative aggregates by micro-CT scanning followed by image analysis. We define the term of "bioaccessible porosity" as portion of aggregate volume attributed to pores with diameters larger than 4 μm, for *D. maris*. The CT results indicated that significant portion of soil aggregate volume was attributed to bioaccessible pores inside the aggregates. The "bioaccessible porosity" was 26-27% in clayey aggregates and was comparable to 24% in case of aggregates from another site with sandy soil which was used for comparison. This can explain significant extent of biodegradation of non-volatile petroleum hydrocarbons as observed in biodegradation experiments of clayey (50% degradation) and sandy (65% degradation) soils. We propose the employed approach as a useful tool to estimate the "bioaccessible porosity" in contaminated soils as well as predicting the bioremediation effectiveness.

## 4.1. INTRODUCTION

Hydrocarbon compounds are utilized by bacteria through a variety of mechanisms. Most commonly, hydrocarbons are degraded by bacteria when they are dissolved in the aqueous phase. For hydrocarbons that have very low aqueous solubility (e.g., high molecular weight aliphatic hydrocarbons), bacterial degradation involves direct uptake of oil phase hydrocarbons at the oil-water interface through contact between the bacterial cell and the oil-water interface. Furthermore, some bacterial strains can produce biosurfactant compounds that enhance the solubility of hydrocarbons in the bulk aqueous phase through micellar solubilisation and/or emulsification and direct uptake of emulsions by bacteria. These mechanisms are three major uptake modes for hydrocarbons in non-aqueous phase liquids (NAPL) such as petroleum oils [1].

For NAPL components of low aqueous solubility, the importance of diffusion distance of hydrocarbons from NAPL phase to bacteria increases with decreasing compound solubility. Adhesion of the bacterial cell to the oil-water interfacial area can increase the uptake rate by decreasing the diffusion distance, even when there is no direct uptake [2]. For sparingly soluble polycyclic aromatic hydrocarbon (PAH) such as anthracene and phenanthrene, when present as components of NAPLs it has been reported that biofilm formation or bacterial cell-NAPL adhesion occur during biodegradation, as well as, the extent of biofilm formation on PAH crystals was inversely related to PAH solubility [3]. MacLeod and Daugulis studied the biodegradation of pyrene and phenanthrene in two-phase partitioning bioreactor (TPPB) by ***Mycobacterium*** PYR-1 strain and found that biodegradation involved adhesion of the cell on the organic side of the oil-water interface [4]. When NAPL compounds are entrapped in soil micro-pores which are smaller than bacterial size, interfacial uptake and consequently bioavailability of less soluble fractions of NAPL (such as PAHs) and essentially insoluble

fractions of NAPL (such as high molecular weight aliphatic hydrocarbons) would be severely limited. This highlights the importance of soil pore network characteristics on the extent of bioremediation of less soluble fraction of petroleum hydrocarbons.

Aged NAPL is mainly composed of high molecular weight, less soluble, non-volatile compounds, and are more viscous than fresh NAPL. Moreover, it has been shown that with prolonged contact with soils, NAPLs can migrate into micro-pores of clayey soils (kaolinite) by altering the wettability of soil mineral domains through a multi-step process which starts with binding of polar fraction of oil with soil mineral [5]. This is demonstrated by Karimi-Lotfabad and Gray, who used confocal laser scanning microscopy to show that NAPLs are mainly associated with clay fractions of soils and fills the micro-pores (< 5 μm) inside the aggregates of kaolinite particles [6].

Different approaches have been employed for characterization of soil pore network micro-structure such as microscopic-imaging of resin-impregnated soil sections [7], gas adsorption based methods or X-ray scanning. Sectioning based methods suffer from being time consuming, limited number of sections which could be imaged, perturbation of the aggregate structure and destructive testing, and relatively large thickness of sections and thus limited 3-D characterization. Micro scale computed tomography (Micro-CT) has been successfully employed as a non-destructive three-dimensional method to study the pore structure of soil aggregates. A combination of scanning and appropriate image analysis procedure provide information on distribution, length, connectivity as well as neck diameter of pores inside the aggregate.

In this study, we examined the effect of pore size on biodegradation of a model NAPL compound in the carefully designed bioreactors with fixed NAPL-water interface separated from

microbial solution by membranes with specific pore diameters. The environmental implications of findings from the cell experiments were later evaluated by characterizing the *in-situ* pore network of aggregates from a hydrocarbon contaminated sites. More than 85% of total identified bacterial community of the site was belong to *Corynebacterineae* sub-order and so, the *Dietzia maris*, a strain isolated from the same site was used as representative of this sub-order in the cell experiments. The specific design of the cells allowed the investigation of the role of pore diameter on biodegradation rates and extents. A few studies have evaluated the effect of intra-aggregate pore diameter on biodegradation of hydrocarbons with artificially contaminated beads [8, 9]. Since the pore size of the beads span over a wide range and moreover some NAPL will coat the surface of beads rather than inside the pores, we designed the reactors with membranes. The pore network of representative aggregates from these two soils were characterized with a combination of micro-CT scanning and BET analysis. BET analysis was used to characterize the nano-range pores in soil aggregates, which ae smaller than maximum attainable resolution by micro-CT scanning, whereas CT-scanning provides the characteristics of fraction of pore network which are larger than bacteria size. We propose the employed approach as a useful tool to predict the effectiveness of bioremediation in different soils, by direct characterization of the "bioaccessible porosity" of the aggregates.

## 4.2. MATERIAL AND METHODS

### 4.1.1. Bioreactor Experiments Using Different Pore Size Membranes

Bioaccessibility of bacteria to a hexadecane layer through different pore sizes, and subsequent biodegradation of hexadecane (2.5×104 dpm/µL), as a representative insoluble NAPL compound, was assessed in specially designed bioreactors. The schematic of the bioreactor is presented in Fig. 4.1, and shows a vial fitted with membranes of specific pore sizes that separate

the oil-water interface in the top chamber from the bacterial suspension in the bottom chamber. Nucleopore membranes with pore sizes of ~12 μm, 3.0 μm, and 0.4 μm were used. Nucelopore membranes have channel like pores with uniform diameter. The pore density of the membranes with 12 μm, 3.0 μm and 0.4 μm pore diameter were 1.1×105, 2×106, and 1.01×108 pores/cm2 , respectively. The membrane thickness was approximately 9 μm.

A bacterial pure culture, *Dietzia maris* (*Dietzia maris*; CA160; GQ870425 (99%)), isolated as major hydrocarbon degrader from a weathered petroleum hydrocarbon contaminated clayey soil from a sub-arctic site in Northwest Territories (NWT, Canada) and capable of using hexadecane as a sole carbon source was used to inoculate the bioreactors. The cells were coccus shaped with low motility in terms of both swimming motility (migration diameter in 0.3% agar plates: 11 mm) or swarming motility (migration diameter on 0.5% agar plates: 7 mm). Details on the preparation of the bacterial culture are provided in the Supporting Information. An inoculum of washed cells ($1.5\times10^8$ CFU/mL) in 13 mL Bushnell-Haas mineral medium [10] was introduced into the lower chamber of bioreactor through the side arm. After inoculation, 40 mL of hexadecane containing $10^6$ dpm of $^{14C}$-1, 2, hexadecane was introduced at the air-water interface of the top chamber of the bioreactor. Sterile techniques were used in handing the bioreactors and experiments were repeated three times.

The biomineralization kinetics of hexadecane was determined by monitoring the $^{14C}CO_2$ production rate. A headspace trap containing 2 mL of 2 M NaOH solution attached to the upper chamber of the bioreactor as shown in Fig. 4.1, was periodically sampled to determine the amount of $^{14C}CO_2$ produced. Further details are provided in the Supporting Information section. At the end of each set of experiments, $^{14}C$ mass balance was performed by sampling from NAPL phase, and aqueous phase, and accounting for the $^{14}CO_2$ produced, and more than 90% of initial

activity was recovered in all systems confirming that there were no significant abiotic losses of hexadecane.

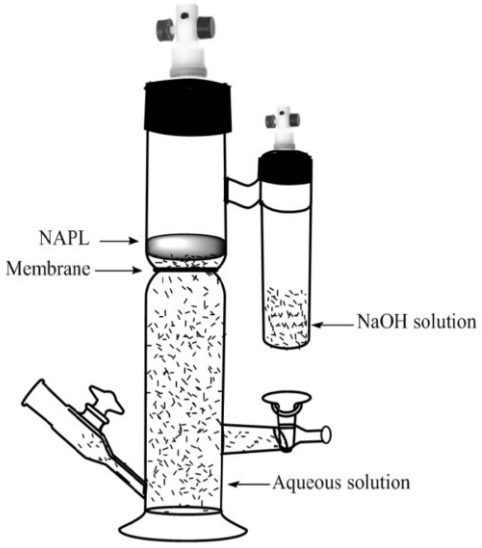


**Fig. 4.1.** The schematic of bioreactors with fixed oil-water interfacial areas fitted with membranes with different pore sizes, the aqueous phase was bacterial suspension in mineral nutrient solution without carbon source. Hexadecane was used as model NAPL phase.

### 4.1.2. Micro-CT Scanning of Soil Aggregates

A high resolution micro X-ray CT scanner (SkyScan 1172) was employed to delineate the interior architecture of several soil aggregates and to characterize their pore size distributions and pore volumes. Representative air-dried aggregates were carefully fixed in pipette and were mounted on the turning base inside the micro-CT. The average intensity of X-ray transmission was adjusted at 50% by tuning the voltage and exposure time at desired resolution. The resolution of acquired images was in the range of 0.9-3.28 μm, depending on the size of aggregate. A 0.5 mm aluminum filter was used to reduce scanning artefacts arising from beam hardening, which results from preferential absorption of low energy X-rays occur in the outer layer of scanning object, leading to a distorted image of the object. During scanning, the sample

was rotated over 360˚ at 0.28˚ step intervals, and at each angular position, at least 3 images were obtained. The acquired projections were reconstructed using NRECON software (SkyScan).

### 4.1.3. Image Analysis

To extract the pore network of soil aggregates, the reconstructed 2D images should be segmented into two populations as the object (soil) and pores (air). In case of natural porous media, segmentation is particularly complex in case of clayey soil aggregates. These aggregates are extremely heterogeneous and are composed of soil constituents of various densities, such as organic material and clay minerals. Moreover, the resolution of the image is in the same order of magnitude of clay particles, which can lead to pixels with intermediate gray scale values. Thus, thresholding is a crucial step in image analysis procedure and suggest that simple global single thresholding methods cannot adequately address this issue.

Indicator kriging (IK) is a thresholding method which is based on minimizing the spatial variance of the indicator kriging to segment the image [11]. In a survey of several common segmentation methods, Wang et al. found that IK method produced the closet binarized image to ground-truth when the histogram of gray scale image had distinguishable peaks [12]. The algorithm involves four major steps of initial threshholding, kiriging and two filtering steps and starts with partial assignment of pixels to the background and the object using two low and high threshold values $(T_0, T_1)$, which is followed by kriging and two filtering steps. The method is quite sensitive to the threshold values. Two different automated methods (Entropy method and bi-normal mixture method) have been originally proposed to determine the low and high threshold values [13], however we obtained better results when we manually adjusted the thresholded values.

After initial thresholding, in the second pass, the rest of the population was segmented based on kriging. Based on Equation 1 the probability of the pixel belonging to the any of two populations in a circular window with fixed radius of 3 pixels was calculated. In the equation, $P(T_i; x_0 \mid n)$ is the probability of the unknown pixel belonging to the population $\pi_i$, $i$ is the indicator variable, $\lambda_\alpha$ is the weight assigned to pixels in the kriging window, $x_o$ denotes the spatial location of an unclassified pixel and $x_\alpha$ represent the neighbouring pixels. The weights were determined by solving a set of constrained equations of ordinary kriging system formed based on semi-variogram model.

$$P(T_i; x_0 \mid n) = \sum_{\alpha=1}^{n} \lambda_\alpha (T_i; x_0) i(T_i; x_0) \qquad i = 0,1$$

Other than initial thresholding and kriging steps, two further filtering steps were employed as part of IK method for removing uncorrelated isolated noises inside the aggregate pores. These steps known as majority filtering are applied before and after the kriging step to those pixels whose population is assigned in thresholding step. The majority filtering window ignores unclassified pixels and is centred only on previously thresholded pixels. If the majority of the pixels in the window belong to the opposite population then the assignment of the pixel is reversed. To preserve micro-pores as a characteristic of the fine texture of soil, majority filtering was applied only to those pixels initially assigned as object.

Furthermore, we developed a multi-step procedure to distinguish the intra-aggregate porosity from surrounding air of the object while retaining the whole aggregate structure and to remove noise outside the aggregate. Firstly, each image was thresholded by a single low global threshold value to retain the object pixels as much as possible, and then in order to remove the

noise from thresholded image, objects smaller than 0.01% of largest aggregate body were removed. The value of 0.01% was chosen based on maximum size of noises observed in air surrounding the aggregate body in the thresholded images. Next, the holes inside and in between of major object components was filled as solid object. Then the alpha-shape of the aggregate body was determined. The alpha-shape as introduced by Edelsbrunner et al. is a formal shape of point sets by sculpting Delauny triangulation [14]. The alpha value (1/maximum radius of triangles) was set at 0.01 which found to be optimum value in terms of providing both representative boundaries as well as computing time. The alpha-shape with alpha value of 0, (radius of infinity) represents the convex-hul of an object. To minimize any artifacts arising from edge determination and specifically to avoid accounting the outer aggregate porosity as intra-aggregate porosity, the resulted alpha-shape was shrunk to 95% of original polygon and then new polygon was applied as a mask to the IK binarized image. Fig. 4.2 presents an example of a reconstructed image and the processed image with the above mentioned procedure. The pore network information was extracted from these thresholded 2-d images assuming each image represents the information of $\pm$ ½ pixel height from aggregate. An advantage of applying this sequence of processes is to preserve the whole aggregate structure rather than considering just a core of aggregate as several previous studies [15, 16]. Considering just the core of aggregate may significantly bias the calculated porosity as the core of aggregates is usually denser than outer layers of aggregate. This was further confirmed by the fact that the calculated porosity for 100 consecutive slices of aggregate varied from 37% to 16% from top (outer layer of aggregate) to the middle (central cross section of aggregate) layers of aggregate. Moreover, the developed image analysis procedure provides precise characterization of open pores versus closed pore pockets inside the aggregate, as well as connectivity of pore network. The image analysis

processes were programmed with Matlab. The analysis was performed using *Guillimin* cluster at the CLUMEQ HPC centre, a part of Compute Canada High Performance Computing facilities. The volume rendering and 3-D representation of soil aggregate structure was performed by Paraview [17].

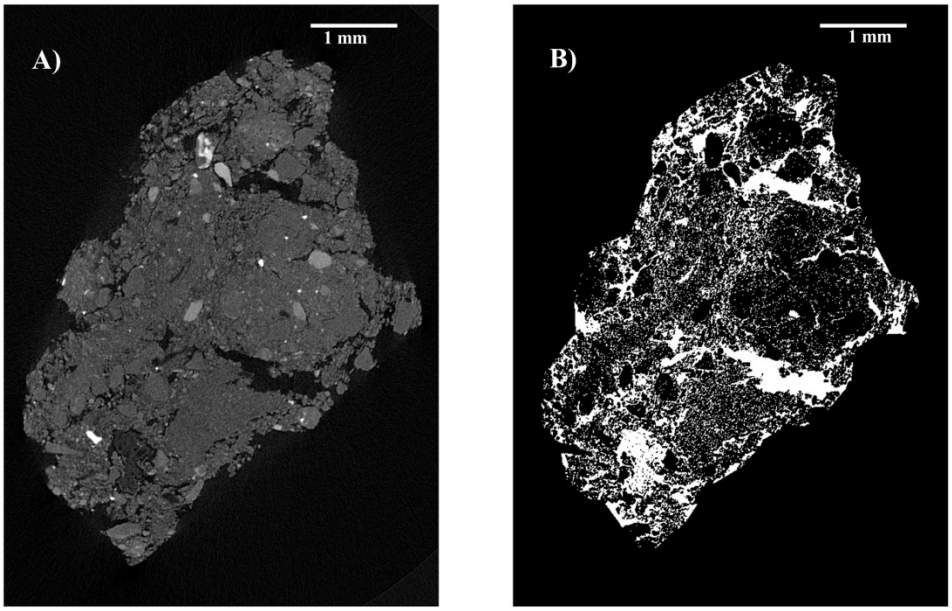


**Fig. 4.2.** A) A sample reconstructed cross section from acquired raw projections from Micro-CT scanning. B) The same image after thresholding with indicator kriging and follow up processes to preserve the whole aggregate body

## 4.3. RESULTS AND DISCUSSION

### 4.1.4. Effect of Pore Size on Hydrocarbon Biodegradation

The results of pore experiments are presented in Fig. 4.3, which shows the percent of radio-labelled hexadecane mineralized to $CO_2$ by bacteria during the experiments in different systems with different membranes. As shown in Figure 4.3, based on produced $CO_2$ more than 10% of hexadecane was mineralized in 41 days in the systems without membrane and in bioreactors where the hexadecane and inoculated bacteria were separated with 12 μm pore diameter

membranes. In contrast, in the reactors fitted with 0.4 μm and 3 μm membranes, no statistically significant mineralization of hexadecane was observed.

In bioreactors without membrane or with membranes with 12 μm pore diameter membranes, the attachment and growth of bacteria on oil-water interface was clearly visible after about two weeks. Fig. 4.3 shows the exponential pattern of mineralization of hexadecane as observed in these systems. It is likely that following bacterial attachment to the NAPL-water interface and interfacial uptake of hexadecane, there was exponential growth of bacteria, which led to an exponential increase in hexdecane mineralization. Thus, direct attachment of *Dietzia maris* to oil controls the biodegradation of hexadecane. Similarly van Hamme et al. [18] reported that *Rhodococcus* Sp. Strain F9-D79 was able to degraded hexadecane only after attachment to oil-water interface, followed by producing mycolic acid-containing capsule and oil emulsification.

The lack of biodegradation in the systems with 0.4 μm pore sizes was expected given the very low solubility of hexadecane on one hand and the size of bacteria on the other hand. Although the 3 μm pore diameter is significantly larger than the size of single cell of bacteria (about 1 μm), the bacteria appear to have been unable to access the hexadecane-water interfacial area. Long range forces such as van der Waals, electrostatic and steric interactions have been proposed to have a strong effect on trapping of bacteria near a solid surface [19]. Moreover, physiological parameters of bacteria such as motility, length of flagellum and aspect ratio have also been suggested to affect interaction between microorganisms and surfaces [20].

Several previous studies have reported unhindered motility in case of micro channels with widths as small as 2 μm [21-23]. Mannik et al. studied swimming speed of *E. coli* and *B. subtilis* in micro channels etched in silicon wafers with widths between 0.3 to 5 μm under nutrient

gradients that resulted in bacterial transport by chemotaxis. They found that bacterial average velocity was not decreased in channels of width up to 1.1 μm [23]. Similar findings have been reported by Binz et al. for motility of *S. marcescens* bacteria in microfluidic structure made of polydimethylsiloxane. The authors reported that the motility of bacteria was reduced only in the case of channels with tortuous paths and widths of 10 μm and lower, whereas in straight channels with widths ranging from 2 to 10 μm or wider channels with tortuous paths the velocity of the bacteria was unchanged [21]. Mannik et al. have also reported that in the densely packed chambers which bacteria are pressed into the channels, bacteria can penetrate pores as small as 0.4 μm, as a result of growth and deformation (flattening) inside the pores [23]. In our experiments, considering the very low solubility of hexadecane, chemotaxis driven motility of bacteria is unlikely. The membranes were not in direct contact of hexadecane, and hexadecane does not solubilize and diffuse towards the membrane, and thus initial adhesion or the growth of bacteria inside the pores was not possible. Moreover, in contrast to the above-mentioned studies, this study was performed with a less motile strain.

Environmental SEM images of bacteria, as acquired with minimum perturbation (without dehydration) revealed significant aggregation of bacterial cells, with aggregates as large as 5 μm (Fig. 4.4). It has been shown that in real soil environments, bacteria are mainly present in aggregated forms rather than single cells. The location of these patches of bacterial community has been suggested to be correlated to nutrient gradient, spatial location of soil organic matter and pores [24]. Our results suggest that effective bacterial transport of *Dietzia maris* (passing through the pores followed by motility, and attachment to oil and finally uptake of hydrocarbon) was severely limited in case of pores with 3 μm and 0.4 μm. Given the aggregation and motility

properties of *Dietzia maris*, this strain would provide a conservative estimation of hexadecane bioaccessible porosity for the hydrocarbon degrading microbial community in the site soil.

Other than the above-mentioned well-controlled studies on bacterial motility on constrained micro environments there are other studies which have evaluated the effect of pore size on biodegradation of contaminants. Noordman et al. studied the biodegradation rate of hexadecane in matrices with different pore sizes in the shake flask slurry bioreactors with beads with known pore size distributions and found that while in 150 hours complete biodegradation was observed in matrices with pore diameters of 300 nm or larger, the extent of biodegradation was limited to 27% in case of matrices with pore sizes of 6 nm [8]. The experimental condition as employed in Noordman et al. study is different than our study in several ways, including the type of porous media and bacterial properties. In their study, the entire surface of beads, including inside the pores as well as outer surface of beads was coated with hexadecane. Consequently, a fraction of hexadecane is readily available to bacteria, and moreover, as a result of growth on bead surface, bacteria would penetrate into the pores. This can explain the different obtained results in their study compared to ours. Our results, however, are in agreement with findings by Strong et al. [25] who examined the decomposition of plant organic matter in soils with different pore size distributions and found that the maximum biological activity in terms of carbon decomposition rate occurred in soils with large volume of pores with 15 μm to 60 μm neck diameters whereas organic matter was mostly protected in soils with large volume of pores with neck diameters less than 4 μm.

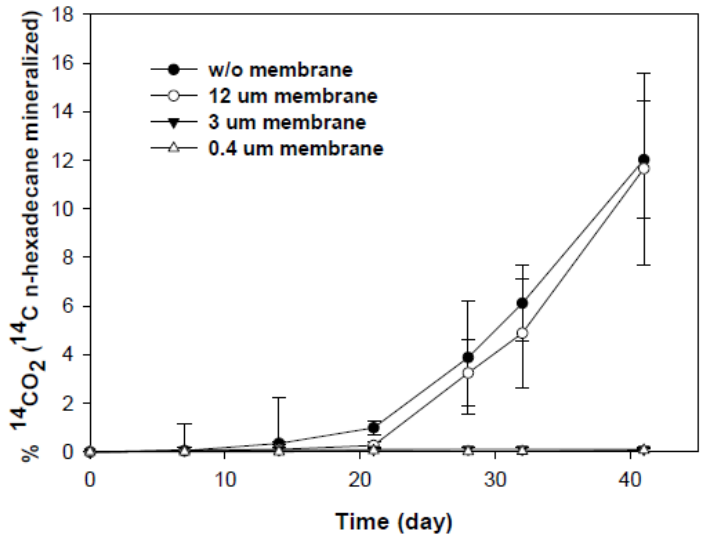


**Fig. 4.3.** The mineralization of n-hexadecane to $CO_2$ during pore experiments with bioreactors with fixed oil-water interfacial areas with membranes with different pore sizes. The error bars indicate the standard deviation of the mean of three replicate samples from three different bioreactors (n=9).

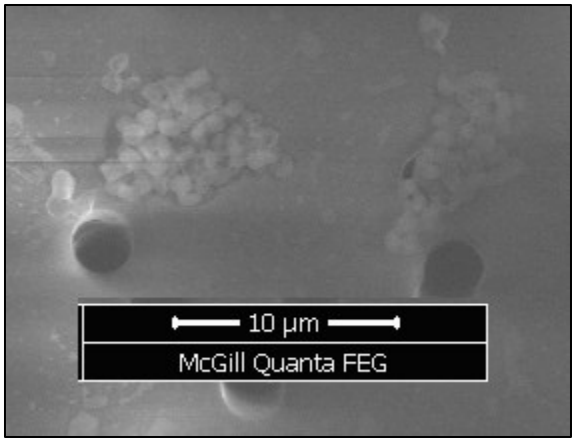


**Fig. 4.4.** The SEM image of *Dietzia maris* on 3 µm membranes, illustrating the aggregation of bacteria

### 4.1.5. Characterization of Aggregate Micro-Structure

To evaluate the significance of findings from pore experiments in bioremediation of contaminated soil, we characterized the pore network of soil aggregates from NWT petroleum contaminated site, the same soil as the *Dietzia maris* strain used in pore experiments was isolated

from. The soil is a fine-grained soil classified as a clay (mostly kaolinite) loam according to the USDA classification system (sand: 33%; silt: 33%; clay: 34%). For comparison aggregates from another petroleum contaminated sub-arctic site at Resolution Island (RI), Nunavut, Canada with a very different texture, classified as sand (gravel: 27%, sand: 72%, silt and clays: 1%) were also characterized. The detailed bioremediation results of NWT and RI soil was reported before [26, 27].

NAPL distribution in different pore sizes is a function of entry pressure as well as wettability of soil minerals [28]. Given the fact that soil minerals are predominantly water wet, NAPLs introduced into soil tend to reside in larger pores. However, it has been shown that direct contact and interfacial bonding between polar components of NAPL and soil minerals can change a water wet domain to an oil wet domain, over time [29]. The change in wettability will further facilitate the entry of NAPL to soil micro-pores. This suggests that aged NAPL could be present at different pore sizes, depending on the residual saturation of NAPL, surface properties of soil minerals and also contact time of NAPL and soil minerals.

We characterized the bioaccessible pore diameters and associated pore volume of representative soil aggregates with micro-CT scanning to estimate "bioaccessible porosity" as the fraction of total aggregate volume attributed to pores larger than 4 μm. The size of 4 μm is assumed to be bioaccessible because in our bioreactor experiments it was found that *Dietzia maris* as major hydrocarbon degrader in NWT clayey soil could not pass through 3 μm pores and access hexadecane, yet the literature discussed above suggests that bacteria should be able to pass into pore sizes slightly larger than its diameter of a single cell.

Figure 4.5A shows the frequency of pore cross sections with specific diameters as determined for whole aggregate cross sections. The values are normalized to aggregate total volume. The pore volumes with diameters larger than specific pore diameters as percent of total aggregate volume are presented in Fig 4.5B. It was found that in case of C1 and C2 aggregates 92-96% of total pore volume was interconnected as a large open pore, however, the pore volume were classified to different pore size classes based on pore diameter of cross sections, which is more meaningful for the purpose of characterization of accessible pore volumes by bacteria. We present the data for two macro-aggregates (dp > 2mm), as C2 and S3 from NWT and RI soils and a meso-aggregate (C1, dp < 2mm) from NWT soil. Given the larger size of macro-aggregates, the minimum attainable pixel sizes were about 2.96 μm and 3.28 μm for NWT and RI macro-aggregates. However, in case of meso-aggergate the scanning was performed with resolution of 1.04 μm. The X-ray scanning of meso-aggregate at this resolution took about 6 hours, and would have been significantly more time consuming for the larger aggregates. The 3D representation of C1 and S3 aggregates is shown in Fig. 4.6. The 3D volume rendering of C2, was computationally very demanding and thus is shown for only two aggregates among several scanned aggregates. A cross section of C2 aggregate is shown in Fig 2.

As shown in Fig 4.5A the number of small micro-pores (<100 μm) was generally higher in NWT aggregates (C1 and C2) than RI aggregate (S3). However, in case of sandy soil relatively small number (< 100) of very large pores (>1000 μm) was present. A very large number of imaged soil pores in C1 aggregate were in the range of 1-3 μm, as shown in Fig 4.5B they contributed to about 1% of total aggregate volume. Based on pore experiment results, this fraction could be considered as part of the potentially non-bioaccessible fraction of the aggregate volume. On the other hand Fig 4.5B shows almost comparable “bioaccessible porosity” of clayey

and sandy aggregates as 26-27% in case of clayey aggregate compared to 24% in case of aggregate from sandy soil.

Biodegradation results for of NWT and RI soil as reported in earlier studies [26, 27]. indicates significant biodegradation of non-volatile fraction of petroleum hydrocarbons (F3: >C16–C34) in case of both NWT (50%) and RI (65%) soils, confirming that significant fraction of aggregate volume and residual NAPL were accessible to soil microbial community as the extent of biodegradation is regulated by their accessibility to microorganisms [30].

While the bioaccessible porosity was comparable in RI and NWT soils, the volume of pores smaller than 1 μm which are non-accessible by bacteria was order of magnitude higher in case of NWT soils than RI soils as determined by BET analysis; the BJH Adsorption cumulative volume of pores between 17Å and 10000Å width was $0.036 \pm 0.016$ $cm^3/g$ for clayey NWT aggregates versus $0.005 \pm 0.000$ $cm^3/g$ for sandy RI aggregates. Strong et al. also reported that volume of pores smaller than 1.2 μm was highly correlated with clay content of soil (correlation factor: 0.96). Furthermore, Karimi-Lotfabad and Gray showed that in kaolinite clayey soil, NAPL could penetrate to soil micro-pores smaller than 5 μm [6]. This can explain the higher remediation endpoint in case of NWT soil ($620.9 \pm 90.3$ mg/kg), compared to the sandy soils ($105.8 \pm 24.1$ mg/kg). This is in agreement with those of Nocentini et al. who reported significant effect of soil texture on biodegradation extent of high molecular weight hydrocarbons. They reported about 45% biodegradation of a high-molecular weight, poorly soluble oil in a coarse grained porous soil (68% sand, 32% silt) compared to 27% reduction in fine grained soil (19% sand, 66% silt, 15% clay) in 277 days.[31] Overall, these results are in line with the hypothesis of our pore experiments that in case of fine textured soils where significant number of pores is smaller than bacterial size and hence direct attachment of oil and bacteria is not possible, the bioavailability of

poorly soluble hydrocarbons is severely limited. The employed approach as introduced here in case of a clayey contaminated soil could be considered as a useful to firstly estimate the "bioaccessible porosity" as well as predicting the effectiveness of bioremediation for each specific case.

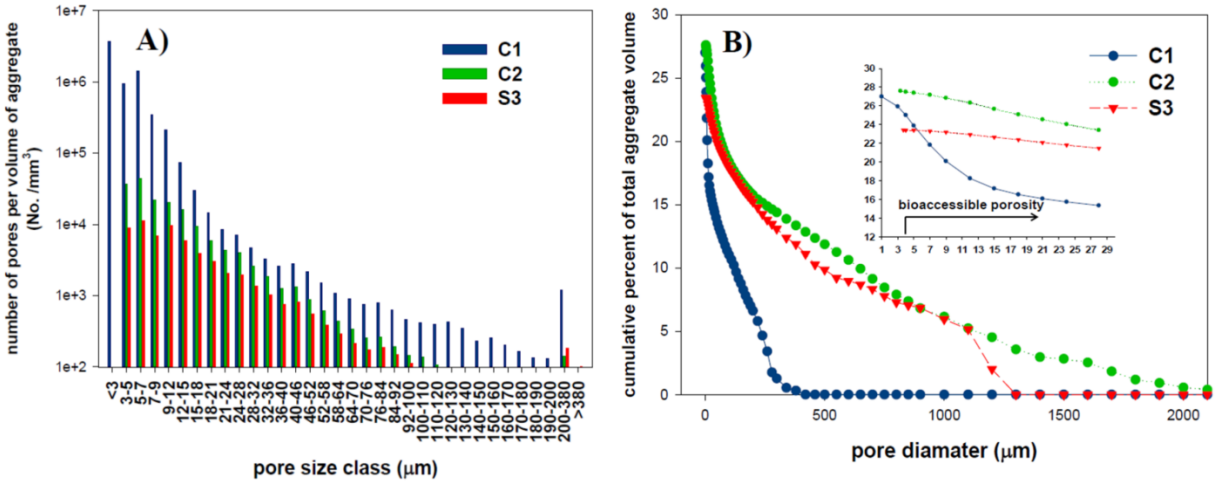


**Fig 4.5.** A) The two-dimensional void pore size distributions in terms of numbers as calculated from all cross sections (500-1500 cross sections for different aggregates). B). Cumulative volume of pores larger than specific pore sizes as present of total aggregate volume. Due to resolution of scanning, data of pores smaller than 3 µm are not available for C2 and S3.

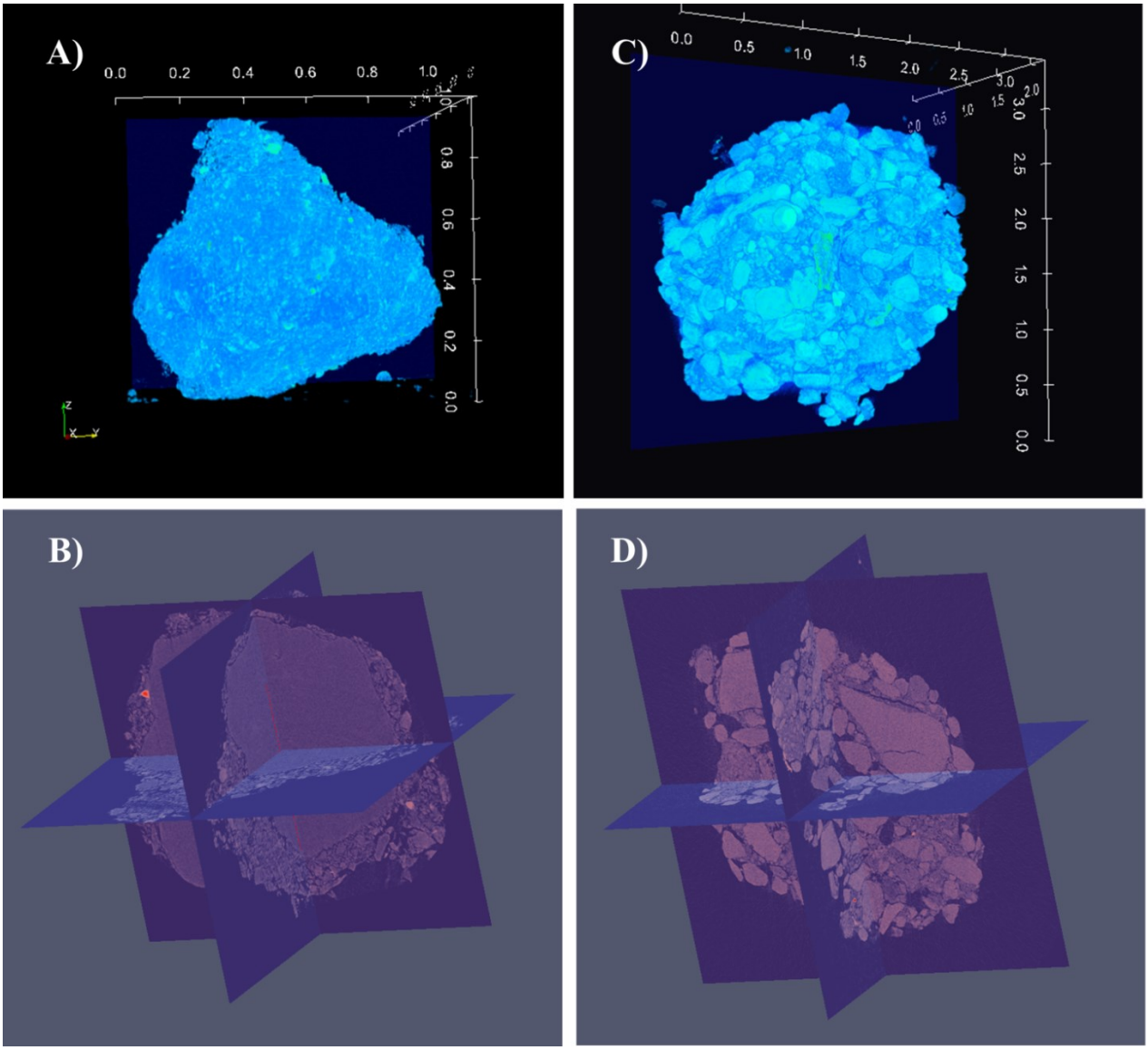


**Fig. 4.6** A) Three dimensional representation of a clayey soil aggregate (C1) B. cross section of clayey aggregate (C1) C. Three dimensional representation of a sandy soil aggregate (S3) D. cross section of sandy aggregate (S3)

## 4.4. CONCLUSION

The results of this study highlight the role of soil characteristics and specifically pore size on biodegradation extent of petroleum hydrocarbon compounds with high molecular weights. The results of the experiments with bioreactors with fitted membranes with different pore sizes indicated no biodegradation of n-hexadecane as a model of poorly soluble hydrocarbon by *Dietzia maris* in case of pore diameters of 3 μm and 0.4 μm, whereas 12 μm pore sizes imposed no limiting effect on biodegradation rate and extent. The characterization of aggregate microstructure of two different site contaminated soil (clayey and sandy) revealed that significant volume of aggregate were bioaccessible for soil microorganisms. Also, calculated "bioaccessible porosity" in sand and clayey aggregates were comparable (24 and 26-27%). Significant biodegradation extent of petroleum hydrocarbons in clayey (50%) and sandy soil (65%) confirmed that significant fraction of NAPL were bioaccessbile to soil microorganisms.

## 4.5. SUPPLEMENTARY INFORMATION

### Materials and Methods

Bacterial culture was grown at 25 ˚C in a 150-mL flask containing 75 mL mineral medium of Bushnell-Haas (magnesium sulfate 0.2 g/L, calcium chloride 0.02 g/L, monopotassium phosphate 1.0 g/L, dipotassium phosphate 1.0 g/L, ammonium nitrate 1.0 g/L, ferric chloride 0.05 g/L) and 1% v/v n-hexadecane as carbon source. After four days, the bacterial solution was centrifuged at 5000 g for 10 min. The precipitated cells were washed twice with the same Bushnell Haas mineral solution three times to remove hexadecane droplets before introducing to the cells.

The biodegradation kinetics of hexadecane was determined by monitoring $^{14C}O_2$ production rate. The basic NaOH solution from traps was sampled at sampling days, was mixed thoroughly and then three subsamples were taken and were transferred to scintillation vials containing *Ultimagold* scintillation cocktail (Perkinelmer), and finally activation was measured using a LS3600 scintillation counter (Beckman Coulter).

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# Chapter 5:

# Summary and Conclusions

The overall goal of this thesis is to better understand the limiting factors in bioremediation of petroleum hydrocarbon contamination in fine grained soil from a sub-arctic site, and to examine the effect of temperature regime as a critical environmental factor affecting biodegradation rate and extent.

The effects of nutrient and moisture amendment on biodegradation rate and extent of petroleum hydrocarbon contamination of an aged site contaminated soil from a sub-arctic site in pilot scale biopiles was evaluated. The experiments were carried out for more than a year to determine the remediation endpoint level and composition of residual petroleum hydrocarbons. The results indicated that, although low level nutrient amendment- low level moisture amendment (95 mg-N/ kg soil, 17.5% moisture) didn't affect the biodegradation rate and extent compared to low level moisture amendment and aerated control biopile (17.5% moisture), however, applying high dose of nutrients (1340 mg-N/kg soil, 23.5% moisture) showed inhibiting effect on biodegradation rate and extent compared to associated aerated control with high level moisture amendment (23.5% moisture). Except the high level nutrient amended system, significant biodegradation extent was occurred in three other systems. The soil microbial community in different systems was studied using TRFLP method for both 16S rRNA and *alkB* genes. The results suggest that significant biodegradation in high level moisture amended system is associated with a significant shift in microbial community; whereas inhibited biodegradation in high level nutrient amended system is associated with a stable microbial community. The analysis of residual TPH (Total Petroleum Hydrocarbon) levels in soil indicated that 85% of residual TPH were consisted of non-volatile petroleum hydrocarbon fraction and remaining 15% were semi-volatile fraction of petroleum hydrocarbons. The fact that even after a year of treatment, semi-volatile fraction, which are known to be more easily biodegradable compared to

non-volatile fraction, were still present in soil samples suggests that limited bio-accessibility is controlling the extent of biodegradation. Also to verify if the lack of enhancement due to nutrient amendment at low doses is due to limited bioavailability of nutrients to bacteria, a short term slurry experiments were carried out, however no nutrient effect was observed, suggesting that background nitrogen and phosphorous are available and likely enough to support the soil biological activity.

Temperature is a critical factor for biodegradation of petroleum hydrocarbons. The experiments were carried out in microcosm scale and involved studying the effect of constant incubation at 5 ˚C, 15 ˚C and the effect of incubation at diurnal temperature variation between 5 ˚C and 15 ˚C. The diurnal variation between 5 ˚C and 15 ˚C is typical temperature profile of the Northwestern Territories site (NWT), where contaminated soil was shipped from. The concentration of semi-volatile (F2; >C10-16) and non-volatile (F3; >C16-C32) fractions of TPH were determined. The results indicated comparable biodegradation extent of F2 (41-48%) and F3 (16-20%) fractions in the systems incubating at 15 ˚C and those experiencing diurnal temperature variation between 5 ˚C and 15 ˚C. The extent of F2 reduction was significantly lower at 5 ˚C (11%), and no statistically significant reduction was observed in F3 level at 5 ˚C. Also, the soil microbial community of 16S rRNA and *alkB* gene harboring bacteria were characterized using pyro-sequencing method which provides in depth phylogenetic information about composition of microbial community. Soil microbial community data showed that 6 major phylum of *Actinobacteria*, *Proteobacteria*, *Chloroflexi*, *Bacteroidetes*, *Acidobacteria*, and *Verrucomicrobia*, were present with relative abundances more than 1% in soil samples. Moreover, the results revealed high similarity between soil samples incubated at 15 ˚C and variable 5 ˚C to 15 ˚C both in terms of 16S rRNA and *alkB* results. γ- *proteobacteria* phylum

was enriched in samples incubated at 15 ˚C and 5 to 15 ˚C. *Actinobacteria,* the other important phylum of hydrocarbon degraders, were relatively abundant at all three temperature regimes. The effect of temperature on soil respiration as indicator of soil biological activity was also studied in small scale microcosm experiments. Highest respiration was observed in the systems incubated at maximum temperature of 15 ˚C. In variable temperature mode, the respiration rate was significantly lower than 15 ˚C systems and was significantly higher than 5 ˚C systems.

The effect of pore diameter on biodegradation and bio-accessibility of hydrocarbons to microorganism was evaluated in well controlled experiments in bioreactors fitted with membranes with selected pore sizes. The key advantage of the design of bioreactors is that it allowed us to precisely elucidate the role of pore size for biodegradation compared to previous studies with similar objective. The results of the experiments indicate about 10% biodegradation of n-hexadecane as model high molecular weight hydrocarbon in systems with membranes with 12 μm pore size and without membrane. On the other hand in systems with membranes with 3 and 0.4 μm pore diameters no biodegradation occurred in 30 days. To further investigate the environmental implications of pore experiment results, we characterized the soil aggregate micro-structure of two different petroleum contaminated soil samples with coarse (gravel: 27%, sand: 72%, silt and clays: 1%) and fine (sand 34%, silt: 32%, clay: 33%) textures. The three-dimensional internal aggregate micro-structure was delineated by X-ray micro-CT scanning at high resolutions. An image analysis procedure was developed to initially segment the reconstructed images acquired from CT-scanning to object (soil) and background (air), to define the aggregate boundaries and finally determine the pore size distribution of aggregates. The pore network information was then used for interpretation of the results of bioremediation studies of these two soil samples. It was demonstrated that significant fraction of aggregate volume either

in case of clayey aggregate (27%) or in case of sandy (24%) aggregates were larger than 4 μm, and consequently assumed to be available to soil bacterial community. Significant biodegradation extent was observed in both clayey soil (50% of non-volatile fraction) and sandy soil (65% of non-volatile fraction) showed that significant fraction of NAPL in soil aggregates were accessible to soil microorganisms.