Stress-tolerant bioremediation strategy for cold-climate sites: laboratory, modelling, and field studies for extending petroleum hydrocarbon biodegradation to seasonally freezing and frozen contaminated soil phases

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ABSTRACT

Bioremediation of petroleum hydrocarbon-contaminated soils in cold climates is challenging due to low temperatures, limited field accessibility, and low microbial activity. Conventional bioremediation practices have focused on short summers in cold climates, but the feasibility of extending hydrocarbon biodegradation to sub-zero temperatures has not been extensively explored. How biodegradation can be enhanced in freezing and frozen soils is unknown. Through laboratory, modelling, and field studies, this research investigated the enhancement of hydrocarbon biodegradation during phase changes in cold soils subjected to seasonal freeze-thaw conditions. In a pilot-scale biopile (3.5 tonnes) of nutrient-amended hydrocarbon-contaminated fine-grained soils operated over the winter at a cold-climate site, hydrocarbon biodegradation was enhanced during seasonal freezing and thawing in the field. The retention of unfrozen water was greater in the treated vs. untreated biopile. The removals of F2 (C10–C16) and F3 (C16–C34) hydrocarbons in the treated biopile were 57 and 58%, respectively, of which 26 and 39% were achieved between November and early March. To delineate the biodegradation activity below 0 °C, the generalized respiration model (GRESP) was modified to cover the soil phase change in hydrocarbon-contaminated northern soils during bioremediation. The Michaelis-Menten equation was modified and incorporated into GRESP to produce a new URESP model. Using soil temperature and unfrozen water content in frozen contaminated soil, URESP accurately estimated soil respiration related to hydrocarbon utilization. How soil amendments manipulate unfrozen water retention was also assessed and linked to hydrocarbon biodegradation during the freezing (4 to -5 °C) and frozen (-5 to -10 °C) soil phases. Nutrients significantly affected the freezing phase by inducing a freezing-point depression, shifting microbial communities, and stimulating biodegradation activity. Unfrozen water content had a significant effect during the frozen phase, which correlated with enhanced biodegradation. F3 hydrocarbons were significantly removed during the freezing and subsequent frozen phases (22–37%), and F3 biodegradation below 0 °C was positively correlated to the α-value (soil freezing index) of conventional soil freezing characteristic curves indexed for various soil types. This research informs the effective management and remediation of seasonally freezing and thawing petroleum hydrocarbon-contaminated sites in northern environments.

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DEDICATION

To my family, my supervisor and everyone who supported me

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LIST OF ABBREVIATION

ANOVA: Analysis of Variance

BET: Brunauer–Emmett–Teller

BRESP: Modified GRESP by the temperature component of GRESP

CCME: Canadian Council of Ministers of the Environment

CFU: Colony-Forming Unit

CWS PHC: Canada-Wide Standard for Petroleum Hydrocarbons in Soil

DCM: Dichloromethane

DWE: Distant Early Warning

FPD: Freezing-point Depression

FRESP: Modified GRESP by including a moisture dependent Q_{10}

GC: Gas Chromatography

GC-FID: Gas Chromatography with Flame-Ionization Detector

GC-MS: Gas Chromatography–mass spectrometry

GRESP: Generalized Respiration

HMW: High Molecular Weight

LMW: Low Molecular Weight

MAE: Mean Absolute Error

MARE: Mean Absolute Relative Error

MSE: Mean Squared Error

NAPL: Non-Aqueous Phase Liquid

NCBI: National Center for Biotechnology Information

ND: Not Detected

NPK: N-P-K based 20:20:20 fertilizer

OTU: Operational Taxonomic Unit

QA: Quality Assurance

QC: Quality Control

PBS: Phosphate Buffer Solution

PCR: Polymerase Chain Reaction

PCoA: Principal Coordinate Analysis

PE: Percent Error

PM: Peat moss

RMSE: Root Mean Square Error

RQ: Respiration Quotient

SD: Standard Deviation

SEM: Standard Error of the Mean

SFCC: Soil Freezing Characteristic Curve

SK: Saskatchewan

SRC: Saskatchewan Research Council

TOC: Total Organic Carbon

TPH: Total Petroleum Hydrocarbon

UCM: Unresolved Complex Mixture

UWC: Unfrozen Water Content

URESP: Modified GRESP by applying an unfrozen water content term

USCS: Unified Soil Classification System

USDA: United States Department of Agriculture

VWC: Volumetric Water Content

XRF: X-ray Fluorescence

ZEO: Zeolite

CHAPTER 1

INTRODUCTION

1.1 Petroleum hydrocarbon contaminated soil

Petroleum hydrocarbons are the most commonly identified contaminants in cold environments, including polar and sub-polar regions such as those in northern Canada (Princz et al., 2012). Hydrocarbon spills in cold environments have occurred due to oil extraction, storage, and transportation on account of infrastructure failure and human error (Camenzuli and Freidman, 2015). Hydrocarbon contamination has become a widespread issue because the above processes are ubiquitous (Kvenvolden and Cooper, 2003; Rohrbacher and St-Arnaud, 2016).

To date, more than 22,000 contaminated or suspected contaminated sites have been identified across Canada by the Federal Contaminated Sites Inventory (Fig. 1.1). In about 80% of these contaminated sites, the soil environments (including surface and subsurface soils, sediments, and groundwater) are contaminated. Approximately 60% of the contaminants at these sites have been identified as petroleum hydrocarbons (Canadian Council of Ministers of the Environment, 2008). Petroleum hydrocarbons that are the most frequently identified contaminants are non-aqueous phase liquids (NAPLs), which includes toxic and carcinogenic hydrophobic organic compounds (Vaughan et al., 2012), i.e., hydrocarbons that threaten human health and ecosystems (Siciliano et al., 2008).

How to deal with contamination in *freezing ground* is one of the most challenging but common questions in environmental remediation. The management of environmental remediation for the contamination that happens in cold climates needs to be extremely efficient and effective due to the low temperatures and low remediation efficiency. Spills in cold soils are considered more harmful than those in soils in temperate environments due to the separation and mobility of hydrocarbons during seasonal freezing and thawing (Barnes and Biggar, 2008). The remediation of hydrocarbon-contaminated sites in polar regions is very costly due to the remoteness and isolation of the locations (Harvey et al., 2012). For example, the remediation cost for the abandoned Distant Early Warning (DEW) contaminated sites that are located in remote Arctic and subarctic regions of northern Canada can exceed $500 million (Hird, 2016).

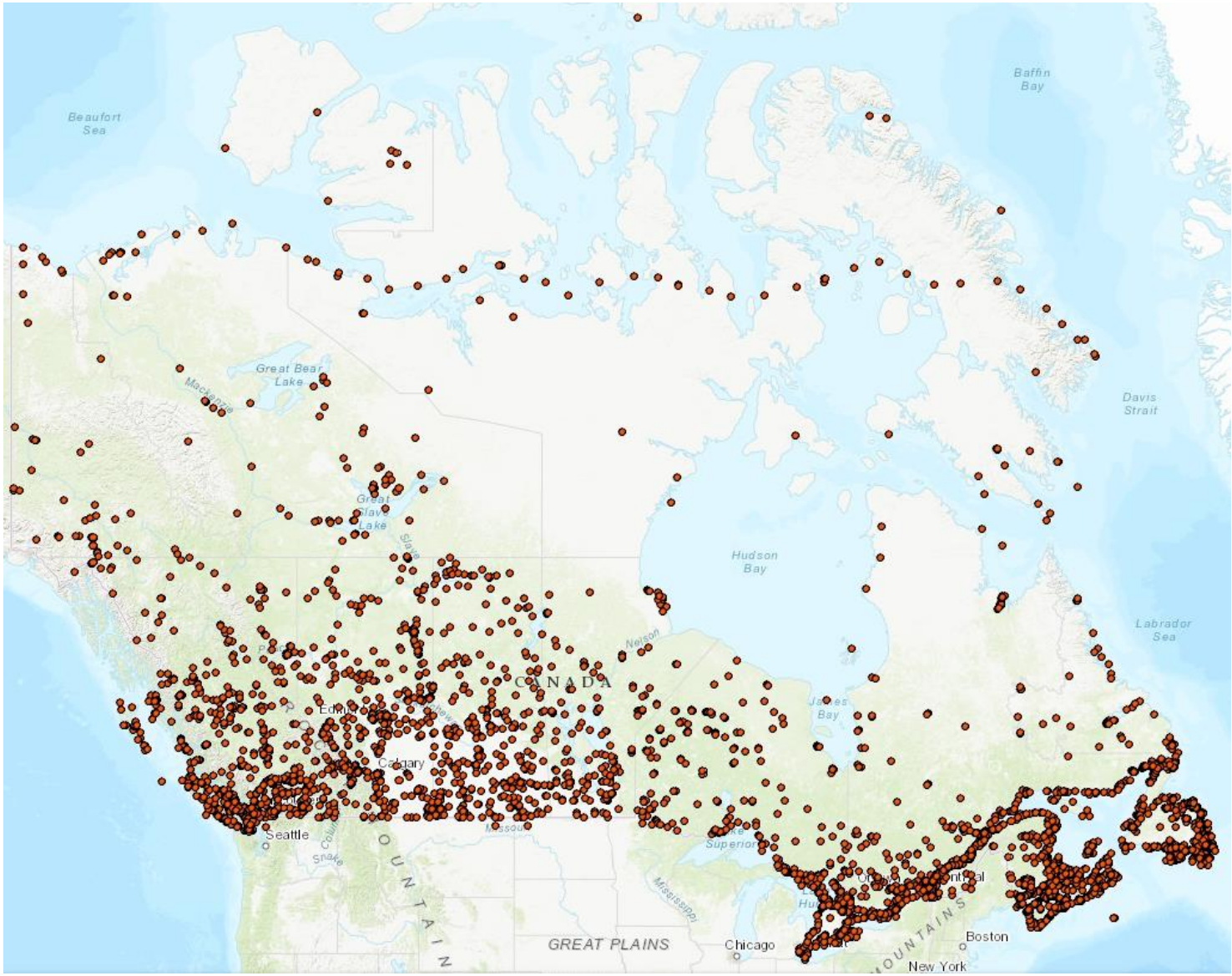

Figure 1.1. Canada Federal Contaminated Sites Inventory (Image source: DATA BASIN, Conservation Biology Institute).

1.2 Bioremediation of petroleum hydrocarbon-contaminated soils in cold climates

Bioremediation refers to the use of living organisms to reduce or remove contaminants such as hydrocarbons, and has been applied to contaminated sites in cold climates as an eco-friendly and cost-effective remediation technology (Aislabie and Foght, 2008; Margesin and Schinner, 1999). The average temperatures of cold climates range from above 10 °C even in the hottest month to below 0 °C in the coldest month (Beck et al., 2018; Peel et al., 2007). Cold climates generally occur above 40° North latitude. Due to the remote locations, limited accessibility, and harsh cold environments, bioremediation in cold climates has typically been applied during summer treatment periods (Yang et al., 2009). However, the summer periods in the cold regions only last two to four months and so are alone insufficient for effective and significant biodegradation that meets the relevant environmental criteria (Mohn and Stewart, 2000; Whyte et al., 2001). This limitation has generated interest in longer timeframes for remediation practices at hydrocarbon-contaminated cold sites (Camenzuli and Freidman, 2015).

1.2.1 Laboratory assessment at low positive temperatures

Hydrocarbon biodegradation has been examined at summer treatment temperatures in the laboratory. In cold regions such as the Alpine, Antarctic, and Arctic, contaminated soils are often nutrient deficient and hence biostimulation by adding nutrients has been employed. Margesin and Schinner (1997) performed hydrocarbon biodegradation fixed at 10 °C. They showed that 90% of total petroleum hydrocarbons (TPHs) were removed from Alpine soils by adding nutrients for 155 days. Aislabie et al. (1998) investigated hydrocarbon biodegradation using ^{14}C-labeled hexadecane and naphthalene fixed at 8 °C. Their results indicated that 20% of ^{14}C-hexadecane and 45% of ^{14}C-naphthalene were removed in 90 days by nutrient treatment of Antarctic desert soils. Mohn and Stewart (2000) also studied hydrocarbon biodegradation fixed at 7 °C and demonstrated that approximately 30% of TPH was removed in 180 days from Arctic tundra soils. Whyte et al. (2001) set the experimental temperature as low as 5 °C for hydrocarbon biodegradation and noted TPH removals of up to 47% for 16 weeks in nutrient-treated Arctic soils. The above studies show the feasibility of biodegradation at low temperatures from 10 to 5 °C in cold climates.

1.2.2 Laboratory assessment at sub-zero temperatures

Hydrocarbon biodegradation under rapid and repeated freezing and thawing conditions has also been investigated. Leszkiewicz (2001) demonstrated that frequent fluctuations of temperature from below to above freezing inhibits biodegradation activity. Eriksson et al. (2001) and Børresen et al. (2007) applied freezing and thawing conditions from −5 to 7 °C and −5 to 5 °C, respectively. Their results indicated hydrocarbon biodegradation can significantly occur during repeated freezing and thawing temperatures in cold climate soils. The rapid and repeated freezing and thawing stress did not inhibit microbial activity and stimulated hydrocarbon biodegradation with the addition of nutrients. The direct mechanism of enhanced biodegradation under repeated freezing and thawing remains unanswered.

In a laboratory experiment fixed at −5 °C, significant hydrocarbon removals were reported using nutrients and biochar (Karppinen et al., 2019; Karppinen et al., 2017a; Karppinen et al., 2017b). However, the experimental setting used a fixed sub-zero temperature for the cold contaminated site soils, which are instead subjected to seasonal freezing and thawing in field conditions (Or et al., 2007). Therefore, how biodegradation occurs in seasonally freezing and frozen contaminated soils in the field remained unexplored.

Hydrocarbon biodegradation under seasonally freezing and thawing conditions has rarely been investigated. Chang et al. (2011) demonstrated significant TPH removals that occurred under site-representative seasonal freezing and thawing conditions in a pilot-scale biodegradation experiment. They conducted the experiment in the laboratory by representing the site temperature changes as slow seasonal freezing (−0.12 °C/day) and thawing (+0.16 °C/day). This study correlated the changes in unfrozen water content to soil respiration activities (CO_2 and O_2). As shown in Table 1.1, hydrocarbon biodegradation at various temperatures has been extensively studied.

1.2.3 Field experiments and implementation

Hydrocarbon biodegradation has also been tested in the field by focusing on the summer period. Field practices for biodegradation have been performed at contaminated sites in the Arctic (McCarthy et al., 2004; Thomassin-Lacroix et al., 2002; Zytner et al., 2001) and Antarctic (Álvarez et al., 2020; Martínez Álvarez et al., 2017; Ruberto et al., 2003) during summer. The summer

periods in cold regions are too short to meet the environmental criteria (Chang et al., 2011; Whyte et al., 2001). Thus, field practices have been performed annually (Delille et al., 2004; McWatters et al., 2016; Mohn et al., 2001; Paudyn et al., 2008; Robichaud et al., 2019; Sanscartier et al., 2009a). McWatters et al. (2016) showed hydrocarbons were significantly removed by adding nutrients for five years in the field in Antarctica. Robichaud et al. (2019) also demonstrated significant hydrocarbon removals for five years in northern Canada using phytoremediation with compost. These studies demonstrate that hydrocarbon removals on a large scale have been achieved in cold climates.

The potential of hydrocarbon biodegradation at sub-zero temperatures in the field has also been considered. Rike et al. (2005) measured soil gases (O_2 and CO_2) in the Arctic field during winter and indirectly demonstrated that biodegradation activity was available between −1 and −3 °C during seasonal thawing at frozen contaminated sites. This was the first study to indirectly report the potential of extended biodegradation activity at sub-zero temperatures in the field.

1.3 Hydrocarbon biodegradation in freezing and frozen soils

1.3.1 Microbial activity in freezing and frozen soils

Microbial activity in freezing and frozen soils associated with unfrozen water content has also been extensively investigated. Unfrozen water content is a crucial factor for the sustainable and extended microbial activity of cold-adapted microorganisms in freezing and frozen soils. Liquid unfrozen water is a prerequisite to transport substrates, nutrients, oxygen, and byproducts (Harvey et al., 2012; Öquist et al., 2009; Tucker, 2014). Eutectophiles were observed at the interface of water and ice (Deming, 2002). How cold-adapted bacteria can survive and remain active in freezing and frozen soils is due to anti-freeze adaptation, which inhibits intracellular ice formation by anti-freeze proteins (Bore et al., 2017).

Various substrates to support microbial activity in freezing and frozen soils have been examined. Tuorto et al. (2014) showed DNA replication using ^{13}C-labeled acetate at temperatures down to −20 °C. Metabolic transformation has been observed at −5 and −20 °C using ^{13}C-labeled glucose (Bore et al., 2017; Bore et al., 2019). Cell growth has been detected at fixed temperatures

of −4 °C using ^{13}C-labeled cellulose (Segura et al., 2017). Catabolic and anabolic activity were also detected at fixed temperatures of −3 and −5 °C using ^{13}C-labeled glucose (Segura et al., 2019).

The change in microbial activity relates to the change of unfrozen water content. Rivkina et al. (2000) reported that the temporal decrease in unfrozen water film thickness during soil freezing is significantly correlated to the gradual depression of microbial metabolic activity as tracked by ^{14}C-radiolabeled acetate. Panikov et al. (2006) described how soil respiration (CO_2 production) is related to the change of unfrozen water content in thin unfrozen water films at temperatures down to −39 °C. Öquist et al. (2009) also demonstrated that CO_2 production was related to the unfrozen water content when the temperature was fixed at −4 °C. Tilston et al. (2010) reported that the inflection point (rapid change) of soil respiration at around −2 °C was lower than at 0 °C, and might be correlated to the change of unfrozen water content rather than soil temperature. The evaluation of microbial activity in freezing and frozen soils is summarized in Table 1.2.

1.3.2 Hydrocarbon biodegradation kinetics at low temperatures

Hydrocarbon degradation kinetics at summer temperatures in cold regions have been analyzed using linear and first order relationships in the laboratory and the field. The TPH degradation rate constant has been analyzed under rapid and repeated freezing and thawing conditions from −5 to 7 °C (Eriksson et al., 2001), resulting in a linear constant of the TPH degradation rate, i.e., 12.9 mg TPH/kg/day for 48 days. Using a landfarm in the sub-Arctic region at various field temperatures from 2 to 25 °C, Zytner et al. (2001) reported first-order TPH degradation constants of 0.022 to 0.0043 d^{-1} for 30 days. In another on-site biodegradation study using a biopile, Thomassin-Lacroix et al. (2002) noted a linear TPH degradation rate of 90 mg TPH/kg/day for 14 days at field temperatures from 0 to 10 °C. Paudyn et al. (2008) performed hydrocarbon biodegradation using a landfarm in the Canadian Arctic region. When the summer temperature was average 3 °C, the TPH degradation rate constant was 0.015 to 0.026 d^{-1}. McWatters et al. (2016) also performed hydrocarbon biodegradation using large-scale on-site biopiles in Antarctic regions. When the biodegradation periods were less than 100 days, the TPH degradation rate constant ranged from 2.6 to 60 mg TPH/kg/day. After 100 days of the

experimental period, the TPH degradation constant was generally lower, from 13 to 26 mg TPH/kg/day.

Biodegradation rate constants at sub-zero temperatures have also been investigated. Chang et al. (2011) applied seasonal freezing and thawing temperature conditions and reported TPH degradation rate constants of 0.0046 to 0.017 d^{-1} when soil temperatures ranged from −5 to 10 °C. At a fixed temperature of −5 °C, Karppinen et al. (2017b) showed that the F3 (C16–C34) degradation rate constant was 0.0036 d^{-1}. As summarized in Table 1.3, hydrocarbon degradation in freezing and frozen soils is still compatible with above zero temperature conditions.

1.4 Freezing and frozen soil microenvironments

1.4.1 Low temperature and cold-adapted bacteria

Temperature plays a significant role in terms of controlling the degree of microbial metabolism. Microbial activity is typically proportional to temperature (Delille et al., 2007). Thus, current conventional remedial strategies and on-site activities for cold sites, regardless of the type of remediation technology, have often been planned or assumed to be effective only during the short summers of northern climates. Microbial activity and mass transfer processes of substrates and nutrients are affected by low temperatures (Harvey et al., 2012). Several studies have investigated the significance of low-temperature effects on soil microbial metabolic activity (Eriksson et al., 2001; Sanscartier et al., 2009b; Walworth et al., 2001).

Table 1.1. Bioremediation studies at low temperatures (F2: C10 – C16, F3: C16 – C34).

Laboratory/Field	Temperature	Experiment duration	Hydrocarbon removal	Reference
Laboratory	Fixed at 10 °C	155 d	90%	Margesin and Schinner (1997)
Laboratory	Fixed at 8 °C	90 d	45%	Aislabie, et al. (1998)
Laboratory	Fixed at 7 °C	180 d	30%	Mohn and Stewart (2000)
Laboratory	Fixed at 5 °C	112 d	47%	Whyte et al. (2001)
Field	Variable in summer	60 d	58%	Zytner et al. (2001)
Laboratory	Variable from -5 to 7 °C	48 d	3.5 mg TPH/kg/day	Eriksson et al. (2001)
Field	Variable in summer	365 d	92%	Mohn et al. (2001)
Field	Variable from 0 to 10 °C	65 d	90 mg TPH/kg/day	Thomassin-Lacroix et al. (2002)
Field	Variable from -2 to 10 °C	50 d	75%	Ruberto et al. (2003)
Field	Variable from 0 to 20 °C	730 d	95%	Delille et al. (2004)
Field	Variable from 1.3 to 4.9 °C	55 d	From 8300 to 500 mg diesel/kg	McCarthy et al. (2004)

Laboratory	Variable from -5 to 5 °C	48 d	54 mg hexadecane/kg/day	Børresen et al. (2007)
Field	Variable	725 d	80%	Paudyn et al. (2008)
Field	Variable	1460 d	73%	Sanscartier et al. (2009)
Laboratory	Variable from -5 to 14 °C	160 d	F2: 32%, F3: 16%	Chang et al. (2011)
Field	Variable from -30 to 10 °C	1825 d	From 3531 to 907 mg TPH/kg	McWatters, et al. (2016)
Field	Variable from 0 to 10 °C	50 d	75%	Martínez Álvarez et al. (2017)
Laboratory	Fixed at -5 °C	90 d	From 266 to 61 mg F3/kg	Karppinen et al. (2017)
Laboratory	Fixed at -5 °C	90 d	From 656 to 473 mg F3/kg	Karppinen et al. (2017)
Laboratory	Fixed at -5 °C	90 d	84%	Karppinen et al. (2019)
Field	Variable	1825 d	75%	Robichaud et al. (2019)
Field	Variable from 0 to 10 °C	50 d	75%	Álvarez et al. (2020)

Table 1.2. Petroleum degradation rate constant related to low temperatures.

Laboratory/Field	Temperature	Hydrocarbon degradation rate constant	Reference
Laboratory	Variable from -5 to 7 °C	12.9 mg TPH/kg/day	Eriksson et al. (2001)
Field	Variable	TPH: 0.022 to 0.0043 d^{-1}	Zytner et al. (2001)
Field	Variable from 0 to 10 °C	90 mg TPH/kg/day	Thomassin-Lacroix et al. (2002)
Field	Variable	TPH: 0.015 to 0.026 d^{-1}	Paudyn et al. (2008)
Laboratory	Variable from -5 to 14 °C	TPH: 0.0046 d^{-1}	Chang et al. (2011)
Field	Variable from -30 to 10°C	2.6 to 60 mg TPH/kg/day (< 100 days) 13 to 26 mg TPH/kg/day (> 100 days)	McWatters et al. (2016)
Laboratory	Fixed at -5 °C	F3: 0.0036 d^{-1}	Karppinen et al. (2017b)

Table 1.3. Microbial activity in freezing and frozen soils.

Experimental temperatures (°C)	Substrate	Microbial activity evaluation	Reference
Dynamic from 5 to -20 (Laboratory)	^{14}C-labeled acetate	^{14}C-CO_2	Rivkina et al. (2000)
Dynamic from 10 to -40 (Laboratory)	^{14}C-labeled glucose	^{14}C-CO_2	Panikov et al. (2006)
Fixed at -4 (Laboratory)	Soil organic matter	CO_2 production	Öquist et al. (2009)
From 10 to -8 at 2 °C interval (Laboratory)	Glucose and sucrose	CO_2 production	Tilston et al. (2010)
From 0, -3, -6, -9, -12 to -20 (Laboratory)	^{13}C-labeled acetate	^{13}C-CO_2 DNA replication	Tuorto et al. (2014)
Fixed +5 (control) −5, and −20 (Laboratory)	^{13}C-labeled glucose	^{13}C-CO_2 Metabolic transformation	Bore et al. (2017)
Fixed at -4 (Laboratory)	^{13}C-labeled cellulose	^{13}C-CO_2 Cell growth	Segura et al. (2017)
Fixed +5 (control) −5, and −20 (Laboratory)	^{13}C-labeled glucose	^{13}C-CO_2 Metabolic transformation	Bore et al. (2019)
Fixed at -3 and -5 (Laboratory)	^{13}C-labeled glucose	^{13}C-CO_2 Anabolic and catabolic activity	Segura et al. (2019)

1.4.2 Unfrozen water content and solutes

When soil freezes, the unfrozen water content and water availability decrease, which significantly affects the microbial activity (Öquist et al., 2009; Sparrman et al., 2004). Unfrozen water content is one of the critical factors necessary for microbial metabolism and chemical reactions such as substrate transfer and waste product removal. Complex porous microstructures associated with the retention of unfrozen water in freezing soils provide physically protective niches for microbial populations, especially for eutectophilic bacteria that are active at the water-ice interface (Deming, 2002; Or et al., 2007). The change of unfrozen water content is positively proportional to microbial respiration (Öquist et al., 2009; Panikov et al., 2006; Rivkina et al., 2000). Chang et al. (2011) reported the emergence of *Corynebacterineae*-related bacterial populations, which are hydrocarbon degraders, in partially frozen soils. They were able to indicate an increase in copy numbers of *alkB* genes responsible for hydrocarbon biodegradation in the areas where unfrozen water was still available. In frozen soils, unfrozen water content can exist as a thin water film surrounding soil particles. The thickness of this unfrozen water film is related to surface areas, adsorptive and capillary forces (Sparrman et al., 2004), and the presence of organic matter and petroleum hydrocarbons (Drotz et al., 2010; Siciliano et al., 2008).

Unfrozen water content as a prerequisite for microbial activity is influenced by osmotic potential and matric potential (Drotz et al., 2009). The osmotic potential is dependent on solute concentrations (Bing and Ma, 2011); when solute concentrations increase, the freezing temperature of solutions decrease (Bing and Ma, 2011; Han et al., 2018), and the soil freezing process is retarded due to the lower freezing temperature with high unfrozen water retention (Ma et al., 2017; Wu et al., 2015). Under seasonally slow freezing conditions (less than −3 °C/day), the solutes are excluded from ice and partitioned to unfrozen water (Konrad and McCammon, 1990). Solute concentrations in the remaining unfrozen water content increase during slow freezing. Thus, osmotic potential can further decrease with more unfrozen water content. The matric potential is dependent on soil surface areas, particle sizes, and pore sizes and decreases with decreasing temperature (Wen et al., 2012). The decreasing matric potential increases the adsorptive water on the soil surface and capillary water between soil particles (Wen et al., 2012). For example, clay with high surface areas and small particle sizes retains more unfrozen water content than sand (Ma et al., 2017; Ren et al., 2017).

Low temperatures increase the viscosity of petroleum hydrocarbons and decrease their volatilization and solubility (Atlas, 1981). Dissolved hydrocarbons can also be excluded during slow freezing from ice growth, and the hydrocarbon concentrations in the unfrozen water content will increase (Barnes et al., 2004). When hydrocarbon concentrations are higher than the solubility, the excluded hydrocarbons will form NAPLs (Barnes et al., 2004). The formation of NAPLs may affect microbial activity with high toxicity and retard the onset of microbial activity (Margesin and Schinner, 2001).

The change in unfrozen water retention based on various soil conditions can be expressed using a soil freezing characteristic curve (SFCC) (Andersland and Ladanyi, 2003). The SFCC exhibits the relationship between negative soil temperatures and corresponding unfrozen water content. SFCCs can be variously formulated as a power form (Anderson and Tice, 1972), a non-linear empirical function (Kozlowski, 2007), and an exponential function (McKenzie et al., 2007).

1.5 Knowledge gap

During seasonal freezing and thawing in contaminated site soils, phase changes occur from freezing to deeply frozen, to thawing, as shown in Fig. 1.2. In winter, contaminated soils are characterized by sustained periods of partially frozen soil conditions, in which liquid unfrozen water content is retained and supports microbial activity. Under these dynamic environmental conditions, how soil water content changes, what microbial responses transpire, and whether hydrocarbon biodegradation occurs/can be enhanced in freezing and frozen soils are topics that remain unexplored.

Unfrozen water content is a critical driver of microbial activity in soils. However, how biodegradation activity is related to unfrozen water availability at sub-zero temperatures and where unfrozen water content is dramatically changing during soil phase changes have not been demonstrated. How unfrozen water retention can be manipulated to extend biodegradation remains unaddressed.

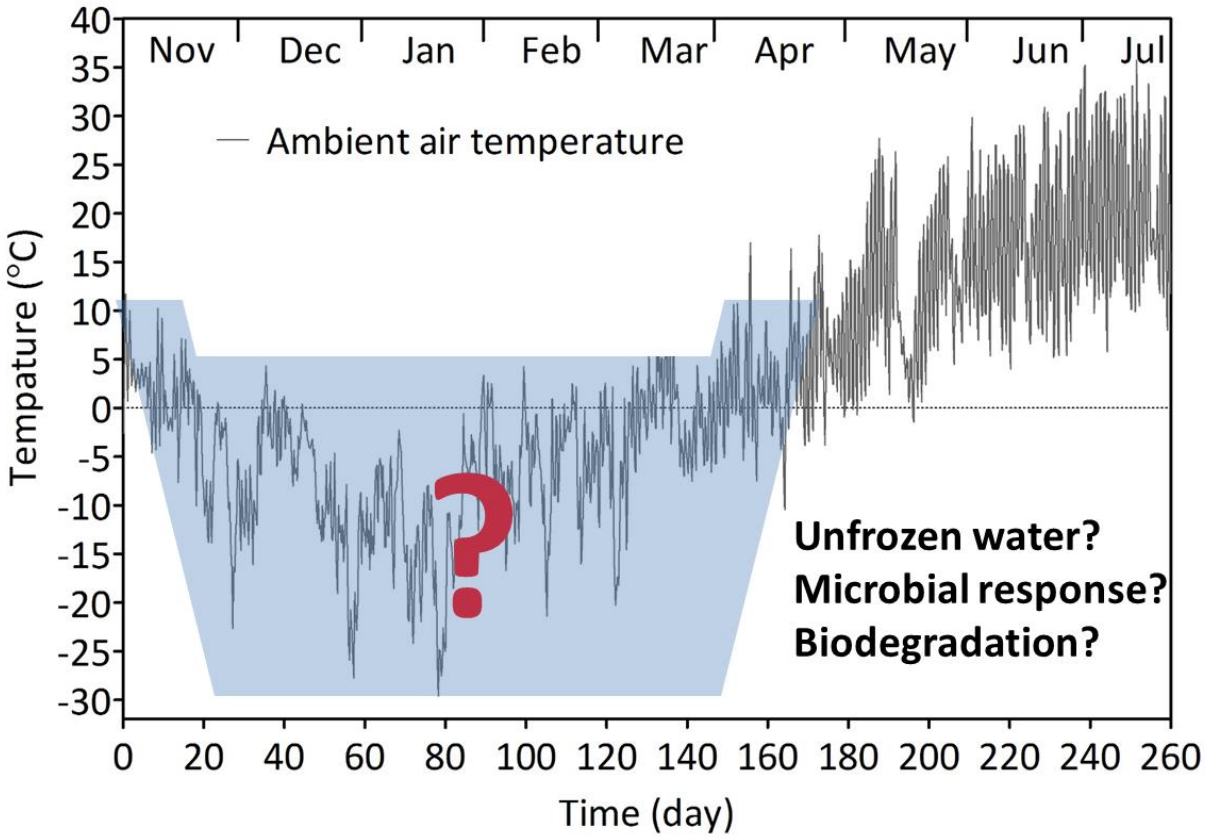

Figure 1.2. Monitored ambient air temperature at a landfarm facility near Saskatoon.

Previous studies performed hydrocarbon biodegradation at fixed sub-zero temperatures or during rapid freezing and thawing. As the soils freeze at *in situ* seasonal rates, the soil microenvironments are subjected to multiple stresses such as sub-zero temperatures, osmotic stress, and water scarcity. Microbial survivors in frozen soils are likely adapted to these multiple stresses as shown in Fig. 1.3. However, actual seasonal freeze/thaw in soils is a *slow* process, and is very different from the fixed temperature or rapid freeze/thaw cycles that have been conventionally adopted in some previous laboratory studies.

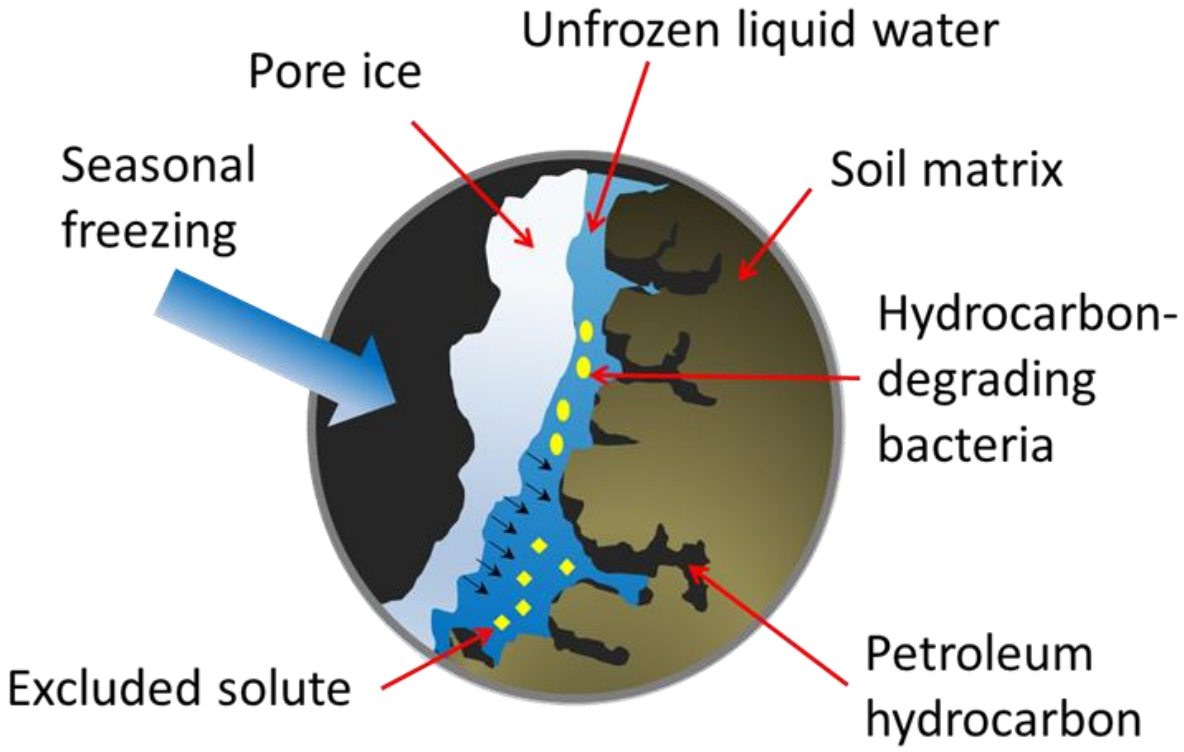

Figure 1.3. Conceptual model of the microenvironment in freezing and frozen soils.

1.6 Rationale for bioremediation of petroleum hydrocarbon-contaminated soils

Cold-adapted hydrocarbon-degrading bacteria can remain metabolically active in hydrocarbon-contaminated cold-climate soils. Bioremediation (the use of microorganisms to degrade or detoxify contaminants) has thus been frequently considered an appropriate remediation technology for cold sites. However, the ground in cold regions remains *partially frozen* and *frozen* for 6 to 9 months of the year, which significantly shortens the active treatment period compared to temperate regions. The present conventional remediation strategies and on-site activities for cold sites have often been planned or assumed to be effective only during the short summers of northern climates (2 to 4 months). These short treatment seasons are insufficient for meeting remediation targets and criteria, especially where contamination is extensive. The planning, remediation, and management of contaminated sites need to be extremely efficient in these regions.

Considering the large number of contaminated sites and intensified extraction of natural resources in northern Canada, there is a strong demand for more cost-effective, efficient, and low-

energy remediation technologies. This research is directed toward developing a novel site remediation strategy and management tool especially designed for environmentally challenging conditions in cold climates.

1.7 Research objective and thesis organization

Petroleum hydrocarbon biodegradation in cold soils was comprehensively investigated during the soil phase changes under seasonal freeze-thaw conditions in the field and in laboratory and modelling studies. The specific objectives were to:

- Enhance hydrocarbon biodegradation in contaminated soils during natural seasonal freeze-thaw at a cold site (Obj. 1: *Field experiment*, Chapter 2),
- Investigate the seasonal activation and inactivation of biodegradation activity and estimate soil respiration using soil temperature and water content (Obj. 2: *Modelling study*, Chapter 3),
- Manipulate unfrozen water content via soil amendments before seasonal freezing and enhance hydrocarbon biodegradation in freezing and frozen soils (Obj. 3: *Laboratory study*, Chapter 4).

This thesis is organized into five chapters, including published and submitted manuscripts. Chapter 1 contains an extensive introduction emphasizing a review of previous studies related to the research topics. Chapters 2 to 4 are stand-alone manuscripts. Chapter 2 describes the feasibility of hydrocarbon biodegradation during seasonal freezing and thawing in the field. Chapter 3 describes the development of a soil microbial respiration model using soil temperature and unfrozen water content while biodegradation occurred. Chapter 4 describes the manipulation of unfrozen water content by soil treatment and the link between soil freezing behaviors and petroleum hydrocarbon biodegradation at freezing and frozen phases. Chapter 5 summarizes and concludes the individual research studies, including a discussion of future work.

1.8 Scope of the research

- Unsaturated, field-aged, petroleum hydrocarbon-contaminated soils were used in the research.
- Representative temperatures for seasonal freezing and thawing in the field were obtained from the measured ambient temperature data at a landfarm facility near Saskatoon, Canada.
- Analytical protocols of petroleum hydrocarbon analyses were based on the Canadian standard developed by the Canadian Council of Ministers of the Environment (CCME).
- The target petroleum hydrocarbons for assessing biodegradation were biodegradable hydrocarbons contained in semi-volatile (CCME F2: C10–C16) and non-volatile (CCME F3: C16–C34) hydrocarbon fractions.

1.9 References

Aislabie, J., Foght, J., 2008. Hydrocarbon-degrading bacteria in contaminated cold soil. In: Filler, D. M., Snape, I., Barnes, D. L., (Eds), Bioremediation of Petroleum Hydrocarbons in Cold Regions. Cambridge University Press, New York, pp. 69-83.

Aislabie, J., McLeod, M., Fraser, R., 1998. Potential for biodegradation of hydrocarbons in soil from the Ross Dependency, Antarctica. Appl. Microbiol. Biotechnol. 49, 210-214.

Álvarez, L.M., Ruberto, L., Gurevich, J., Mac Cormack, W., 2020. Environmental factors affecting reproducibility of bioremediation field assays in Antarctica. Cold Reg. Sci. Technol. 169, 102915.

Andersland, O.B., Ladanyi, B. 2003. Frozen ground engineering: John Wiley & Sons,

Anderson, D.M., Tice, A.R., 1972. Predicting unfrozen water contents in frozen soils from surface area measurements. Highway research record 393, 12-18.

Atlas, R.M., 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiological reviews 45, 180.

Børresen, M., Barnes, D., Rike, A., 2007. Repeated freeze–thaw cycles and their effects on mineralization of hexadecane and phenanthrene in cold climate soils. Cold Reg. Sci. Technol. 49, 215-225.

Barnes, D., Biggar, K., 2008. Movement of petroleum through freezing and frozen soils. Bioremediation of petroleum hydrocarbons in cold regions. Cambridge University Press, Cambridge, UK, 55-68.

Barnes, D.L., Wolfe, S.M., Filler, D.M., 2004. Equilibrium distribution of petroleum hydrocarbons in freezing ground. The Polar Record 40, 245.

Beck, H.E., Zimmermann, N.E., McVicar, T.R., Vergopolan, N., Berg, A., Wood, E.F., 2018. Present and future Köppen-Geiger climate classification maps at 1-km resolution. Scientific data 5, 180214.

Bing, H., Ma, W., 2011. Laboratory investigation of the freezing point of saline soil. Cold Reg. Sci. Technol. 67, 79-88.

Bore, E.K., Apostel, C., Halicki, S., Kuzyakov, Y., Dippold, M.A., 2017. Microbial metabolism in soil at subzero temperatures: adaptation mechanisms revealed by position-specific 13 °C labeling. Front Microbiol. 8, 1-10.

Bore, E.K., Halicki, S., Kuzyakov, Y., Dippold, M.A., 2019. Structural and physiological adaptations of soil microorganisms to freezing revealed by position-specific labeling and compound-specific 13 C analysis. Biogeochemistry 143, 207-219.

Camenzuli, D., Freidman, B.L., 2015. On-site and in situ remediation technologies applicable to petroleum hydrocarbon contaminated sites in the Antarctic and Arctic. Polar Res. 34, 1-19.

Chang, W., Akbari, A., David, C., Ghoshal, S., 2018. Selective biostimulation of cold- and salt-tolerant hydrocarbon-degrading Dietzia maris in petroleum-contaminated sub-Arctic soils with high salinity. J. Chem. Technol. Biotechnol. 93, 294-304.

Chang, W., Klemm, S., Beaulieu, C., Hawari, J., Whyte, L., Ghoshal, S., 2011. Petroleum hydrocarbon biodegradation under seasonal freeze−thaw soil temperature regimes in contaminated soils from a sub-Arctic Site. Environ. Sci. Technol. 45, 1061-1066.

Delille, D., Coulon, F., Pelletier, E., 2004. Effects of temperature warming during a bioremediation study of natural and nutrient-amended hydrocarbon-contaminated sub-Antarctic soils. Cold Reg. Sci. Technol. 40, 61-70.

Delille, D., Pelletier, E., Coulon, F., 2007. The influence of temperature on bacterial assemblages during bioremediation of a diesel fuel contaminated subAntarctic soil. Cold Regions Science and Technology 48, 74-83.

Deming, J.W., 2002. Psychrophiles and polar regions. Curr. Opin. Microbiol. 5, 301-309.

Drotz, S.H., Sparrman, T., Schleucher, J., Nilsson, M., Öquist, M.G., 2010. Effects of soil organic matter composition on unfrozen water content and heterotrophic CO2 production of frozen soils. Geochim. Cosmochim. Acta 74, 2281-2290.

Drotz, S.H., Tilston, E.L., Sparrman, T., Schleucher, J., Nilsson, M., Öquist, M.G., 2009. Contributions of matric and osmotic potentials to the unfrozen water content of frozen soils. Geoderma 148, 392-398.

Eriksson, M., Ka, J.-O., Mohn, W.W., 2001. Effects of low temperature and freeze-thaw cycles on hydrocarbon biodegradation in Arctic tundra soil. Appl. Environ. Microbiol. 67, 5107-5112.

Han, Y., Wang, Q., Kong, Y., Cheng, S., Wang, J., Zhang, X., Wang, N., 2018. Experiments on the initial freezing point of dispersive saline soil. Catena 171, 681-690.

Harvey, A.N., Snape, I., Siciliano, S.D., 2012. Changes in liquid water alter nutrient bioavailability and gas diffusion in frozen antarctic soils contaminated with petroleum hydrocarbons. Environ. Toxicol. Chem. 31, 395-401.

Hird, M., 2016. The DEW line and Canada's arctic waste: Legacy and futurity. Northern Review, 23–45.

Karppinen, E.M., Mamet, S.D., Stewart, K.J., Siciliano, S.D., 2019. The Charosphere Promotes Mineralization of 13C-Phenanthrene by Psychrotrophic Microorganisms in Greenland Soils. J. Environ. Qual.

Karppinen, E.M., Siciliano, S.D., Stewart, K.J., 2017a. Application method and biochar type affect petroleum hydrocarbon degradation in northern landfarms. J. Environ. Qual. 46, 751-759.

Karppinen, E.M., Stewart, K.J., Farrell, R.E., Siciliano, S.D., 2017b. Petroleum hydrocarbon remediation in frozen soil using a meat and bonemeal biochar plus fertilizer. Chemosphere 173, 330-339.

Konrad, J., McCammon, A., 1990. Solute partitioning in freezing soils. Can. Geotech. J. 27, 726-736.

Kozlowski, T., 2007. A semi-empirical model for phase composition of water in clay–water systems. Cold Reg. Sci. Technol. 49, 226-236.

Kvenvolden, K., Cooper, C., 2003. Natural seepage of crude oil into the marine environment. Geo-marine letters 23, 140-146.

Leszkiewicz, C.G., 2001. The effect of freeze/thaw temperature fluctuations on microbial metabolism of petroleum hydrocarbon contaminated Antarctic soil.

Ma, T., Wei, C., Xia, X., Zhou, J., Chen, P., 2017. Soil freezing and soil water retention characteristics: connection and solute effects. J. Perform. Constr. Fac. 31, D4015001.

Margesin, R., Schinner, F., 1997. Bioremediation of diesel-oil-contaminated alpine soils at low temperatures. Appl. Microbiol. Biotechnol. 47, 462-468.

Margesin, R., Schinner, F., 1999. Biological decontamination of oil spills in cold environments. J. Chem. Technol. Biotechnol. 74, 381-389.

Margesin, R., Schinner, F., 2001. Biodegradation and bioremediation of hydrocarbons in extreme environments. Appl. Microbiol. Biotechnol. 56, 650-663.

Martínez Álvarez, L., Ruberto, L., Lo Balbo, A., Mac Cormack, W., 2017. Bioremediation of hydrocarbon-contaminated soils in cold regions: Development of a pre-optimized biostimulation biopile-scale field assay in Antarctica. Sci. Total Environ. 590, 194-203.

McCarthy, K., Walker, L., Vigoren, L., Bartel, J., 2004. Remediation of spilled petroleum hydrocarbons by in situ landfarming at an arctic site. Cold Reg. Sci. Technol. 40, 31-39.

McKenzie, J.M., Voss, C.I., Siegel, D.I., 2007. Groundwater flow with energy transport and water–ice phase change: numerical simulations, benchmarks, and application to freezing in peat bogs. Advances in water resources 30, 966-983.

McWatters, R., Wilkins, D., Spedding, T., Hince, G., Raymond, B., Lagerewskij, G., Terry, D., Wise, L., Snape, I., 2016. On site remediation of a fuel spill and soil reuse in Antarctica. Sci. Total Environ. 571, 963-973.

Miri, S., Naghdi, M., Rouissi, T., Kaur Brar, S., Martel, R., 2019. Recent biotechnological advances in petroleum hydrocarbons degradation under cold climate conditions: A review. Crit. Rev. Environ. Sci. Technol. 49, 553-586.

Mohn, W., Radziminski, C., Fortin, M.-C., Reimer, K., 2001. On site bioremediation of hydrocarbon-contaminated Arctic tundra soils in inoculated biopiles. Appl. Microbiol. Biotechnol. 57, 242-247.

Mohn, W.W., Stewart, G.R., 2000. Limiting factors for hydrocarbon biodegradation at low temperature in Arctic soils. Soil Biol. Biochem. 32, 1161-1172.

Öquist, M.G., Sparrman, T., Klemedtsson, L., Drotz, S.H., Grip, H., Schleucher, J., Nilsson, M.,

2009. Water availability controls microbial temperature responses in frozen soil CO2 production. Global Change Biol. 15, 2715-2722.

Or, D., Smets, B.F., Wraith, J., Dechesne, A., Friedman, S., 2007. Physical constraints affecting bacterial habitats and activity in unsaturated porous media–a review. Adv. Water Resour. 30, 1505-1527.

Panikov, N., Flanagan, P., Oechel, W., Mastepanov, M., Christensen, T., 2006. Microbial activity in soils frozen to below -39 °C. Soil Biol. Biochem. 38, 785-794.

Paudyn, K., Rutter, A., Rowe, R.K., Poland, J.S., 2008. Remediation of hydrocarbon contaminated soils in the Canadian Arctic by landfarming. Cold Reg. Sci. Technol. 53, 102-114.

Peel, M.C., Finlayson, B.L., McMahon, T.A., 2007. Updated world map of the Köppen-Geiger climate classification.

Princz, J.I., Moody, M., Fraser, C., Van der Vliet, L., Lemieux, H., Scroggins, R., Siciliano, S.D., 2012. Evaluation of a new battery of toxicity tests for boreal forest soils: assessment of the impact of hydrocarbons and salts. Environmental toxicology and chemistry 31, 766-777.

Ren, J., Vanapalli, S.K., Han, Z., 2017. Soil freezing process and different expressions for the soil-freezing characteristic curve. Sci. Cold. Arid. Reg. 9, 221-228.

Rike, A.G., Haugen, K.B., Engene, B., 2005. In situ biodegradation of hydrocarbons in arctic soil at sub-zero temperatures—field monitoring and theoretical simulation of the microbial activation temperature at a Spitsbergen contaminated site. Cold Reg. Sci. Technol. 41, 189-209.

Rike, A.G., Schiewer, S., Filler, D., 2008. Temperature effects on biodegradation of petroleum contaminants in cold soils. In: Filler, D. M., Snape, I., Barnes, D. L., (Eds), Bioremediation of Petroleum Hydrocarbons in Cold Regions. Cambridge University Press, Cambridge, UK, Cambridge, pp. 84-108.

Rivkina, E., Friedmann, E., McKay, C., Gilichinsky, D., 2000. Metabolic activity of permafrost bacteria below the freezing point. Appl. Environ. Microbiol. 66, 3230-3233.

Robichaud, K., Stewart, K., Labrecque, M., Hijri, M., Cherewyk, J., Amyot, M., 2019. An ecological microsystem to treat waste oil contaminated soil: Using phytoremediation assisted by fungi and local compost, on a mixed-contaminant site, in a cold climate. Sci. Total Environ. 672, 732-742.

Rohrbacher, F., St-Arnaud, M., 2016. Root exudation: the ecological driver of hydrocarbon rhizoremediation. Agronomy 6, 19.

Ruberto, L., Vazquez, S.C., Mac Cormack, W.P., 2003. Effectiveness of the natural bacterial flora, biostimulation and bioaugmentation on the bioremediation of a hydrocarbon contaminated Antarctic soil. Int. Biodeterior. Biodegradation 52, 115-125.

Sanscartier, D., Laing, T., Reimer, K., Zeeb, B., 2009a. Bioremediation of weathered petroleum hydrocarbon soil contamination in the Canadian High Arctic: Laboratory and field studies.

Chemosphere 77, 1121-1126.

Sanscartier, D., Zeeb, B., Koch, I., Reimer, K., 2009b. Bioremediation of diesel-contaminated soil by heated and humidified biopile system in cold climates. Cold Reg. Sci. Technol. 55, 167-173.

Segura, J.H., Nilsson, M.B., Haei, M., Sparrman, T., Mikkola, J.-P., Gräsvik, J., Schleucher, J., Öquist, M.G., 2017. Microbial mineralization of cellulose in frozen soils. Nat. Commun. 8, 1154.

Segura, J.H., Nilsson, M.B., Schleucher, J., Haei, M., Sparrman, T., Székely, A., Bertilsson, S., Öquist, M.G., 2019. Microbial utilization of simple carbon substrates in boreal peat soils at low temperatures. Soil Biol. Biochem.

Siciliano, S.D., Schafer, A.N., Forgeron, M.A., Snape, I., 2008. Hydrocarbon contamination increases the liquid water content of frozen Antarctic soils. Environ. Sci. Technol. 42, 8324-8329.

Sparrman, T., Öquist, M., Klemedtsson, L., Schleucher, J., Nilsson, M., 2004. Quantifying unfrozen water in frozen soil by high-field 2H NMR. Environmental science & technology 38, 5420-5425.

Thomassin-Lacroix, E., Eriksson, M., Reimer, K., Mohn, W., 2002. Biostimulation and bioaugmentation for on-site treatment of weathered diesel fuel in Arctic soil. Appl. Microbiol. Biotechnol. 59, 551-556.

Tilston, E., Sparrman, T., Öquist, M., 2010. Unfrozen water content moderates temperature dependence of sub-zero microbial respiration. Soil Biol. Biochem. 42, 1396-1407.

Tucker, C., 2014. Reduction of air-and liquid water-filled soil pore space with freezing explains high temperature sensitivity of soil respiration below 0 °C. Soil Biol. Biochem. 78, 90-96.

Tuorto, S.J., Darias, P., McGuinness, L.R., Panikov, N., Zhang, T., Häggblom, M.M., Kerkhof, L.J., 2014. Bacterial genome replication at subzero temperatures in permafrost. ISME J. 8, 139-149.

Vaughan, S., Sloan, B.C., Shaw, T.R., Hilier, R. 2012 Spring Report of the Commissioner of the Environment and Sustainable Development, CHAPTER 3—Federal Contaminated Sites and Their Impacts., 2012.

Walworth, J., Braddock, J., Woolard, C., 2001. Nutrient and temperature interactions in bioremediation of cryic soils. Cold Regions Science and Technology 32, 85-91.

Wen, Z., Ma, W., Feng, W., Deng, Y., Wang, D., Fan, Z., Zhou, C., 2012. Experimental study on unfrozen water content and soil matric potential of Qinghai-Tibetan silty clay. Environ. Earth Sci. 66, 1467-1476.

Whyte, L., Goalen, B., Hawari, J., Labbé, D., Greer, C., Nahir, M., 2001. Bioremediation treatability assessment of hydrocarbon-contaminated soils from Eureka, Nunavut. Cold Reg. Sci. Technol. 32, 121-132.

Whyte, L.G., Hawari, J., Zhou, E., Bourbonnière, L., Inniss, W.E., Greer, C.W., 1998. Biodegradation of variable-chain-length alkanes at low temperatures by a psychrotrophic Rhodococcus sp. Appl. Environ. Microbiol. 64, 2578-2584.

Wu, M., Tan, X., Huang, J., Wu, J., Jansson, P.-E., 2015. Solute and water effects on soil freezing characteristics based on laboratory experiments. Cold Reg. Sci. Technol. 115, 22-29.

Yang, S.-Z., Jin, H.-J., Wei, Z., He, R.-X., Ji, Y.-J., Li, X.-M., Yu, S.-P., 2009. Bioremediation of oil spills in cold environments: a review. Pedosphere 19, 371-381.

Zytner, R., Salb, A., Brook, T., Leunissen, M., Stiver, W., 2001. Bioremediation of diesel fuel contaminated soil. Can. J. Civ. Eng. 28, 131-140.

CHAPTER 2

ENHANCED BIOREMEDIATION OF NUTRIENT-AMENDED, PETROLEUM HYDROCARBON-CONTAMINATED SOILS OVER A COLD-CLIMATE WINTER: THE RATE AND EXTENT OF HYDROCARBON BIODEGRADATION AND MICROBIAL RESPONSE IN A PILOT-SCALE BIOPILE SUBJECTED TO NATURAL SEASONAL FREEZE-THAW TEMPERATURES

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This chapter was published as Kim, J., Lee, A.H., Chang, W., 2018. *Enhanced bioremediation of nutrient-amended, petroleum hydrocarbon-contaminated soils over a cold-climate winter: The rate and extent of hydrocarbon biodegradation and microbial response in a pilot-scale biopile subjected to natural seasonal freeze-thaw temperatures*. *Science of the Total Environment*, 612 (2018) 903–913. Dr. Chang (supervisor) provided the funding, developed the experimental design, and suggested the motivation behind enhanced biodegradation during seasonal freezing and thawing. Dr. Lee assisted with the culture-independent microbial analyses and the operation of the 3D PCR platform. Jihun Kim performed all the field work, such as the biopile construction, site monitoring and sampling. Physicochemical analyses and culture-dependent microbial analyses of contaminated site soils were also conducted by Jihun Kim. He also carried out physicochemical and microbial data interpretation under Dr. Chang's supervision and prepared the manuscript draft, figures and tables.

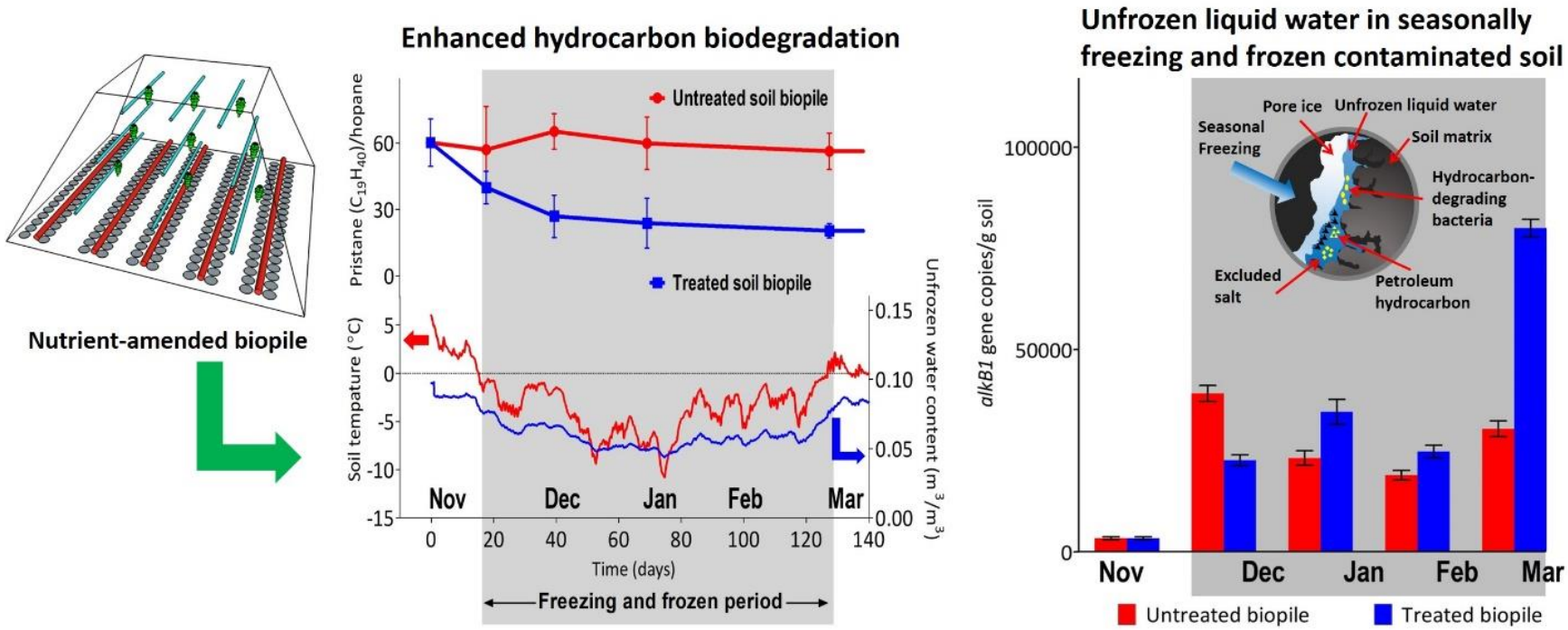

Figure 2.1. Graphical abstract of Chapter 2.

Highlights

- Pilot-scale soil biopiles operated from winter to summer at a cold-climate site.
- Nutrient amendment enhanced hydrocarbon biodegradation during seasonal freeze-thaw.
- Unfrozen water remained detectable in the partially and deeply frozen biopiles.
- The *alkB1* gene copy numbers increased in the partially thawed soil below 0 °C.
- The microbial community compositions shifted during seasonal soil freeze-thaw.

2.1 Abstract

A pilot-scale biopile field experiment for nutrient-amended petroleum-contaminated fine-grained soils was performed over the winter at a cold-climate site. The rate and extent of hydrocarbon biodegradation and microbial responses were determined and corresponded to the on-site soil phase changes (from unfrozen to partially frozen, deeply frozen, and thawed) associated with natural seasonal freeze-thaw conditions. Treated and untreated biopiles were constructed (~3,500 kg each) on an open outdoor surface at a remediation facility in Saskatoon, Canada. The treated biopile received N-P-K-based nutrient and humate amendments before seasonal freezing. Real-time field monitoring indicated significant unfrozen water content in the treated and untreated biopiles throughout the freezing period, from the middle of November to early March. Unfrozen water was slightly more available in the treated biopile due to the aqueous nutrient supply. Soil CO_2 production and O_2 consumption in the treated biopile were generally greater than in the untreated biopile. Total removal percentages for F2 (>C10-C16), F3 (>C16-C34), and total petroleum hydrocarbons (TPH) in the treated biopile were 58, 57, and 58%, respectively, of which 26, 39, and 33% were removed during seasonal freezing and early thawing between November to early March. F3 degradation largely occurred during freezing while F2 hydrocarbons were primarily removed during thawing. Biomarker-based hydrocarbon analyses confirmed enhanced biodegradation in the treated biopile during freezing. The soil treatment increased the first-order rate constants for F2, F3, and TPH degradation by a factor of 2 to 7 compared to the untreated biopile. Shifts in bacterial community appeared in both biopiles as the biopile soils seasonally froze and thawed. Increased *alkB1* gene copy numbers in the treated biopile, especially in the partially thawed phase during early thawing, suggest extended hydrocarbon biodegradation to the seasonal freeze-thaw season, due to the nutrients supplied prior to seasonal freezing.

2.2 Introduction

Cold-climate soil environments are vast, unique, and environmentally sensitive. Large numbers of cold-climate sites, including those in sub-polar and polar regions, have been impacted by petroleum hydrocarbons mainly associated with intensified industrial and human activities

(Aislabie et al., 2004; Filler et al., 2015; Siciliano et al., 2008; Snape et al., 2008). Bioremediation has been frequently considered a less destructive remediation technology for petroleum hydrocarbon-contaminated soils in cold climates, including sites in the high Arctic and Antarctica (Aislabie et al., 2006; Camenzuli and Freidman, 2015; Chang et al., 2010; Mair et al., 2013; Margesin, 2004; McWatters et al., 2016; Paudyn et al., 2008; Whyte et al., 2001). Stimulating indigenous cold-adapted hydrocarbon-degrading microbial populations by supplying nutrients (typically nitrogen and/or phosphorous) to cold site soils is effective for active summer remediation at many cold-region sites (e.g., >0 to ~20 °C) (Braddock et al., 1997; Delille et al., 2004; Ferguson et al., 2003; Martínez Álvarez et al., 2017; McCarthy et al., 2004; Thomassin-Lacroix et al., 2002; Walworth et al., 2001; Walworth et al., 2007; Zytner et al., 2001) and for extended treatment periods spanning several annual seasonal cycles (Leewis et al., 2013; McWatters et al., 2016; Paudyn et al., 2008; Sanscartier et al., 2009a).

Field observations of *in situ* soil CO_2 production and O_2 consumption during seasonal transition periods at northern petroleum-contaminated sites (Norway), and the associated modeling studies, suggest hydrocarbon biodegradation occurs in frozen soils during winter (Rike et al., 2003; Rike et al., 2005). A large-scale biopile remediation project at Antarctic sites demonstrated the successful biostimulation of petroleum hydrocarbon-contaminated soils exposed to an extreme cold climate over a 5-year period (McWatters et al., 2016). Laboratory-controlled soil microcosm experiments conducted at fixed sub-zero temperatures (e.g., -5 °C) and under repeated, cyclic soil freeze-thaw conditions have consistently indicated significant petroleum hydrocarbon biodegradation in nutrient-amended, petroleum-contaminated cold-climate soils (Børresen et al., 2007; Eriksson et al., 2001; Freidman et al., 2016; Karppinen et al., 2017). Chang et al. (2011)'s pilot-scale laboratory bioremediation experiment indicated hydrocarbon biodegradation in nutrient-amended petroleum-contaminated sub-Arctic soils subjected to site-representative seasonal freeze-thaw temperatures, which *slowly* decreased from 2 to -5 °C and then increased to 4 °C at estimated site-specific seasonal rates using a temperature-programmable cold room.

Fixed sub-zero temperatures, rapid soil freeze-thaw cycles, and natural seasonal freeze-thaw cycles might all create different soil microenvironments, especially in terms of unfrozen water availability in the soil matrices (Henry, 2007; Olsson et al., 2003). The rates of soil freezing and thawing (slow or rapid), along with the soil type, composition, surface area, and salinity,

regulate the retention of unfrozen liquid water, pore ice formation, and solute partitioning into unfrozen liquid water (Andersland and Ladanyi, 2004; Konrad and McCammon, 1990; Marion, 1995). Natural seasonal soil freeze-thaw processes foster a progressive transition in the thermal phases of cold-region soils, with the exception of surface soils in the topmost few centimeters of cold ground that are most affected by daily temperature fluctuations and other factors such as sunlight and wind speed. In deeper soils subjected to slow seasonal freeze-thaw cycles, solutes (i.e., nutrients) might be excluded from pore ice and potentially remain available in the unfrozen water (Marion, 1995). The average rate of *in situ* seasonal freezing and thawing in on-site soils is generally slow (Chang et al., 2011; Henry, 2007; Konrad and McCammon, 1990; Olsson et al., 2003). Unfrozen water in freezing and frozen soils is regarded as a prerequisite for microbial survival at sub-zero temperatures (Clein and Schimel, 1995; Panikov et al., 2006; Rivkina et al., 2000). Decreasing unfrozen water content (or unfrozen water film thickness) during soil freezing is correlated with decreasing microbial metabolic activity (Rivkina et al., 2000).

To date, hydrocarbon biodegradation potential has not been studied in detail in contaminated cold-climate soils subjected to seasonal freeze-thaw conditions characterized by gradual thermal phase changes from unfrozen to partially frozen, deeply frozen, partially thawed, and completely thawed using field data. Field bioremediation studies for remote sites in extreme cold climates (e.g., sub-Arctic, high Arctic, and Antarctica) rely largely on short summers to access the sites for soil treatment, sampling and monitoring campaigns, typically once per year (McWatters et al., 2016; Sanscartier et al., 2009a). Few detailed time-dependent datasets exist for hydrocarbon degradation and microbial responses corresponding to sequential changes in soil thermal phases, despite the fact that such field data could reveal an important link between hydrocarbon-degrading microbial survivors and the potential to extend biodegradation activity in contaminated soils during their natural seasonal soil freeze-thaw processes. Such detailed datasets for various cold-climate seasonalities, from moderate to extreme, are lacking.

This field study aimed to demonstrate the rate and extent of hydrocarbon biodegradation, as well as microbial community responses, corresponding to on-site soil thermal phase changes in outdoor pilot-scale biopiles subjected to natural seasonal freeze-thaw temperatures. The treated and untreated (control) biopiles were installed using field-aged, petroleum hydrocarbon-contaminated soils on a large open-surface area of a soil remediation facility in Saskatoon

(52º08´N 106º41´W; Saskatchewan, Canada). Sampling soil at each soil thermal phase, defined based on ambient air and soil temperatures and corresponding unfrozen water content, is very critical in soil freeze-thaw experiments for addressing biodegradation potential throughout a winter season (Henry, 2007). Here, real-time field monitoring of ambient air and soil temperatures and volumetric unfrozen water content in both biopiles was conducted, allowing responsive soil sampling for hydrocarbon and microbial community analyses when the biopile soils were unfrozen, partially frozen, deeply frozen, partially thawed, and completely thawed in the field. To the best of the authors' knowledge, such hydrocarbon and microbial field data in association with detailed observations of on-site seasonal soil thermal phase changes are currently lacking.

2.3 Materials and methods

2.3.1 Contaminated soil

The physicochemical and microbial properties of the site soils are presented in Table 2.1. Briefly, the clayey fine-grained soil (46% w/w coarse-grained and 54% w/w fine-grained soil particles based on the Unified Soil Classification System) had a gravimetric water content and soil pH of 15.3% (w/w) and 7.6, respectively. Soluble inorganic nitrogen species from soil extracts, including nitrite, nitrate, and ammonia, were deficient whereas total phosphorus was not limited (432.5 ± 12.6 mg/kg). Viable hydrocarbon-degrading bacteria were enumerated at approximately 2.3×10^4 CFU (colony forming units) per gram of soil using Bushnell Haas nutrient-media plates spiked with 1% (v/v) diesel as the sole carbon source.

Based on the Canada-Wide Standard for Petroleum Hydrocarbons in Soil (CWS PHC) Tier 1 Method, hydrocarbon concentrations in soils are determined for specified hydrocarbon fractions: F1 (C6-C10), F2 (>C10-C16), F3 (>C16-C34), and F4 (>C34) (CCME, 2001). Semi-volatile hydrocarbons (F2) and non-volatile hydrocarbons (F3) were the dominant hydrocarbon fractions in the site soils at concentrations of 2385 ± 477.3 mg/kg and 2811 ± 507.5 mg/kg, respectively, while F1 and F4 hydrocarbons were negligible.

Table 2.1. Physicochemical and microbial characteristics of the field-aged petroleum hydrocarbon-contaminated site soil.

Soil type	Coarse grain (%, w/w) Fine grain (%, w/w)	46 54
Gravimetric soil moisture	%	15.4
Soil pH	As $CaCl_2$ extract	7.6
Petroleum hydrocarbons (mg/kg)	F1: >C6 – C10 F2: >C10 – C16 F3: >C16 – C34 F4: >C34 – C40	ND[b] 2,385 ± 477.3 2,811 ± 507.5 ND[b]
Nutrient (nitrogen and phosphorus)	Total organic carbon (%) Soluble nitrite (mg/kg) Soluble nitrate (mg/kg) Soluble ammonia (mg/kg) Total phosphorous (mg/kg)	0.53 ± 0.11 ND[b] 0.18 ± 0.29 0.62 ± 0.07 433 ± 12.6
Viable hydrocarbon-degrader[a]	CFU/g	$2.3 \pm 0.58 \times 10^4$
Nutrient amendment	N (mg N/kg) P (mg P/kg) Humate	166 505 2% (w/w)

[a]: Bushnell Hass plate with 1% (v/v) diesel (sterilized).
[b]: ND: Not detected.

2.3.2 Biopile construction

A set of three pilot-scale biopiles with trapezoidal cross-sections were constructed on the ground at an outdoor soil treatment facility (PINTER & Associates, Saskatoon, Saskatchewan, Canada) (Fig. 2.2). Saskatoon, Saskatchewan is a Canadian prairie city where the climate is generally cold semi-arid (BSk) and humid continental (Dfb), based on the Köppen-Geiger classification (Peel et al., 2007).

Approximately 3500 kg of wet site soil (measured bulk density of 1.29 g/cm^3) were used to construct each biopile measuring approximately 1 m high, 2 m wide at the bottom, and 1 m wide at the top (Fig. 2.2). The biopiles were well homogenized when they were constructed layer-by-layer. A total of nine perforated soil gas collection tubes with two-stage valves were horizontally embedded in each biopile. A drainage collection system consisting of clean gravel and five polyvinyl chloride (PVC) pipes was installed at the bottom of the biopiles.

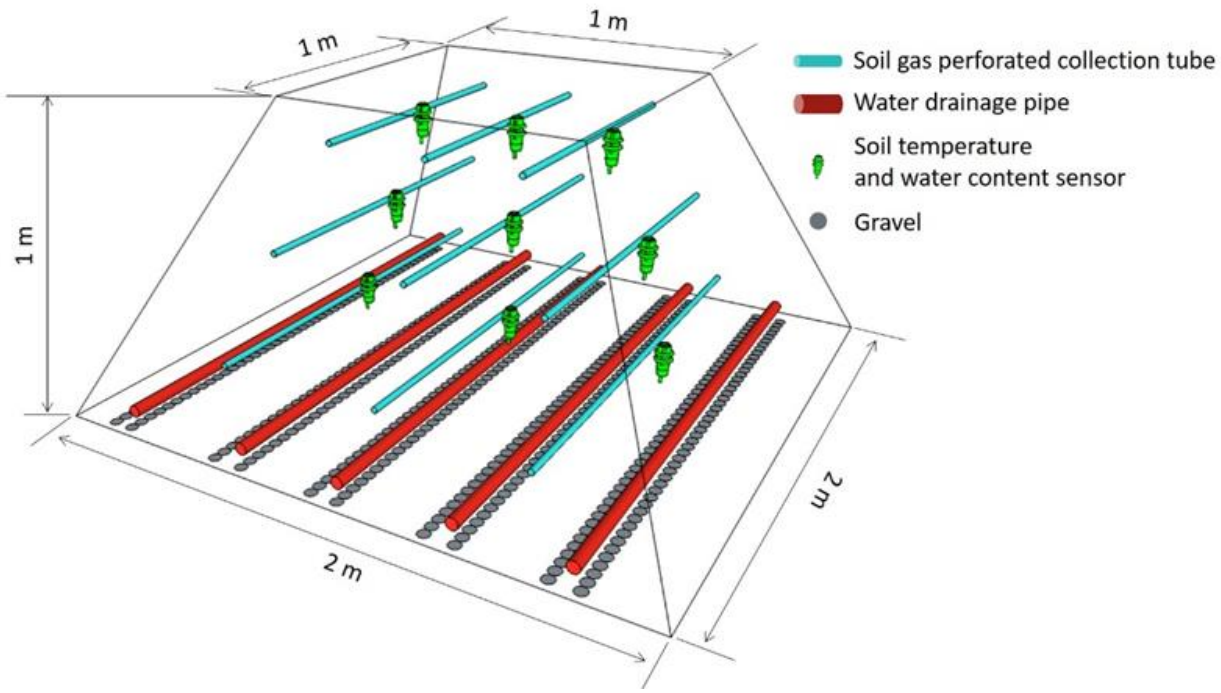

Figure 2.2. Schematic diagram of the pilot-scale biopile.

The three biopiles included one treated, one untreated, and one dummy biopile. The treated biopile received a water-based fertilizer solution and a humate soil amendment (Section 2.3.3). The untreated biopile was unamended and used as a control. The treated and untreated biopiles were loosely covered with tarpaulin covers. The dummy biopile was also untreated and had no tarpaulin cover. The soil temperatures and unfrozen water contents of the uncovered (dummy) and covered biopiles were nearly identical. No effect from the tarpaulin cover was indicated, and therefore this paper henceforth only reports data for the treated vs. untreated biopiles. The field experiment started at the beginning of November 2015 and ran until mid-July 2016, for a total experiment duration of 260 days. The outdoor biopiles were intended to be operated as passive biopiles requiring no maintenance during the winter. Extra air was not supplied as the biopiles were designed to take advantage of ambient air to maintain aerobic conditions.

2.3.3 Soil treatment

The sterilized nutrient solution and humate amendment (autoclaved at 121 °C, 20 min) were carefully spread layer-by-layer when the treated biopile was constructed. The favorable nutrient doses (between C/N ratios of 100:0.5 and 100:2) specific to the site soils were pre-determined by separate soil microcosm experiments at 25 °C, prior to the present pilot-scale field study (Kim and Chang, 2017). Based on the preliminary work, commercial 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®) was applied to achieve soil nutrient concentrations of 166 mg N/kg and 505 mg P/kg. The humate, with a mean particle size of 1.2 mm (Black Earth Organic Humic and Fulvic Acid, Canada), was used to provide additional surface area for microbial attachment and survival as well as to retain unfrozen water. The humate was minimally added at 2% (w/w) to maintain a neutral soil pH (Fig. C.2). No negative effects or interference with hydrocarbon extraction recovery from the site soils were associated with the minimal humate addition (Figs. C.3 and C.4).

2.3.4 Field monitoring and responsive soil sampling

A total of 18 probes (5TM probes, Decagon Devices, Pullman, USA) with three data loggers (EM50, Decagon Devices, Pullman, USA) were embedded in the treated and untreated

biopiles for real-time monitoring of soil temperature and volumetric water content. The probes were installed in triplicate in the top (60-90 cm), middle (30-60 cm), and bottom (0-30 cm) layers of both biopiles, and took measurements at 6-h intervals for the entire experimental period. Ambient air temperature surrounding the biopiles was also monitored in six-hour intervals using Temperature Data Loggers (Dickson®, VWR Canada) during the 260-day seasonality-based bioremediation experiment. The snow depth in the field was measured manually (Supplementary Data). Soil gases (CO_2 and O_2) were monitored using portable gas detectors equipped with infrared and electrochemical sensors for CO_2 and O_2 gases, respectively (MX6i multi gas detectors, Industrial Scientific, Pittsburgh, USA), as similarly described in Chang and Ghoshal (2014).

The sampling timing and associated soil phases are specified in Fig. 2.3. The partially frozen phase of the soils refers to the soil phase when free unfrozen water content gradually decreases as the soil temperatures decreased below its freezing-point depression, as suggested by Kozlowski (2003). In the partially frozen soils, unfrozen and frozen water coexist (Chang et al., 2011). As soil temperatures further decrease, the soils become deeply frozen. However, non-freezable water could exist, as indicated by the plateau in the unfrozen water content profile (Chang et al., 2011; Kozlowski, 2003; Olsson et al., 2003). The soil samples were aseptically and randomly collected at the three points within each of the three layers (top, middle and bottom), providing a total of ~1.5 kg of soil in total for each sampling day. The sampling days are associated with the seven soil thermal phase changes that occur seasonally. The soil samples were shipped in an ice box, and stored at -20 °C in the Environmental Engineering Laboratory at the University of Saskatchewan for the downstream analyses.

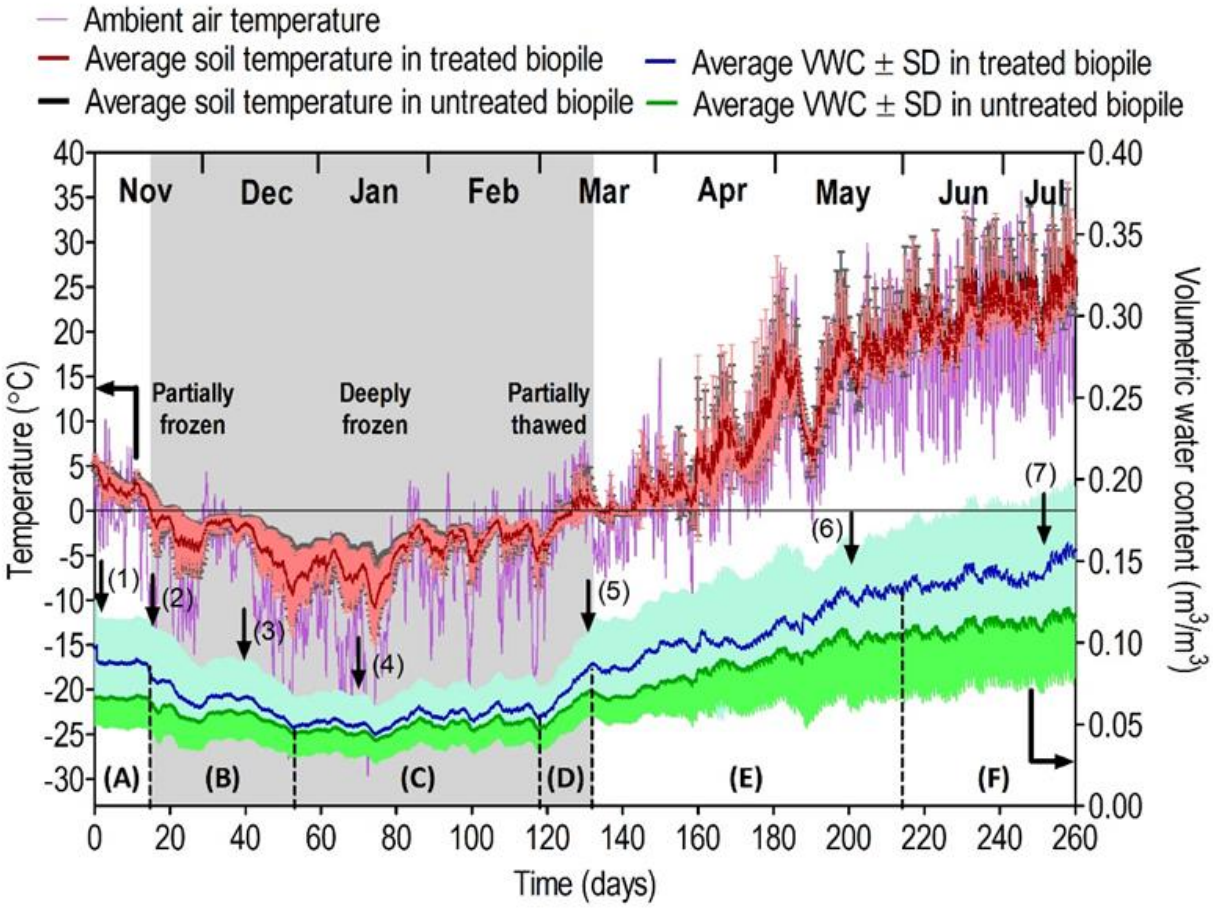

Figure 2.3. The monitored ambient air temperature surrounding the biopiles is presented (purple line). The soil temperature and volumetric water content (VWC) averaged for the surface, middle, and bottom layers of the treated and untreated biopiles. The average soil temperatures of the treated and untreated biopiles were nearly identical (soil temperatures of the untreated biopile are not visible as a result and are therefore presented again in Supplementary Fig. C.1). The portions highlighted in light red and blue or green show the standard deviations (SD) of the mean soil temperature and volumetric unfrozen water content, respectively. The arrows numbered (1) to (7) refer to the soil sampling points related to seasonal soil thermal phase changes. Corresponding time periods for each soil thermal phase are indicated: (A) unfrozen phase, (B) partially frozen, (C) deeply frozen, (D) partially thawed, (E) thawed, and (F) summer.

2.3.5 Petroleum hydrocarbon analyses

For the petroleum hydrocarbon analyses, 9 to 15 replicate soil samples were used per soil layer, per sampling day, per biopile (total of 378 soil samples minimum). The protocol for petroleum hydrocarbon analyses is based on the CWS PHC Tier 1 Method, described in the Supplementary Data section, and further details are available in the author's publications (Chang et al., 2010; Chang et al., 2011).

2.3.6 High-throughput sequencing

Genomic DNA was extracted using PowerSoil® DNA Isolation Kits (MoBio Laboratories, USA) immediately when the homogenized soil samples were shipped to the lab, based on the manufacturer's instructions with minor modifications (Chang et al., 2013). The concentration and purity of DNA extracts were determined by A260/280 and A260/230 ratios using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technology, USA). A total of 14 DNA samples from the middle layer, per biopile, were submitted to RTL Genomics (Lubbock, Texas, USA) for *Illumina* MiSeq analyses of 16S rRNA V4 amplicons (with 515F/806R primer) based on paired-end sequencing (Kennedy et al., 2014), as well as for the downstream bioinformatics analyses. The high-throughput sequencing data obtained were submitted to the BioProject archive under Accession Number PRJNA390770 at the National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/bioproject/). A rarefaction curve plot (species richness vs. sequence reads) was produced using R software (© 2016 The R Foundation). This study focuses only on bacterial communities due to the negligible presence of archaeal members (~0.1%) in both the treated and untreated biopile soils. The species richness of the bacterial communities was determined from the rarefaction plot, subsampling from 500 to 20000 reads in increments of 500 reads (Fig. C.5). The relative abundances of operational taxonomic units (OTUs) from the treated and untreated biopiles, sorted with a sequence similarity cut-off of 97%, were calculated. A heat map of the top 30 bacterial genera was then constructed based on the Bray-Curtis dissimilarity matrix (Clarke et al., 2006) and OTU relative abundances, and aligned with the seasonal phase changes of the biopile soils.

2.3.7 Gene copy numbers

A 3D digital polymerase chain reaction (PCR) system (QuantStudio™, Life Technologies) was used to quantify gene copy numbers (also called copy number variants, CNVs). The 3D Digital PCR system uses a high-density nanofluidic chip containing 20,000 reaction wells to partition a sample into thousands of independent PCR reactions, and generates the absolute concentrations of target genes (Dong et al., 2015). For each of the 7 sampling days (or soil thermal phases), the 3D digital PCR analyses were conducted for the 3 samples from the middle layer (triplicated runs), using 5 replicated individual DNA samples, resulting in a total of 105 DNA samples. The TaqMan® assay (Thermo Fisher Scientific Canada) was used for the target *alkB1* gene with a forward primer (5'- ATC TGG GCG CGT TGG GAT TTG AGC G-3'), a reverse primer (5'-CGC ATG GTG ATC GCT GTG CCG CTG C-3'), and a FAM™-dye labeled probe (5'-ACT CCG GAA GAT CCG GCG A-3'), as described in Whyte et al. (2002). The 14.5-µL reaction samples were prepared with 7.25 µL QuantStudio™ 3D PCR Master Mix v2 (QuantStudio™), 5 µL DNA, and 0.725 µL of TaqMan® assay (primer and probe mix), resulting in target sequence concentrations in the final reactions that generally ranged between 200 and 2,000 copies/µL. The reaction samples were then loaded onto the chips using a QuantStudio™ 3D Digital chip loader. The chips were sealed and loaded into a thermal cycler (ProFlex™ 2x Flat PCR System, Applied Biosystems). The PCR reactions were carried out using the following temperature conditions: 94 °C for 10 mins, followed by 39 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 2 mins. The data analysis was then performed using QuantStudio™ 3D Analysis Suite Cloud Software v3.0.2.2, which produced absolute concentrations of the target gene per µL of input DNA.

2.4 Results and discussion

2.4.1 Air and soil temperature regime

Fig. 2.3 presents the ambient air temperatures, soil temperatures in the biopiles taken as an average of the temperatures measured at three depths in the treated and untreated biopiles (surface, middle, and bottom), along with the corresponding unfrozen water content data. The ambient air temperature at the site decreased from 3 ± 6.5 °C in early November to -30 ± 0.8 °C by mid-

January, then increased to 5 ± 6.2 °C by the end of April and rose further to 29 ± 9.7 °C in the summer (mid-July).

Under the given conditions, the average soil temperatures of the treated and untreated biopiles (gray line: standard deviation) were nearly identical. The soil temperatures of the untreated biopile are not easily visible in Fig. 2.3, and are thus presented separately in Supplementary Fig. C.1. In the treated biopile, the initial soil temperature of 6 ± 0.3 °C in early November decreased to -10 ± 4.1 °C in mid-January, then increased to 19 ± 8.1 °C by the end of April and rose further to 28 ± 4.5 °C in the summer (mid-July). The soil temperature data indicate that the biopiles were subjected to a slow overall seasonal freezing rate of -0.17 °C/day from November and December, as expected, and a seasonal thawing rate of +0.1 °C/day from late February to the end of April.

2.4.2 Seasonal soil thermal phase changes and unfrozen water retention

While the seasonal freeze-thaw temperatures of the treated and untreated biopiles were nearly identical, the retained quantity of unfrozen water was different (Fig. 2.3). The unfrozen water content in the treated biopile was generally greater than in the untreated biopile, which was attributed to the aqueous nutrient supply. The initial mean volumetric water content at the beginning of the experiment in early November was 0.097 ± 0.029 m^3/m^3 in the treated biopile and 0.066 ± 0.017 m^3/m^3 in the untreated biopile ((A) in Fig. 2.3). Liquid water content in the biopile soils gradually decreased during seasonal freezing below near -1.5 °C from November to December ((B) in Fig 2.3), during which time unfrozen and frozen water coexisted, creating a partially frozen state within the contaminated soils. Based on the unfrozen water profiles for the untreated and treated biopiles in this study, along with the corresponding soil temperatures, free unfrozen water was available until at least mid-December (partially frozen, (B) in Fig. 2.3), as indicated by the slow decrease in unfrozen water content. Free water became available again beginning in early March (partially thawed, (D) in Fig. 2.3). When the soils were deeply frozen from January to February ((C) in Fig. 2.3), the plateau in the unfrozen water content profile was observed, suggesting that thin non-freezable unfrozen water films in isolated microsites were likely detected in the deeply frozen soil. Oxygen was not depleted in either biopile during the deeply frozen phase (>20% v/v) of this field study. Partially frozen microbial niches might be available

to cold-adapted hydrocarbon-degrading bacteria during both the freezing and thawing seasons as well as the deeply frozen phase. The lowest volumetric unfrozen water content measured during the deeply frozen phase in the treated biopile (~0.023 m^3/m^3 at around -10 °C) is more than one order of magnitude greater than the 0.001 m^3/m^3 measured at -4.4 °C in a previous study involving coarse-grained contaminated soils amended with similar nutrients (Chang et al., 2011). This is mainly due to the effect of soil type, as fine-grained soils with large surface areas and porous networks tend to retain greater amounts of unfrozen liquid water and exhibit lower freezing-depression temperatures than coarse-grained sandy soils (Andersland and Ladanyi, 2004). Field monitoring studies conducted in the Alaskan Arctic indicate that water may remain unfrozen over extended periods of seasonal freezing (3 to 6 months) (Olsson et al., 2003), during which detectable unfrozen water and ice coexist (Hinkel et al., 2001; Olsson et al., 2003).

As the weather warmed and the gradual thaw began, the unfrozen water content of the treated and untreated biopile soils increased to 0.11 ± 0.04 and 0.09 ± 0.03 m^3/m^3, respectively, by the end of April ((E) in Fig. 2.3). The water content then stabilized as the biopile soils completely thawed and slightly increased during the thawing season and summer (mid-July; (F) in Fig. 2.3) due to melting of snow and rainfall. Higher water content in the treated biopile than in the untreated biopile was maintained throughout the field experiment, especially in the thawing season and summer. The humate amendment may have positively influenced water retention in the treated biopile when additional water became available.

2.4.3 Extended microbial respiration activity

The on-site CO_2-O_2 soil gas data reflect enhanced microbial activity in the treated biopile compared to the untreated biopile. Fig. 2.4 shows the cumulative average CO_2 and O_2 soil gas concentrations measured from the treated and untreated biopiles (three measuring points per top, middle and bottom layer, per biopile). In particular, CO_2 production and O_2 consumption were measurable in the partially frozen soils in the treated biopile at values two to five times higher than in the untreated biopile. Microbial respiration activity in the treated biopile was suppressed in the deeply frozen phase; however, it did not cease, which suggests a delayed seasonal inactivation of microbial respiration activity. An early seasonal activation of microbial respiration activity in the treated biopile was then indicated at sub-zero temperatures as the soil was seasonally thawing in

early March, concurrent with the unfrozen water content beginning to increase (Section 2.4.2). This provides insight into a key factor for enhancing cold-adapted aerobic heterotrophs: they likely remain active longer in partially frozen soils before deep freezing occurs and activate earlier in partially thawed soils prior to the warm season, as suggested by Rike et al. (2003) and Rike et al. (2005). In particular, this study confirms increases in *alkB1* gene copy numbers corresponding to seasonal changes in microbial respiration activity in the partially frozen and thawed states (Fig. 2.9 and Section 2.4.10).

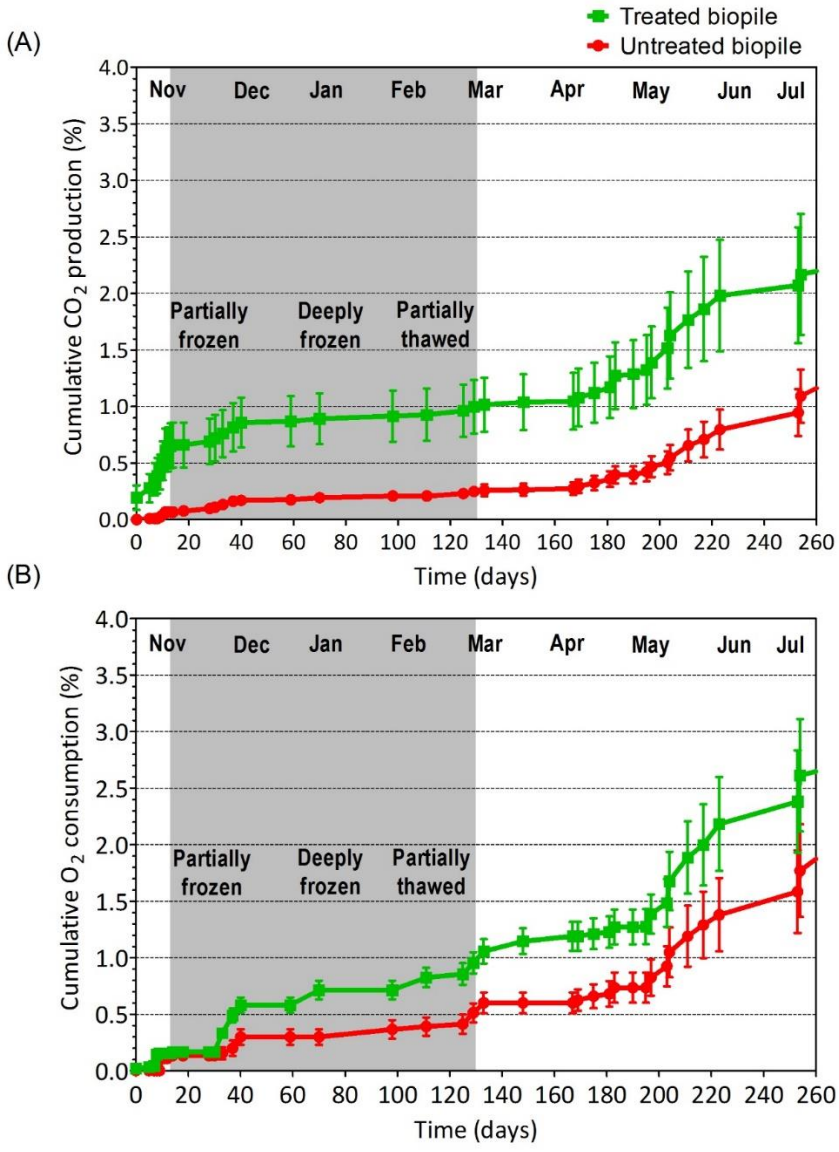

Figure 2.4. Cumulative CO_2 and O_2 soil gas concentrations from the treated and untreated biopiles. The error bars refer to the standard errors of the means (n = 8 or 9).

2.4.4 Enhanced hydrocarbon biodegradation under seasonal freeze-thaw conditions

Fig. 2.5 presents the GC-FID chromatograms for TPHs in soils from the treated and untreated biopiles when they were partially frozen (day 40), deeply frozen (day 70), and partially thawed (day 129). These chromatograms are overlapped with chromatograms for the corresponding initial soil TPHs. Decreases in TPHs at each soil thermal phase are easily visible, even in the unresolved complex mixture (UCM) represented by the 'hump' area of the chromatograms. Hydrocarbon degradation was notably enhanced in the treated biopile during seasonal freezing and early thawing. This is most likely due to the extended availability of unfrozen liquid water in the treated soils, the amended nutrient supply, and oxygen availability, which are required to extend the metabolic activity of cold-adapted hydrocarbon degraders. Remarkably, TPH removal was 33% in the treated soils by the time the soils were partially thawed.

Furthermore, using the robust biomarker 17α(H),21β(H)-hopane, identified in the site soils, the diagnostic hydrocarbon ratios for discerning hydrocarbon biodegradation from abiotic losses (nC16/hopane, nC19/hopane, pristane/hopanes, and phytane/hopane), along with the biomarker and TPH data from the untreated control biopile, support the assertion that biodegradation is the prevailing hydrocarbon removal mechanism in the treated biopile during seasonal freezing (Fig. 2.6).

2.4.5 Seasonality-induced sequential degradation of F3 to F2 hydrocarbons

Interestingly, TPH biodegradation in the seasonal freezing and deeply frozen phases of the treated soils was mainly due to the removal of heavier, non-volatile F3 hydrocarbons rather than lighter, semi-volatile F2 hydrocarbons (Fig. 2.7). The majority of the F2 hydrocarbons were subsequently removed during the thawing phase. F2 degradation was further enhanced in the summer season with both biotic and abiotic loss involved. The differences in the F2, F3, and TPH datasets are statistically significant (Fig. 2.7; two-way ANOVA, $p < 0.0001$). Bonferroni post-tests specifically indicate the statistical difference between hydrocarbon concentrations in the treated and untreated soils for each soil thermal phase. Previous biodegradation studies indicate that, in general, lighter hydrocarbons are more readily biodegradable than heavier hydrocarbons (de Jonge et al., 1997; Sanscartier et al., 2009b; Zytner et al., 2001). For example, de Jonge et al. (1997) reported a sequential degradation pattern from lighter to heavier hydrocarbons based on carbon number.

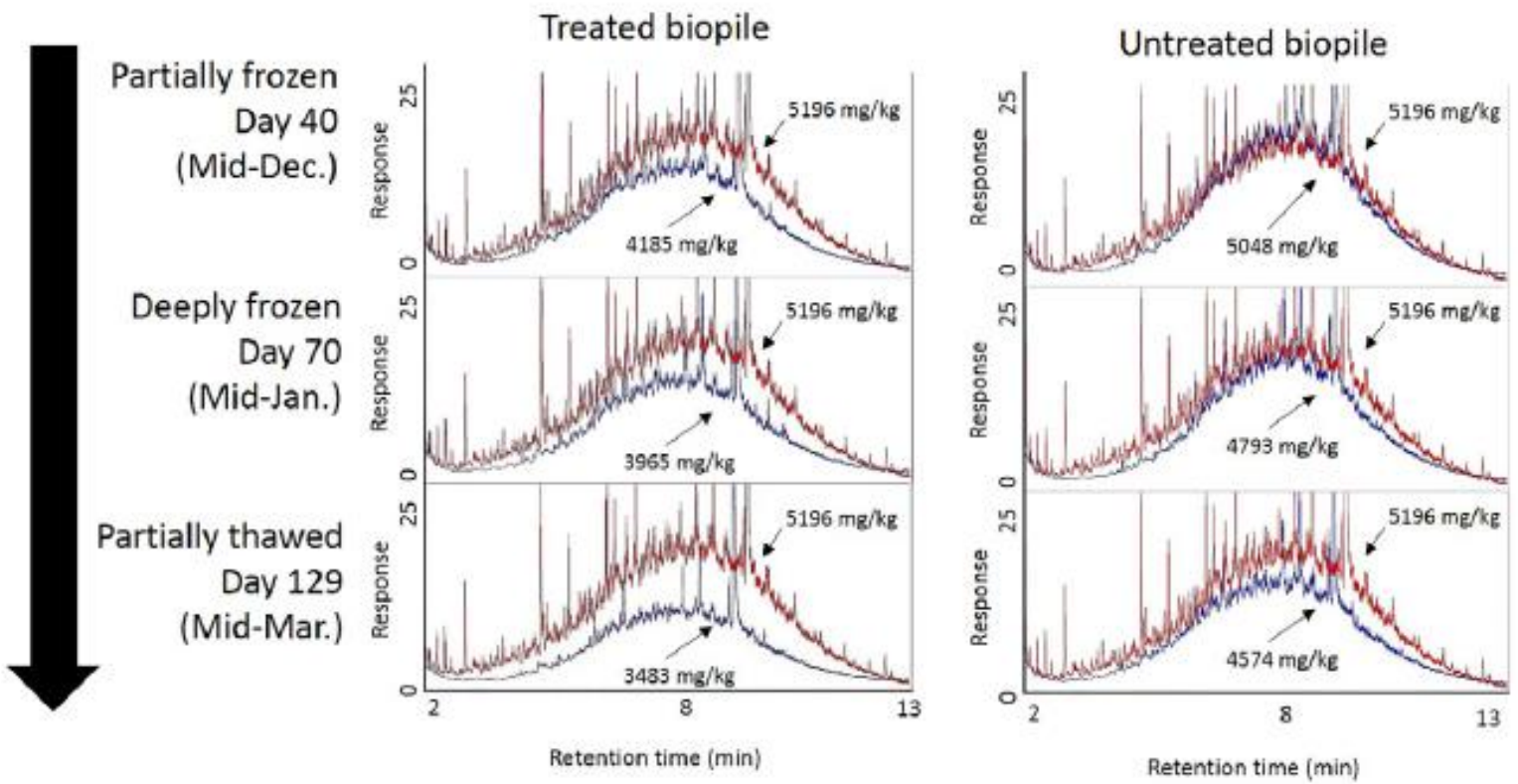

Figure 2.5. Overlapped representative GC chromatograms for hydrocarbons in the initial soil (red), and for partially frozen, deeply frozen and partially thawed soils (blue).

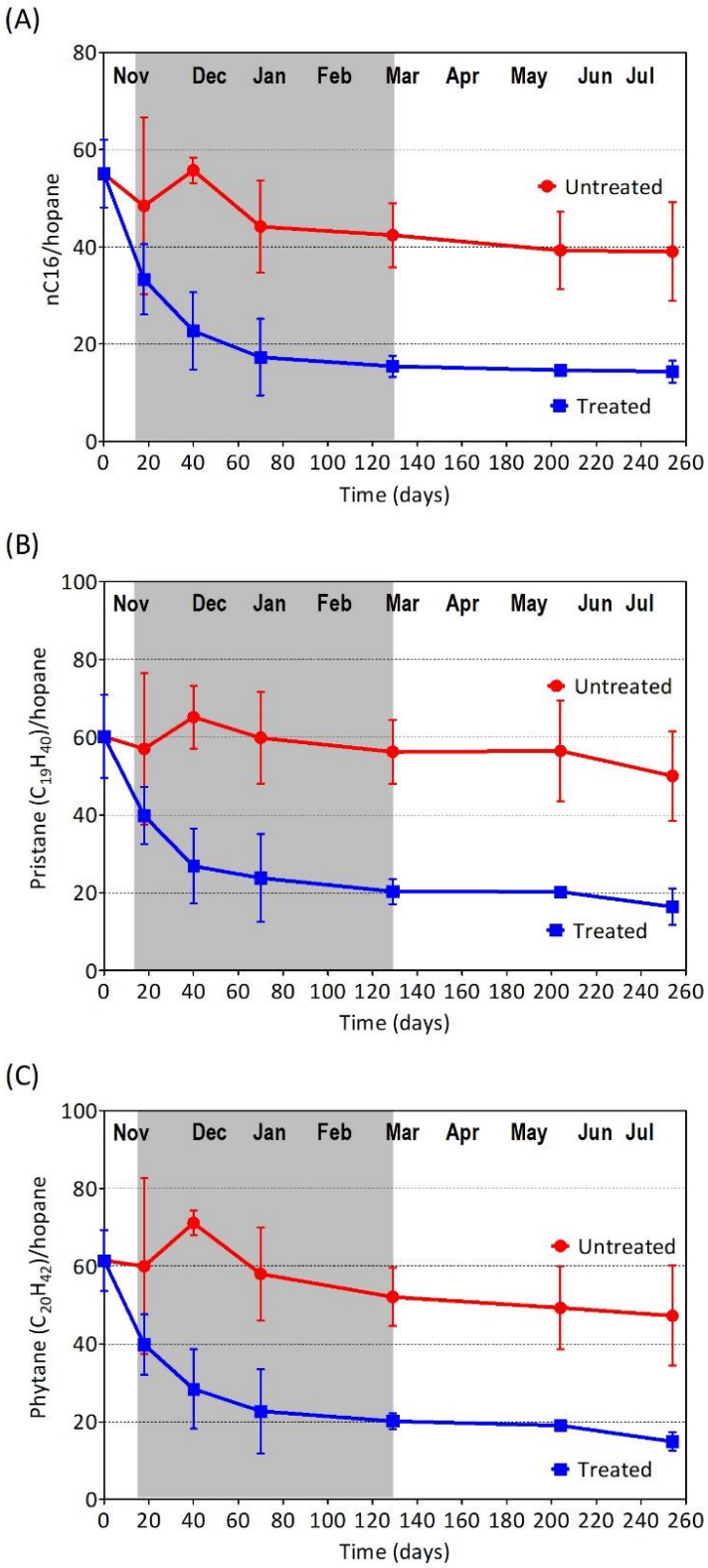

Figure 2.6. Hydrocarbon analyses using n-alkane (nC16) and branched alkanes (pristane and phytane) normalized with hopane. The error bars refer to the standard errors of the means (n = 9 for each soil phase).

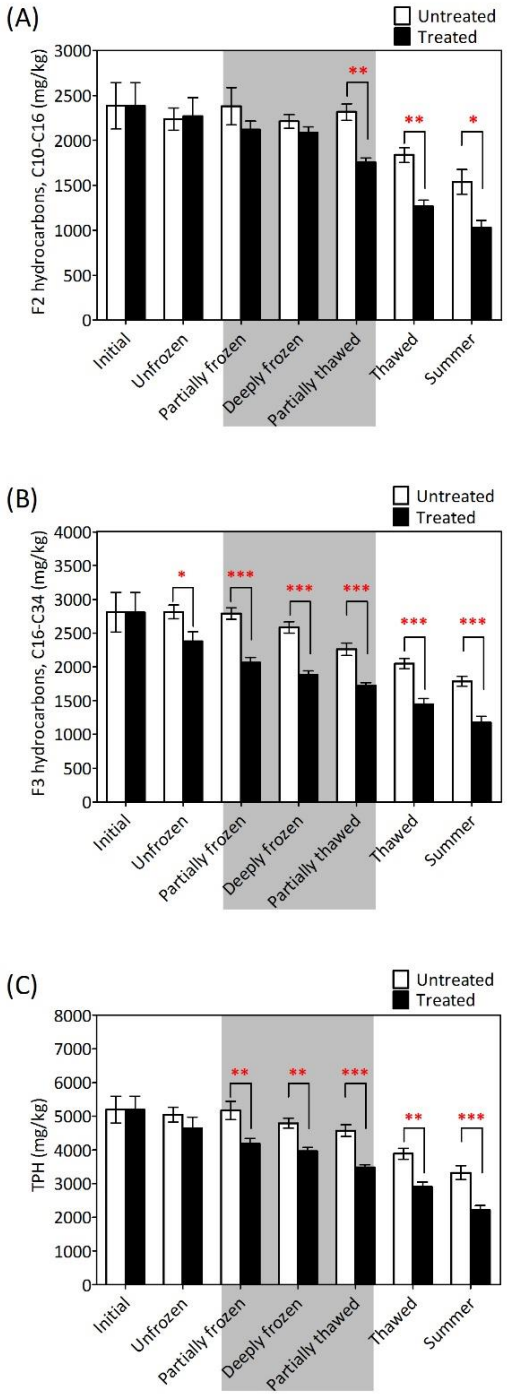

Figure 2.7. Petroleum hydrocarbon analyses for the treated and untreated biopile soils: F2 (A), F3 (B), and TPH (C). The error bars refer to the standard errors of the means (n = 9 for each soil phase). Two-way analysis of variance (ANOVA) with Bonferroni post-tests in GraphPad Prism 5 were used to verify the statistical significance of the soil hydrocarbon datasets. *, ** and *** refer to $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

In contrast, the observation in this study that F3 hydrocarbons were favorably degraded under freeze-thaw conditions suggests an apparent sequential degradation pattern from *heavier* to *lighter* hydrocarbons. Microbial hydrocarbon uptake mechanisms in seasonally freezing and frozen soils are not well understood. However, the pattern observed in this study is likely attributed to the reduced accessibility of more soluble F2 hydrocarbons trapped in frozen pore water during seasonal freezing, which would then become available again to bacteria later in the thawing season. Less soluble F3 hydrocarbons likely interact with unfrozen water in seasonally freezing and frozen soil matrices (Siciliano et al., 2008), and might still be accessible to microbial survivors, as similarly reported in Karppinen et al. (2017) and Chang et al. (2011), studies that indicated the degradation of F3 hydrocarbons in frozen or freezing contaminated soils with limited unfrozen water. Many indirect indicators (e.g., CO_2 production) suggest significant microbial activity in a variety of frozen microbial habitats, including frozen soils (pristine and petroleum-contaminated soils) and Arctic sea ice samples (Garneau et al., 2016; Monson et al., 2006; Rike et al., 2003; Tilston et al., 2010). In addition, increased salt concentrations in the unfrozen water from the nutrient supply in the freezing and frozen soils might cause the emergence of microbial populations tolerant to salinity. Indeed, halotolerant bacteria in Arctic soils have been identified (Mykytczuk et al., 2012). Hydrocarbon degrading *Dietzia maris* strain NWWC4 exhibits a dual tolerance to low temperatures and high salinity and has been isolated from non-saline petroleum-contaminated sub-Arctic soils (Chang et al., 2017) The inhibitory effects of high nutrient doses for the biostimulation of petroleum hydrocarbon-contaminated soils, such as osmotic stress, have been reported mainly for summer remediation (Braddock et al., 1997; Chang et al., 2010; Walworth et al., 2007); off-season soil treatments before long winters have not been extensively explored. Freezing temperatures and increases in salinity might have different effects on winter microbial communities, which shift in composition from the summer microbial communities under seasonal freeze-thaw conditions in cold-climate soils.

2.4.6 Meaningful hydrocarbon removal during the unconventional season

This field study observed high hydrocarbon removal efficiencies (%) in the pilot-scale biopiles over the course of the long winter, which is typically considered the off-season. Detailed F2, F3, and TPH removal efficiencies with respect to each seasonal soil thermal phase are tabulated

in Table C.1. From November to mid-January (from unfrozen to partially frozen and deeply frozen soil phases), the F2, F3, and TPH removal efficiencies were 13, 33, and 24% in the treated biopile, but only 7, 8, and 8% in the control (untreated) biopile. By mid-July, the final removal of F3 hydrocarbons was 58% (without tilling or active aeration), which corresponded to a final concentration of 1177 ± 30 mg/kg that is below the Canadian environmental standard of 1300 mg/kg for agricultural fine-grained soils (CCME, 2008). Furthermore, the remaining total inorganic N was 83 mg N/kg (down from ~166 mg N/kg after the nutrient supply), reflecting nutrient consumption through bioremediation. The soil treatment prior to seasonal soil freezing (e.g., before November) was therefore effective at enhancing indigenous hydrocarbon degraders and achieving meaningful and practical removal efficiencies during the long off-season, without active management of the contaminated soils. In addition to F3 hydrocarbon removal, overall F2 and TPH removal efficiencies were similarly high at 57% (1033 mg/kg) and 58% (2209 mg/kg), respectively.

In the untreated biopile, the final removal efficiency was 36% by mid-July (for all hydrocarbon fractions). The site soils exhibited considerable intrinsic attenuation of hydrocarbons (including abiotic and biotic losses), most likely due to the significant abundance of indigenous hydrocarbon degraders (Figs. 2.8 and 2.9). However, meaningful intrinsic attenuation of petroleum hydrocarbons was only indicated in the untreated biopile during thawing and summer.

2.4.7 Faster biodegradation during freezing than during thawing in the treated biopile

Hydrocarbon degradation kinetics were remarkably increased by a factor of two to seven in the treated vs. untreated biopiles due to the off-season soil treatment. Table 2.2 presents the detailed rate constants (d^{-1}) for hydrocarbon degradation based on the first-order kinetic model. Interestingly, in the treated biopile, the first-order rate constant for F3 degradation during seasonal freezing from November to mid-January (70 days) was 0.0066 d^{-1}, which is five times greater than that determined for the thawing period (0.0013 d^{-1}; 184 days). The lower rate constant for F3 hydrocarbon degradation during the thawing and summer season is due to lower hydrocarbon concentrations (i.e., less bioavailable hydrocarbons) after significant F3 biodegradation during seasonal freezing. The rate constant of 0.0066 d^{-1} for F3 hydrocarbon degradation during the freezing months was obtained when the site soil temperatures decreased from 6 ± 0.27 to -10 ±

4.1 °C in the field. This rate is generally comparable to first-order rate constants obtained from temperature-controlled laboratory studies and other field studies for petroleum-contaminated soils conducted under various low temperature regimes: 0.0036 d^{-1} (highest value) for F3 hydrocarbons at a fixed temperature of -5 °C (Karppinen et al., 2017), 0.009 d^{-1} for TPHs at a fixed 15 °C (Akbari and Ghoshal, 2014), 0.079 to 0.209 d^{-1} at above 0 °C of the biopile (Gomez and Sartaj, 2013), 0.0046 to 0.017 d^{-1} for TPHs at variable temperatures of -5 to 10 °C (Chang, 2013), 0.015 to 0.026 at around 3 °C during a summer season at a sub-Arctic site (Paudyn et al., 2008), and 0.0022 to 0.0043 d^{-1} for TPHs in the field at variable temperatures of 2 to 25 °C (Zytner et al., 2001). Therefore, the hydrocarbon degradation kinetics data produced in this study offer insight into the promising potential advantages of off-season soil treatments that aim to extend bioremediation, with reasonable hydrocarbon degradation rates, to unconventional seasons in cold climates that resemble the climate of the site used in this study.

2.4.8 Shifts in bacterial community compositions

Fig. 2.8 is a heat map constructed with the 30 most abundant genera in the middle layer of the treated and untreated biopiles. No notable bacterial community composition differences were indicated at the genus level across the depth profile in the homogenized biopiles (Fig. C.7). The heat map shows changes in the relative abundances of the top genera with corresponding soil thermal phases (unfrozen, partially frozen, deeply frozen, partially thawed, thawed, and summer). The bacterial community composition visibly responded to the seasonal freeze-thaw temperatures in the treated and untreated biopiles. Genera reported to contain hydrocarbon-degrading species are denoted with black dots next to the genus name. Various relatives of potential hydrocarbon-degrading bacteria or bacteria previously identified in hydrocarbon-contaminated sites were abundant in both the treated and untreated biopile soils, including *Thiobacillus*, *Rhodoferax*, *Pseudomonas*, *Pseudoxanthomonas*, *Sphingomonas*, *Luteimonas*, *Pedobacter*, *Mycobacterium*, *Hydrogenophaga*, *Sphingobium*, *Variovorax*, *Massilia*, *Nocardioides*, *Arthrobacter*, *Rhodococcus*, and *Polaromonas* (Aburto and Peimbert, 2011; Aislabie and Foght, 2008; Alonso-Gutiérrez et al., 2009; Margesin and Schinner, 2001). The high abundance of *Thiobacillus* in the site soils may be related to the considerable amounts of sulfur (3000 mg/kg) and iron (18333 mg/kg) elements detected in the field-aged site soils.

Table 2.2. First-order degradation rate constants, k (d^{-1}), for semi-volatile hydrocarbons (F2), non-volatile hydrocarbons (F3), and total petroleum hydrocarbons (TPH), and hydrocarbon removals percentage (%) in the treated and untreated biopiles.

		First-order degradation rate constant[a]					
		Freezing period[b]		Thawing period[b]		Entire period[b]	
		k (d^{-1})	R^2	k (d^{-1})	R^2	k (d^{-1})	R^2
F2	Treated	0.0022	0.88	0.0037	0.99	0.0029	0.97
	Untreated	0.0010	0.26	0.0015	0.72	0.0013	0.77
F3	Treated	0.0066	0.93	0.0022	0.95	0.0039	0.84
	Untreated	0.0009	0.69	0.0019	0.98	0.0016	0.96
TPH	Treated	0.0044	0.92	0.0027	0.96	0.0033	0.95
	Untreated	0.0009	0.64	0.0017	0.93	0.0015	0.93

		Hydrocarbon removal percentage (%)[c]					
		Unfrozen	Partially frozen	Deeply frozen	Partially thawed	Thawed	Summer
F2	Treated	5	11	13	26	47	57
	Untreated	6	6	7	7	23	36
F3	Treated	15	27	33	39	49	58
	Untreated	0	1	8	20	27	36
TPH	Treated	11	20	24	33	48	58
	Untreated	3	3	8	12	25	36

[a] The kinetic parameters for F2, F3, and TPH removal were determined based on the nonlinear curve-fitting method using GraphPad Prism 5 (GraphPad Software, USA).

[b] Freezing period: from Day 0 to Day 70; Thawing period: from Day 71 to Day 254; and Entire period = Freezing period + Thawing period.

[c] Hydrocarbon removal percentage (%) = $1 - [C]_i/[C_0]$, $[C]_i$ = hydrocarbon concentration, i = soil thermal phase, and $[C_0]$ = initial hydrocarbon concentration).

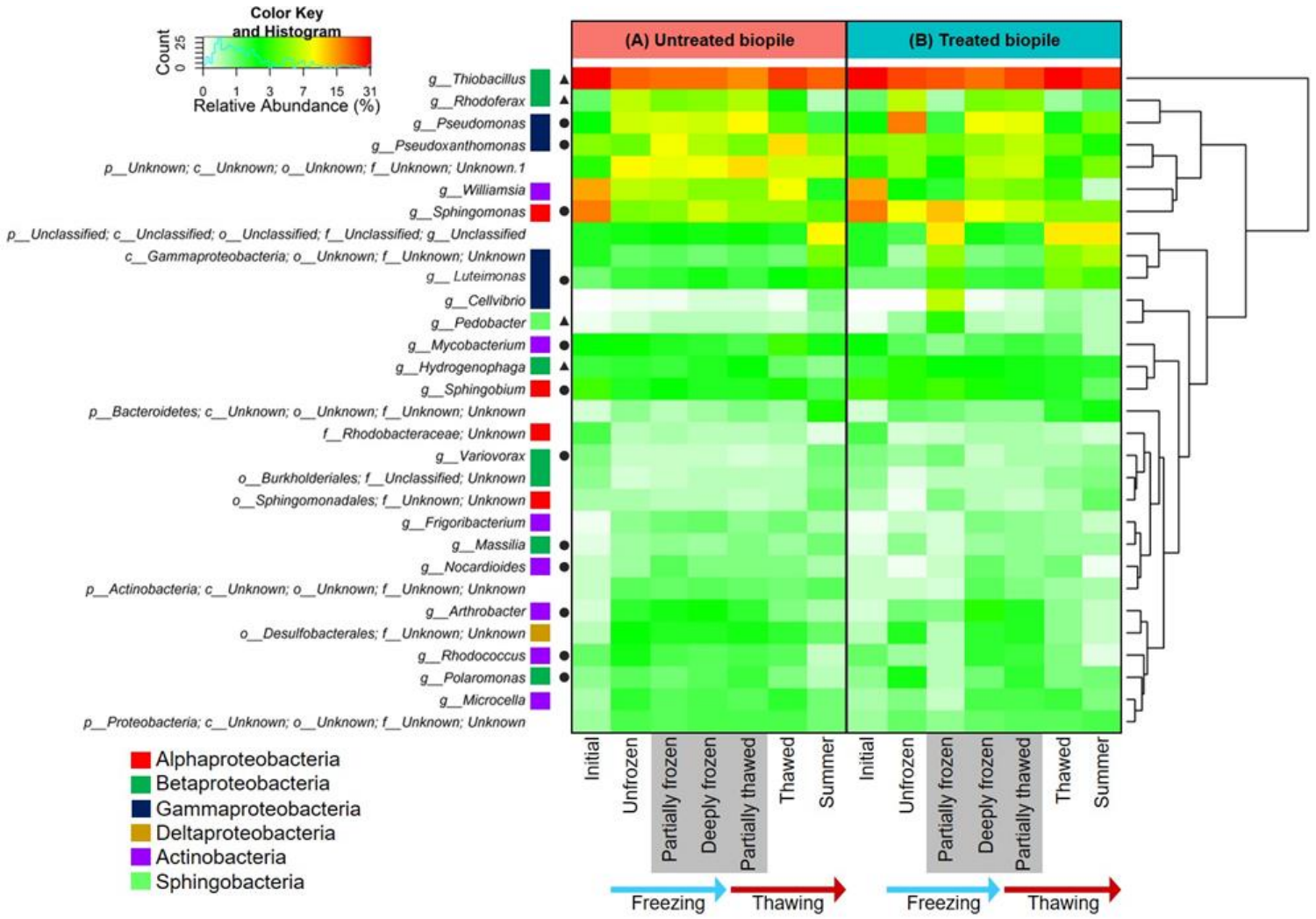

Figure 2.8. The constructed heat map showing the relative abundances of the top 30 bacterial genera. The heat map is aligned with the phases of the seasonal freeze-thaw sequence. Genera that include reported hydrocarbon-degrading species are marked by black dots. Genera that include bacterial species previously identified in hydrocarbon-contaminated environments are marked by black triangles.

In general, microbial community composition was influenced by seasonality. The heat map shows sequential changes in the time-dependent color intensities (relative abundances) of the microbial members across the soil phase changes, reflecting the seasonal shifts in microbial community composition. In the untreated biopile soils, for example, the relative abundance of the genera *Arthrobacter*, *Rhodococcus*, and *Polaromonas*, which include hydrocarbon-degrading species of the Actinobacteria phylum, tended to be greater during the freezing season compared to the summer, while the *Variovorax* genus was more abundant before seasonal freezing and in summer compared to the freezing season; this is a clear indicator of a seasonal shift in the composition of the bacterial community. In the treated biopile, the soil treatment also influenced the abundance of the several genera, especially during the soil phase change from unfrozen to partially frozen, based on the distribution patterns of the members in the heat map (Fig. 2.8). For instance, the relative abundance of *Pseudoxanthomonas*, *Sphingomonas*, *Luteimonas*, *Cellvibrio*, *Pedobacter*, and *Variovorax* appeared to be greater in the treated biopile than in the untreated biopile during the unfrozen or partially frozen phases. In addition to the seasonality, changes in hydrocarbon fractions (substrates) as biodegradation progressed may have influenced the shifts in microbial community compositions (Hu et al., 2017). Note that this study only focuses on qualitatively describing the bacterial community shifts associated with the soil phase changes (seasonality) and soil treatment, based on the changes in the distribution (color-intensity) of the relative abundances of the members specified in the heat map.

2.4.9 Increased functional gene copy numbers under seasonal freeze-thaw conditions

Using the 3D digital PCR technique that provides an absolute quantification of target genes, CNV values for the *alkB1* gene responsible for alkane hydrocarbon degradation were determined (Fig. 2.9). The *alkB1* gene copy numbers notably increased in both the treated and untreated biopile soils in the unfrozen phase after the biopiles were set up (day 18). The *alkB1* gene copy numbers during the seasonal freezing and thawing phases tended to be higher in the treated biopile than in the untreated biopile. Exposure to air and the soil treatment positively influenced hydrocarbon degraders in the initial unfrozen soil phase.

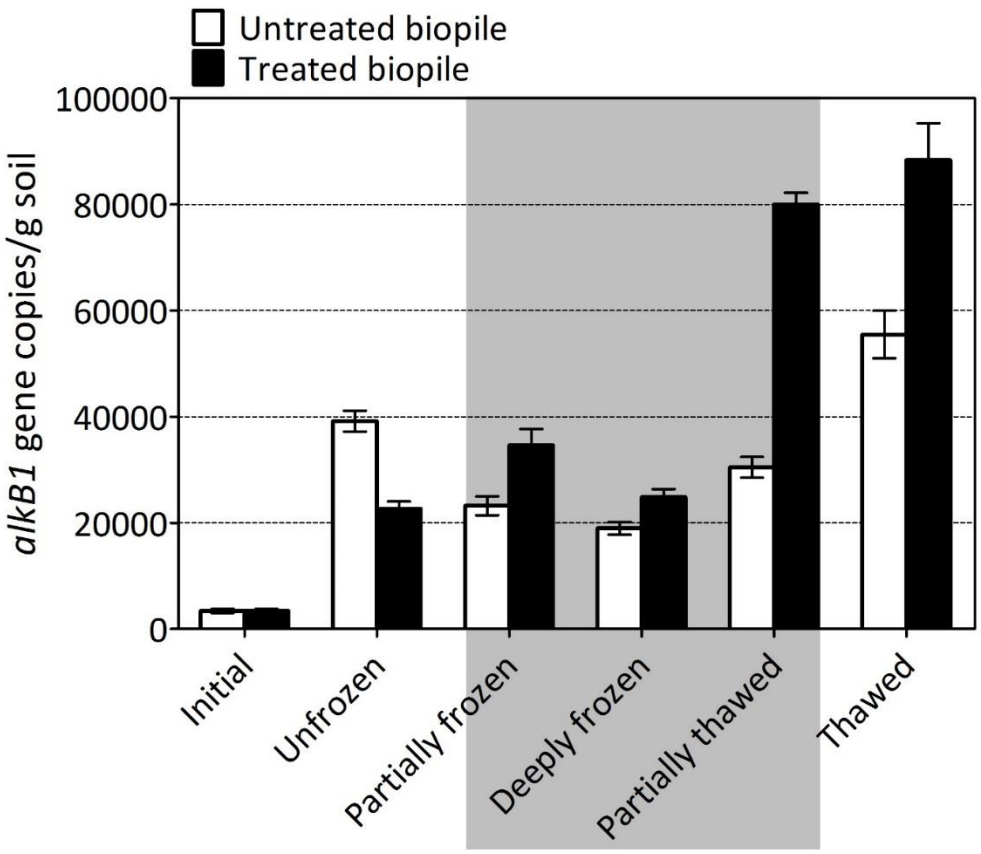

Figure 2.9. Changes in *alkB1* gene copy numbers with respect to the seasonal soil freeze-thaw sequence in the treated and untreated biopiles. *alkB1* gene copy numbers were determined using a 3D digital PCR system. The error bars refer to the standard errors of the means (n = 5 for each soil phase).

After that point, *alkB1* gene copy numbers in the untreated biopile generally decreased and remained lower than in the treated biopile. In the treated biopile, *alkB1* gene copy numbers continued to increase until the partially frozen phase, before slightly decreasing in the deeply frozen phase. However, a remarkable increase in *alkB1* gene copy numbers was observed in the partially thawed phase, reflecting the early seasonal activation of hydrocarbon degraders in the treated soils once free unfrozen water became available, albeit limited, at sub-zero temperatures during the early thawing period. The early start-up of microbial activity in partially thawed soils at sub-zero temperatures is a very important observation drawn from the field data produced in this study. Taking advantage of the prolonged activity and early activation of those populations is

the key requirement for successfully extending hydrocarbon bioremediation beyond the short conventional treatment season. It implies that early seasonal activation is a significant advantage gained from an off-season water-based soil treatment used to stimulate hydrocarbon degraders adapted to partially frozen, deeply frozen, and partially thawed soil environments.

2.5 Conclusions

This field study investigated the rate and extent of petroleum hydrocarbon biodegradation in two pilot-scale biopiles, one treated and one untreated, subjected to the natural seasonal freeze-thaw conditions in the field. The study also observed microbial community responses to seasonal soil thermal phase changes and the nutrient and humate soil amendments of the treated pile. The outcomes support the feasibility of supplying nutrients after summer to effectively extend petroleum hydrocarbon bioremediation to the winter off-season in fine-grained soils under seasonal climate conditions similar to those of the study site. This study provides extensive new field data and associated knowledge for improving bioremediation of petroleum-contaminated soils in cold climates. The key contributions of this study are summarized as follows.

First, the water-based nutrient supply and humate amendment were effective for enhancing biodegradation activity and for maintaining greater unfrozen water availability in the treated biopile during seasonal freeze-thaw, compared to the untreated biopile. On-site CO_2 production and O_2 consumption in the treated biopile did not completely cease during seasonal freeze-thaw. However, considerable abiotic and biotic removal of hydrocarbons occurred in the untreated biopile during the thawing phase and summer. Indigenous hydrocarbon-degrading bacteria were well established in the site soils, which exhibited significant intrinsic attenuation capability for hydrocarbons.

Second, petroleum hydrocarbon degradation continued in the treated biopile under seasonal freeze-thaw conditions, including when the biopile soils were partially frozen, frozen, and partially thawed. Enhanced biodegradation above and beyond abiotic losses was indicated in the treated biopile using biomarker-based hydrocarbon analyses. Heavier F3 hydrocarbons were preferentially degraded during the seasonal freezing period, and lighter F2 hydrocarbons were primarily removed in the subsequent thawing period and summer.

Third, the F2, F3, and TPH removal efficiencies of 57-58% in the treated passive biopile, with no energy requirements, were meaningful in practice. The soil treatment increased the first-order degradation rate constants by a factor of two to seven compared to the untreated biopile.

Fourth, seasonality and the soil treatment both influenced bacterial community composition. Various genera that include known hydrocarbon-degrading species were consistently or selectively abundant as the soils underwent seasonal freezing and thawing. The relative abundances of *Arthrobacter*, *Rhodococcus*, and *Polaromonas* were greater during freezing than in summer.

Fifth, *alkB1* gene copy numbers in the treated biopile generally increased throughout the study, except during the deeply frozen period, and were higher than in the untreated biopile. A notable increase in *alkB1* gene copy numbers was observed in the partially thawed treated soils (at sub-zero temperatures), which suggests an *early* seasonal activation of microbial activity due to the off-season soil treatment.

Overall, the findings of this study suggest a promising new bioremediation strategy for extending the effective duration of bioremediation at petroleum hydrocarbon-contaminated sites subjected to cold seasonal climatic conditions similar to those considered here.

2.6 Acknowledgements

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2.7 References

Aburto, A., Peimbert, M., 2011. Degradation of a benzene–toluene mixture by hydrocarbon-adapted bacterial communities. Ann. Microbiol. 61, 553-562.

Aislabie, J., Balks, M.R., Foght, J.M., Waterhouse, E.J., 2004. Hydrocarbon spills on Antarctic soils: Effects and management. Environ. Sci. Technol. 38, 1265-1274.

Aislabie, J., Foght, J., 2008. Hydrocarbon-degrading bacteria in contaminated cold soils, in: Filler, D.M., Snape, I., Barnes, D.L. (Eds.), Bioremediation of Petroleum Hydrocarbons in Cold Regions. Cambridge University Press, New York, pp. 69-83.

Aislabie, J., Saul, D.J., Foght, J.M., 2006. Bioremediation of hydrocarbon-contaminated polar soils. Extremophiles. 10, 171-179.

Akbari, A., Ghoshal, S., 2014. Pilot-scale bioremediation of a petroleum hydrocarbon-contaminated clayey soil from a sub-Arctic site. J. Hazard. Mater. 280, 595-602.

Alonso-Gutiérrez, J., Figueras, A., Albaigés, J., Jiménez, N., Vinas, M., Solanas, A.M., Novoa, B., 2009. Bacterial communities from shoreline environments (Costa da Morte, Northwestern Spain) affected by the Prestige oil spill. Appl. Environ. Microbiol. 75, 3407-3418.

Andersland, O.B., Ladanyi, B., 2004. Frozen ground engineering, second ed. John Wiley & Sons, Hoboken, New Jersey.

Braddock, J.F., Ruth, M.L., Catterall, P.H., Walworth, J.L., McCarthy, K.A., 1997. Enhancement and inhibition of microbial activity in hydrocarbon-contaminated Arctic soils: Implications for nutrient-amended bioremediation. Environ. Sci. Technol. 31, 2078-2084.

Børresen, M.H., Barnes, D.L., Rike, A.G., 2007. Repeated freeze–thaw cycles and their effects on mineralization of hexadecane and phenanthrene in cold climate soils. Cold Reg. Sci. Technol. 49, 215-225.

Camenzuli, D., Freidman, B.L., 2015. On-site and in situ remediation technologies applicable to petroleum hydrocarbon contaminated sites in the Antarctic and Arctic. Polar Res. 34, 24492.

CCME, 2001. Reference method for the Canada-wide standard for petroleum hydrocarbons in soil - tier 1 method. Canadian Council of Ministers of the Environment (CCME), Winnipeg, Manitoba, Canada.

CCME, 2008. Canada-wide standard for petroleum hydrocarbons (PHC) in soil: Scientific rationale. Canadian Council of Ministers of the Environment (CCME), Winnipeg, MB, Canada.

Chang, W., 2013. Bioremediation in northern climates: How can petroleum hydrocarbon biodegradation be assessed in soils under seasonal freeze-thaw conditions? Proceedings of the 2013 Northern Latitudes Mining Reclamation Workshop and 38th Annual Meeting of the Canadian Land Reclamation Association, Whitehorse, Yukon, pp. 21-31.

Chang, W., Akbari, A., David, C., Ghoshal, S., 2017. Selective biostimulation of cold- and salt-

tolerant hydrocarbon-degrading *Dietzia maris* in petroleum-contaminated sub-Arctic soils with high salinity. J. Chem. Technol. Biotechnol. (In press).

Chang, W., Akbari, A., Snelgrove, J., Frigon, D., Ghoshal, S., 2013. Biodegradation of petroleum hydrocarbons in contaminated clayey soils from a sub-Arctic site: The role of aggregate size and microstructure. Chemosphere. 91, 1620-1626.

Chang, W., Dyen, M., Spagnuolo, L., Simon, P., Whyte, L.G., Ghoshal, S., 2010. Biodegradation of semi-and non-volatile petroleum hydrocarbons in aged, contaminated soils from a sub-Arctic site: Laboratory pilot-scale experiments at site temperatures. Chemosphere. 80, 319-326.

Chang, W., Ghoshal, S., 2014. Respiratory quotients as a useful indicator of the enhancement of petroleum hydrocarbon biodegradation in field-aged contaminated soils in cold climates. Cold Reg. Sci. Technol. 106, 110-119.

Chang, W., Klemm, S., Beaulieu, C., Hawari, J., Whyte, L.G., Ghoshal, S., 2011. Petroleum hydrocarbon biodegradation under seasonal freeze−thaw soil temperature regimes in contaminated soils from a sub-Arctic site. Environ. Sci. Technol. 45, 1061-1066.

Clarke, K.R., Somerfield, P.J., Chapman, M.G., 2006. On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray–Curtis coefficient for denuded assemblages. J. Exp. Mar. Biol. Ecol. 330, 55-80.

Clein, J.S., Schimel, J.P., 1995. Microbial activity of tundra and taiga soils at sub-zero temperatures. Soil Biol. Biochem. 27, 1231-1234.

de Jonge, H., Freijer, J.I., Verstraten, J.M., Westerveld, J., Van der Wielen, F.W.M., 1997. Relation between bioavailability and fuel oil hydrocarbon composition in contaminated soils. Environ. Sci. Technol. 31, 771-775.

Delille, D., Coulon, F., Pelletier, E., 2004. Effects of temperature warming during a bioremediation study of natural and nutrient-amended hydrocarbon-contaminated sub-Antarctic soils. Cold Reg. Sci. Technol. 40, 61-70.

Dong, L., Meng, Y., Sui, Z., Wang, J., Wu, L., Fu, B., 2015. Comparison of four digital PCR platforms for accurate quantification of DNA copy number of a certified plasmid DNA reference material. Sci. Rep. 5, 13174.

Eriksson, M., Ka, J.O., Mohn, W.W., 2001. Effects of low temperature and freeze-thaw cycles on hydrocarbon biodegradation in Arctic tundra soil. Appl. Environ. Microbiol. 67, 5107-5112.

Ferguson, S.H., Franzmann, P.D., Revill, A.T., Snape, I., Rayner, J.L., 2003. The effects of nitrogen and water on mineralisation of hydrocarbons in diesel-contaminated terrestrial Antarctic soils. Cold Reg. Sci. Technol. 37, 197-212.

Filler, D.M., Kennicutt, M.C., Snape, I., Sweet, S.T., Klein, A.G., 2015. Arctic and Antarctic spills, in: Fingas, M. (Ed.), Handbook of Oil Spill Science and Technology. John Wiley & Sons, Inc, Hoboken, pp. 495-512.

Freidman, B.L., Gras, S.L., Snape, I., Stevens, G.W., Mumford, K.A., 2016. Effects of freeze–thaw phenomena on controlled nutrient release: Application to bioremediation. CLEAN–Soil, Air, Water. 44, 1739-1749.

Garneau, M.È., Michel, C., Meisterhans, G., Fortin, N., King, T.L., Greer, C.W., Lee, K., 2016. Hydrocarbon biodegradation by Arctic sea-ice and sub-ice microbial communities during microcosm experiments, Northwest Passage (Nunavut, Canada). FEMS Microbiol. Ecol. 92, fiw130.

Gomez, F., Sartaj, M., 2013. Field scale ex-situ bioremediation of petroleum contaminated soil under cold climate conditions. Int. Biodeterior. Biodegrad. 85, 375-382.

Henry, H.A.L., 2007. Soil freeze–thaw cycle experiments: Trends, methodological weaknesses and suggested improvements. Soil Biol. Biochem. 39, 977-986.

Hinkel, K.M., Paetzold, F., Nelson, F.E., Bockheim, J.G., 2001. Patterns of soil temperature and moisture in the active layer and upper permafrost at Barrow, Alaska: 1993–1999. Glob. Planet. Change. 29, 293-309.

Hu, P., Dubinsky, E.A., Probst, A.J., Wang, J., Sieber, C.M., Tom, L.M., Gardinali, P.R., Banfield, J.F., Atlas, R.M., Andersen, G.L., 2017. Simulation of *Deepwater Horizon* oil plume reveals substrate specialization within a complex community of hydrocarbon degraders. Proc. Natl. Acad. Sci. U.S.A. 114, 7432-7437.

Karppinen, E.M., Stewart, K.J., Farrell, R.E., Siciliano, S.D., 2017. Petroleum hydrocarbon remediation in frozen soil using a meat and bonemeal biochar plus fertilizer. Chemosphere.

Kennedy, K., Hall, M.W., Lynch, M.D.J., Moreno-Hagelsieb, G., Neufeld, J.D., 2014. Evaluating bias of *Illumina*-based bacterial 16S rRNA gene profiles. Appl. Environ. Microbiol. 80, 5717-5722.

Kim, J., Chang, W., 2017. Remediation in challenging environments: Preliminary numerical simulation and measurement of unfrozen water content for an outdoor pilot-scale biopile in cold climate. Proceedings of 2017 Canadian Society for Civil Engineering (CSCE) Annual Conference and Annual General Meeting, Vancouver, Canada.

Konrad, J.M., McCammon, A.W., 1990. Solute partitioning in freezing soils. Can. Geotech. J. 27, 726-736.

Kozlowski, T., 2003. A comprehensive method of determining the soil unfrozen water curves: 1. Application of the term of convolution. Cold Reg. Sci. Technol. 36, 71-79.

Leewis, M.C., Reynolds, C.M., Leigh, M.B., 2013. Long-term effects of nutrient addition and phytoremediation on diesel and crude oil contaminated soils in subarctic Alaska. Cold Reg. Sci. Technol. 96, 129-137.

Mair, J., Schinner, F., Margesin, R., 2013. A feasibility study on the bioremediation of hydrocarbon-contaminated soil from an Alpine former military site: Effects of temperature and biostimulation. Cold Reg. Sci. Technol. 96, 122-128.

Margesin, R., 2004. Bioremediation of petroleum hydrocarbon-polluted soils in extreme temperature environments, in: Singh, A., Ward, O.P. (Eds.), Applied Bioremediation and Phytoremediation. Springer, Berlin, pp. 215-234.

Margesin, R., Schinner, F., 2001. Biodegradation and bioremediation of hydrocarbons in extreme environments. Appl. Environ. Microbiol. 56, 650-663.

Marion, G.M., 1995. Freeze-Thaw Processes and Soil Chemistry, US Army Corps Engineers, Cold Regions Research and Engineering Laboratory (CRREL), Special Report 95-12.

Martínez Álvarez, L.M., Lo Balbo, A., Mac Cormack, W.P., Ruberto, L.A.M., 2017. Bioremediation of hydrocarbon-contaminated soils in cold regions: Development of a pre-optimized biostimulation biopile-scale field assay in Antarctica. Sci. Total Environ. 590, 194-203.

McCarthy, K., Walker, L., Vigoren, L., Bartel, J., 2004. Remediation of spilled petroleum hydrocarbons by in situ landfarming at an Arctic site. Cold Reg. Sci. Technol. 40, 31-39.

McWatters, R.S., Wilkins, D., Spedding, T., Hince, G., Raymond, B., Lagerewskij, G., Terry, D., Wise, L., Snape, I., 2016. On site remediation of a fuel spill and soil reuse in Antarctica. Sci. Total Environ. 571, 963-973.

Monson, R.K., Lipson, D.L., Burns, S.P., Turnipseed, A.A., Delany, A.C., Williams, M.W., Schmidt, S.K., 2006. Winter forest soil respiration controlled by climate and microbial community composition. Nature. 439, 711-714.

Mykytczuk, N.C., Wilhelm, R.C., Whyte, L.G., 2012. *Planococcus halocryophilus* sp. nov., an extreme sub-zero species from high Arctic permafrost. Int. J. Syst. Evol. Microbiol. 62, 1937-1944.

Olsson, P.Q., Sturm, M., Racine, C.H., Romanovsky, V., Liston, G.E., 2003. Five stages of the Alaskan Arctic cold season with ecosystem implications. Arct. Antarct. Alp. Res. 35, 74-81.

Panikov, N.S., Flanagan, P.W., Oechel, W.C., Mastepanov, M.A., Christensen, T.R., 2006. Microbial activity in soils frozen to below −39 °C. Soil Biol. Biochem. 38, 785-794.

Paudyn, K., Rutter, A., Rowe, R.K., Poland, J.S., 2008. Remediation of hydrocarbon contaminated soils in the Canadian Arctic by landfarming. Cold Reg. Sci. Technol. 53, 102-114.

Peel, M.C., Finlayson, B.L., McMahon, T.A., 2007. Updated world map of the Köppen-Geiger climate classification. Hydrol. Earth Syst. Sci. Discuss. 4, 439-473.

Prince, R.C., Elmendorf, D.L., Lute, J.R., Hsu, C.S., Haith, C.E., Senius, J.D., Dechert, G.J., Douglas, G.S., Butler, E.L., 1994. 17α(H),21β(H)-hopane as a conserved internal marker for estimating the biodegradation of crude oil. Environ. Sci. Technol. 28, 142–145.

Rike, A.G., Haugen, K.B., Børresen, M.H., Engene, B., Kolstad, P., 2003. In situ biodegradation of petroleum hydrocarbons in frozen Arctic soils. Cold Reg. Sci. Technol. 37, 97-120.

Rike, A.G., Haugen, K.B., Engene, B., 2005. In situ biodegradation of hydrocarbons in Arctic soil at sub-zero temperatures—field monitoring and theoretical simulation of the microbial activation temperature at a Spitsbergen contaminated site. Cold Reg. Sci. Technol. 41, 189-209.

Rivkina, E.M., Friedmann, E.I., McKay, C.P., Gilichinsky, D.A., 2000. Metabolic activity of permafrost bacteria below the freezing point. Appl. Environ. Microbiol. 66, 3230-3233.

Sanscartier, D., Laing, T., Reimer, K., Zeeb, B., 2009a. Bioremediation of weathered petroleum hydrocarbon soil contamination in the Canadian High Arctic: Laboratory and field studies. Chemosphere. 77, 1121-1126.

Sanscartier, D., Zeeb, B., Koch, I., Reimer, K., 2009b. Bioremediation of diesel-contaminated soil by heated and humidified biopile system in cold climates. Cold Reg. Sci. Technol. 55, 167-173.

Siciliano, S.D., Schafer, A.N., Forgeron, M.A.M., Snape, I., 2008. Hydrocarbon contamination increases the liquid water content of frozen Antarctic soils. Environ. Sci. Technol. 42, 8324-8329.

Snape, I., Acomb, L., Barnes, D.L., Bainbridge, S., Eno, R., Filler, D.M., Plato, N., Poland, J.S., Raymond, T.C., Rayner, J.L., Riddle, M.J., Rike, A.G., Rutter, A., Schafer, A.N., Siciliano, S.D., Walworth, J.L., 2008. Contamination, regulation, and remediation: An introduction to bioremediation of petroleum hydrocarbons in cold regions, in: Filler, D.M., Snape, I., Barnes, D.L. (Eds.), Bioremediation of Petroleum Hydrocarbons in Cold Regions. Cambridge University Press, New York, pp. 55-68.

Thomassin-Lacroix, E.J.M., Eriksson, M., Reimer, K.J., Mohn, W.W., 2002. Biostimulation and bioaugmentation for on-site treatment of weathered diesel fuel in Arctic soil. Appl. Microbiol. Biotechnol. 59, 551-556.

Tilston, E.L., Sparrman, T., Öquist, M.G., 2010. Unfrozen water content moderates temperature dependence of sub-zero microbial respiration. Soil Biol. Biochem. 42, 1396-1407.

Wang, Z., Yang, C., Fingas, M., Hollebone, B., Yim, U.H. and Oh, J.R., 2007. Petroleum biomarker fingerprinting for oil spill characterization and source identification, In: Wang, Z., Stout, S.A. (Eds.), Oil Spill Environmental Forensics, Academic Press, Burlington, pp. 73–146.

Walworth, J., Braddock, J., Woolard, C., 2001. Nutrient and temperature interactions in bioremediation of cryic soils. Cold Reg. Sci. Technol. 32, 85-91.

Walworth, J., Pond, A., Snape, I., Rayner, J., Ferguson, S., Harvey, P., 2007. Nitrogen requirements for maximizing petroleum bioremediation in a sub-Antarctic soil. Cold Reg. Sci. Technol. 48, 84-91.

Whyte, L.G., Goalen, B., Hawari, J., Labbé, D., Greer, C.W., Nahir, M., 2001. Bioremediation treatability assessment of hydrocarbon-contaminated soils from Eureka, Nunavut. Cold Reg. Sci. Technol. 32, 121-132.

Whyte, L.G., Schultz, A., Van Beilen, J.B., Luz, A.P., Pellizari, V., Labbé, D., Greer, C.W., 2002.

Prevalence of alkane monooxygenase genes in Arctic and Antarctic hydrocarbon-contaminated and pristine soils. FEMS Microbiol. Ecol. 41, 141-150.

Zytner, R.G., Salb, A., Brook, T.R., Leunissen, M., Stiver, W.H., 2001. Bioremediation of diesel fuel contaminated soil. Can. J. Civ. Eng. 28, 131-140.

CONNECTING TEXT: CHAPTER 2 AND 3

In Chapter 2, the enhanced hydrocarbon biodegradation during seasonal freezing and thawing was achieved with higher retention of unfrozen water content in the treated biopile. To understand the relationship between microbial activity and unfrozen water content at the soil phase change, soil respiration was estimated using a new soil respiration model that was developed by employing the input of soil temperature and water content in Chapter 3.

CHAPTER 3

MODIFIED SOIL RESPIRATION MODEL (URESP) EXTENDED TO SUB-ZERO TEMPERATURES FOR BIOSTIMULATED PETROLEUM HYDROCARBON-CONTAMINATED SUB-ARCTIC SOILS

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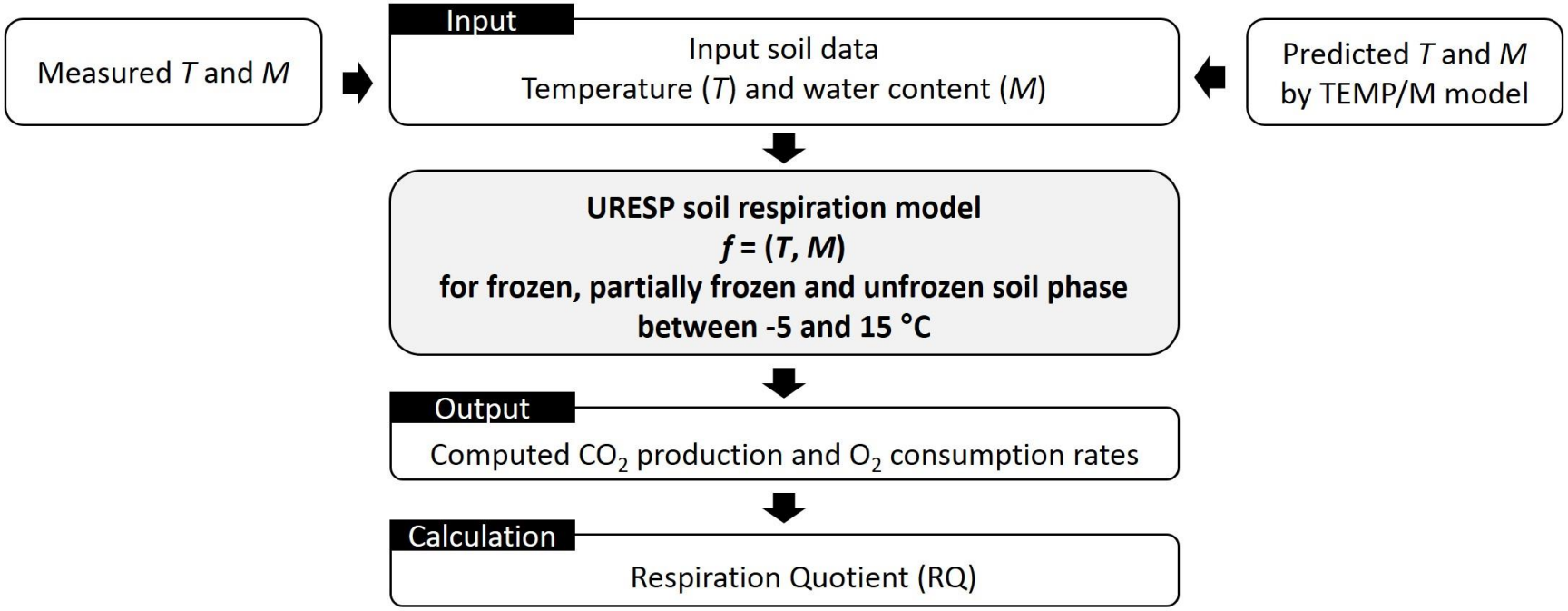

Figure 3.1. Graphical abstract of Chapter 3.

Highlights

- A newly modified soil respiration model (URESP) extends below 0 °C.
- The URESP model couples the effects of soil temperature and unfrozen water content.
- The URESP model was fitted to soil respiration rates measured between −5 and 15 °C.
- Respiration quotients (RQ) were derived from URESP-computed respiration rates.
- The URESP-derived RQs matched experimental RQs related to hydrocarbon degradation.

3.1 Abstract

It has been increasingly reported that aerobic soil respiration activity (CO_2 production and O_2 consumption) is measurable in frozen cold-climate soils. This study modifies the Generalized Respiration (GRESP) model, a function of soil temperature (*T*) and unfrozen water content (*M*), to cover the frozen, partially frozen and unfrozen phases of successfully bioremediated, petroleum hydrocarbon-contaminated, sandy sub-Arctic soils. The Michaelis-Menten equation was modified to express the observable change in unfrozen water content near 0 °C, which is related to soil respiration activity during soil phase changes and at temperatures below the effective endpoint of detectable unfrozen water at -2 °C. The modified Michaelis-Menten equation was further combined with a Q_{10} temperature term, and was then incorporated into the GRESP equation to produce a new URESP model for the engineered soil bioremediation system at sub-zero temperatures. The URESP model was applied to published input data measured from the biostimulated site soils of a pilot-scale soil tank experiment conducted between -5 and 15 °C. The model fit well with the experimental data for CO_2 production (R^2 = 0.96) and O_2 consumption (R^2 = 0.92). A numerical soil thermal model (TEMP/W model) of the thawing biotreated soils in the tank was also used in this study to produce valid alternative (predictive) input *T* and *M* data for the URESP model. The URESP-derived respiration quotients (RQ; 0.695 to 0.698), or the ratios of CO_2 production to O_2 consumption, aligned with the experimental RQ values from the soil tank experiment (0.69) and fell within the theoretical RQ range for aerobic hydrocarbon degradation (0.63–0.80). The URESP model combined with the TEMP/W simulation approximated changes in soil respiration during thawing and characterized the computed soil respiration outputs as related to hydrocarbon utilization, based on their RQ values.

3.2 Introduction

Variable trends in CO_2 production and O_2 consumption (soil respiration) in biologically enhanced contaminated soils generally reflect microbial metabolic activity and substrate utilization. Monitoring CO_2 and O_2 in the soil gas phase has been used in practice to track on-site aerobic microbial activity during bioremediation for petroleum hydrocarbon-contaminated soils in cold

climates (Gomez and Sartaj, 2013; Margesin and Schinner, 2001; Rike et al., 2003; Rike et al., 2005; Sanscartier et al., 2009; Snape et al., 2008b; Whyte et al., 2003).

Modelling soil respiration in natural soil systems is useful for predicting seasonal CO_2 emissions and understanding the metabolic functions of soils (Davidson et al., 2012; Flattery et al., 2018; Lloyd and Taylor, 1994; Luo and Zhou, 2006; Öquist et al., 2009; Schaefer and Jafarov, 2016; Tucker, 2014). Similarly, from a remediation perspective, calibrated soil respiration models specific to contaminated site soils may be useful for approximating the timing of seasonal activation and inactivation of microbial activity at remote cold sites (Rike et al., 2003; Rike et al., 2005). However, the application and modification of soil respiration models extended to sub-zero temperatures in biostimulated petroleum hydrocarbon-contaminated soils has not received significant attention in the literature, as it has for pristine soils (uncontaminated soils).

Microbial activity in frozen soils has been explored more frequently using pristine soils than with contaminated soils. Some indigenous cold-adapted bacteria are metabolically active in frozen soils that still contain unfrozen water films (Aislabie et al., 2006; Chang et al., 2011; Clein and Schimel, 1995; Drotz et al., 2010; Kim et al., 2018; Margesin et al., 2003; Margesin and Schinner, 1999; Mykytczuk et al., 2012; Öquist et al., 2009; Panikov et al., 2006; Raymond-Bouchard and Whyte, 2017; Rivkina et al., 2000; Siciliano et al., 2008). Microbial uptake mechanisms for hydrocarbons in the aqueous and non-aqueous phases in frozen contaminated soils have not been extensively understood, unlike for unfrozen contaminated soils (Abbasnezhad et al., 2011; Ehlers and Luthy, 2003; Ghoshal and Luthy, 1998; Ramaswami et al., 1997). Nonetheless, enhanced hydrocarbon biodegradation in seasonally freezing and thawing contaminated soils, along with the observable relationship between unfrozen water and soil respiration activity, have been reported through laboratory and field experiments (Chang et al., 2011; Karppinen et al., 2017b; Kim et al., 2018; Siciliano et al., 2008). The strong correlation observed near 0 °C between unfrozen water content, aerobic soil respiration, and hydrocarbon biodegradation in previous bioremediation studies may offer a practical rationale for applying soil respiration models to engineered soil bioremediation systems undergoing seasonal freezing and thawing.

Water potential (matric and osmotic potential) and soil temperatures regulate the amount of unfrozen water in frozen soils, which influences microbial activity at sub-zero temperatures (Carson et al., 2010; Drotz et al., 2009; Öquist et al., 2009; Sturm et al., 2003; Suzuki, 2004;

Tucker, 2014; Wilson and Griffin, 1975). Soil physical and chemical properties (particle sizes, pore sizes/surface areas, clay content, organic matter, solute concentrations/salinity, hydrocarbon contamination, and so on) also influence shifts in unfrozen water availability and thus microbial activity in frozen soils (Drotz et al., 2010; Ma et al., 2017; Or et al., 2007; Siciliano et al., 2008; Wen et al., 2012). Integrated soil respiration models that consider a multitude of variables (e.g., temperatures, substrates and oxygen-limiting conditions) have been developed for natural soil systems, including for cold-climate soils (Bunnell et al., 1977; Carlyle and Than, 1988; Davidson et al., 2012; Del Grosso et al., 2005; Öquist et al., 2009; Schlentner and Cleve, 1985; Steinweg et al., 2012; Tucker, 2014).

This study aims to modify the Generalized Respiration (GRESP) equation, originally formulated by Bunnell et al. (1977) as a function of soil temperature (*T*) and water content (*M*), for successfully bioremediated, petroleum hydrocarbon-contaminated soils undergoing simulated seasonal thawing. In addition, soil freezing characteristics expressed as measured soil temperature and unfrozen water content data can be transferred to a high-throughput numerical soil thermal model (e.g., TEMP/W) in the form of the soil freezing characteristic curve (SFCC), along with soil thermal parameters (e.g., soil thermal conductivity and heat capacity). This type of numerical thermal analysis provides a large number of predicted input variables (*T* and *M*) for the modified GRESP model (called URESP in this study). Connecting the calibrated soil respiration model to the numerical soil thermal model may be a practical tool for generating time-variable spatial distributions of soil temperature, water content and respiration data in contaminated soils under different climate scenarios at large scales. The quality of predictive soil respiration data can be verified by comparing their respiration quotients (RQ) to those of the measured respiration rates. The RQ is the ratio of CO_2 production to O_2 consumption used for characterizing substrate utilization (Chang and Ghoshal, 2014; Dilly et al., 2011; Lamy et al., 2013; Sánchez-Cañete et al., 2018; Sobek et al., 2017).

The specific objectives of this study are to (1) evaluate existing GRESP-based models for their applicability to sub-zero temperatures, (2) modify the GRESP model specifically for biostimulated petroleum-contaminated site soils subjected to seasonal thawing temperatures (-5 to 15 °C), (3) explore the feasibility of using a numerical soil thermal modelling tool to predict soil temperature and unfrozen water content in site soils subjected to thawing, and (4) apply the

modified GRESP model to approximate RQ values using *measured* and *predicted* input variables (soil temperatures and unfrozen water content) for the same site soils to verify that the respiration outputs remain characteristic of aerobic hydrocarbon degradation.

3.3 Materials and methods

Figure 3.2 shows a flow chart of the research protocol for this study, which consists of 4 major steps: (1) data acquisition for measured soil temperature (T) and volumetric water content (M); (2) evaluation of several existing soil respiration models; (3) modification of the GRESP model; and (4) application of the modified GRESP model for calculating RQ values. The details of each step are explained in the following sections.

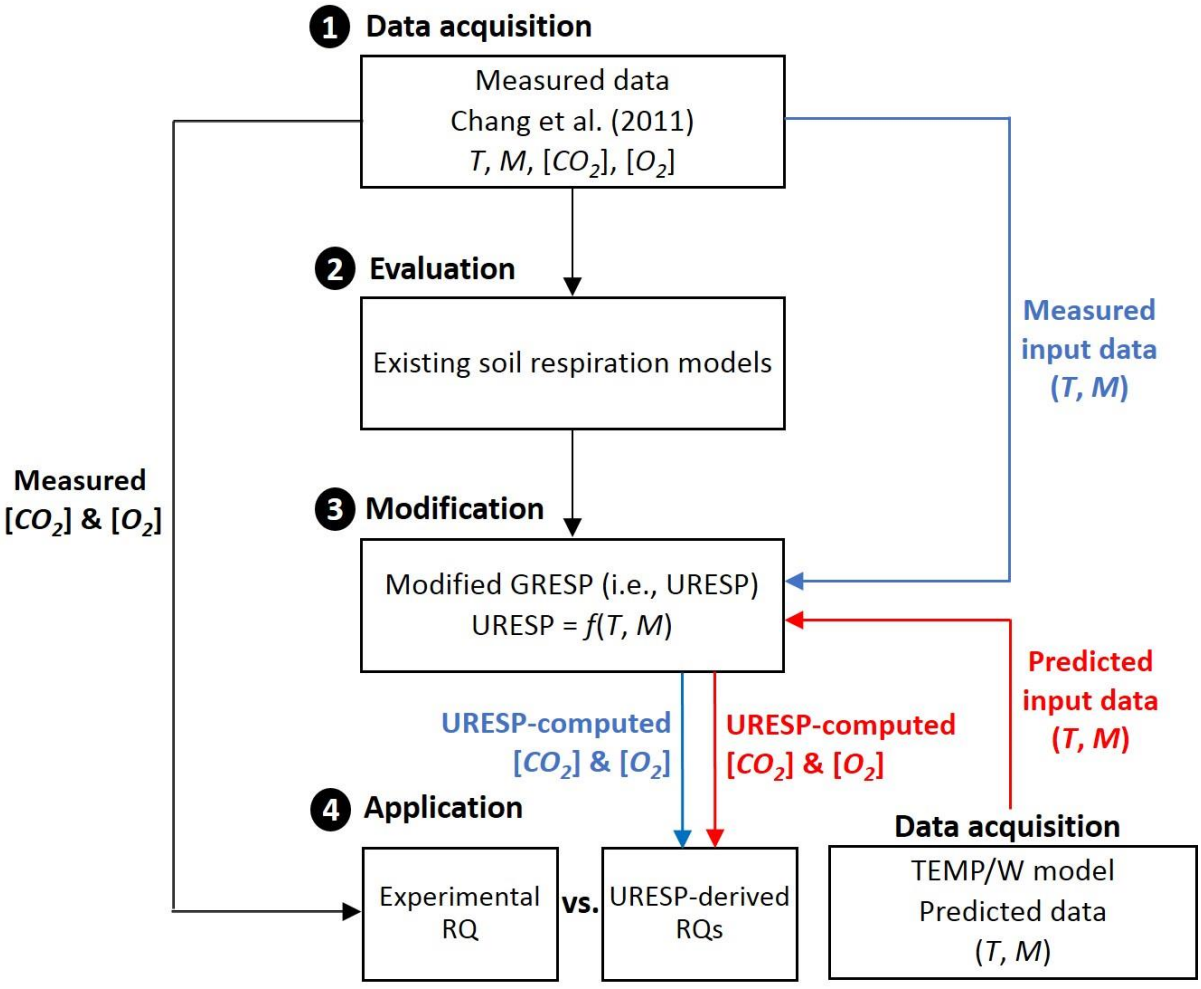

Figure 3.2. Flow chart of the protocol used for this modelling study.

3.3.1 Data acquisition (measured soil data)

This study required an input dataset of measured soil temperatures and volumetric water contents obtained specifically from successfully bioremediated, petroleum hydrocarbon-contaminated soils from a cold site undergoing seasonal thawing, as well as measured soil respiration data (CO_2-O_2) to compare with the output data of the soil respiration models. The measured soil dataset used in this present study was acquired from Chang et al. (2011), an experimental study that reported the occurrence of enhanced hydrocarbon biodegradation in coarse-grained, nutrient-amended, petroleum hydrocarbon-contaminated, sub-Arctic soils under site-representative seasonal freeze-thaw conditions. Briefly, the petroleum-contaminated soils were classified as sand (27% gravel, 72% sand, ad 1% silt and clays) (Chang et al., 2010). The petroleum hydrocarbon contaminants in the site soils are heavily aged and mainly consist of F2 hydrocarbons (semi-volatiles, >C10–C16) and F3 hydrocarbons (non-volatiles, >C16–C34), based on analyses performed in accordance with the Canada-Wide Standard – Tier 1 Method (CCME, 2001). The presence of various catabolic genes for hydrocarbon degradation (*alkB, ndoB, phnAc,* and *xylE*) and viable hydrocarbon-degrading bacteria were indicated in the contaminated soils. The successful biostimulation was achieved using a nutrient amendment and pH adjustment optimized for the site soils through a series of the experiments under low temperature regimes, including seasonal freeze-thaw conditions. The detailed soil characterization, hydrocarbon biodegradation and microbial community data for the site soils are available in the prior experimental studies (Chang et al., 2010; Chang et al., 2011). Pilot-scale soil tanks (1.0 m L × 0.65 m W × 0.35 m H) equipped with aeration systems contained approximately 300 kg of wet contaminated soils and were used for bioremediation experiments carried out in a large-scale temperature-programmable cold room. The soil was treated with a water-based nutrient solution (20% N, 20% P_2O_5, 20% K_2O, Plant-Prod) and 2000 mg $CaCO_3$/kg. The detailed designs of the pilot-scale soil tanks and biostimulation treatment are described in the corresponding publications (Chang et al., 2011).

CO_2 and O_2 concentrations in the soil gas phase were measured in the tanks via four horizontal perforated gas collection tubes embedded in the soil. An infrared-based sensor equipped with internal pumps (ATX 620, Industrial Scientific Co.) was connected to the gas collection tubes and the CO_2 and O_2 concentrations from the soil tanks were measured periodically. Real-time monitoring of soil temperatures and volumetric water contents was carried out using thermocouples and frequency domain reflectometry (FDR) sensors (Decagon Devices). The FDR-

based sensors were validated for measurements in frozen soils as cold as -15 °C (Yoshikawa and Overduin, 2005).

In the temperature-programmable cold room, the soil temperature in the tanks was first gradually raised from -5 to 4 °C at the site-specific seasonal rate of +0.16 °C/day to mimic seasonal thawing, and then up to 15 °C at +3.87 °C/day for a potential seasonal burst in soil respiration activity. This present modelling study considers only the thawing phase (from -5 to 15 °C) of the pilot-scale soil tank experiment in Chang et al. (2011).

3.3.2 Evaluation of existing soil respiration models

3.3.2.1 Arrhenius equation

Arrhenius-based soil respiration models have been broadly employed or modified to address the temperature dependency of soil respiration under various environmental conditions (Arrhenius, 1898; Lloyd and Taylor, 1994; Moinet et al., 2018; Qi et al., 2002). The basic form of the Arrhenius equation considered in this study is shown as Eq. 3.1, where *RESP* is the soil respiration rate (mmol CO_2 or O_2/kg/min), *A* is the Arrhenius coefficient, E_a is the activation energy (J/mol), *R* is the ideal gas constant (8.314 J/K·mol), and *T* is absolute temperature (K). In this study, the Arrhenius equation was applied to the temperature range of -5 to 15 °C.

$$RESP = Ae^{\left(\frac{-E_a}{RT}\right)} \quad \text{(3.1)}$$

3.3.2.2 GRESP-based models

GRESP-based models are founded on a combination of measurement and principle and were considered in this study to address the coupled effects of soil temperature and unfrozen water content. The original form of the GRESP model (Bunnell et al., 1977) is presented as Eq. 3.2, where *GRESP* is the soil respiration rate (mmol CO_2 or O_2/kg/min), *T* is temperature (°C), *M* is water content (% dry weight), a_1 is the percent water content on a dry-weight basis at half field capacity, a_2 is the percent water content at half maximum retentive capacity, a_3 is the theoretical maximum respiration rate at 10 °C, and a_4 is a Q_{10} coefficient.

$$GRESP = \frac{M}{a_1+M} \times \frac{a_2}{a_2+M} \times a_3 \times a_4^{\left(\frac{T-10}{10}\right)} \quad \text{..... (3.2)}$$

The BRESP (Schlentner and Cleve, 1985) and FRESP models (Carlyle and Than, 1988) were developed by modifying the GRESP model. Table 3.1 presents the detailed equations and associated parameters of the GRESP, BRESP and FRESP models, along with the Arrhenius equation.

Table 3.1. Existing soil respiration models considered for comparison in this study.

Equation	Reference
$RESP = A \times e^{\left(\frac{-E_a}{RT}\right)}$	Arrhenius (1898)
$GRESP = \frac{M}{a_1 + M} \times \frac{a_2}{a_2 + M} \times a_3 \times a_4^{\left(\frac{T-10}{10}\right)}$	Bunnell et al., (1977)
$BRESP = \frac{M}{a_1 + M} \times \frac{a_2}{a_2 + M} \times a_3 \times \left(\left(\frac{1}{a_6 + a_4^{-\left(\frac{T-10}{10}\right)}} \right) + a_5 \right)$	Schlentner and Cleve (1985)
$FRESP = \frac{M}{a_1 + M} \times \frac{a_2}{a_2 + M} \times a_3 \times A_4^{\left(\frac{T-10}{10}\right)}$	Carlyle and Than (1988)

RESP: soil respiration rate (mmol CO_2 or O_2/kg/min), *A*: Arrhenius coefficient, E_a: activation energy (J/mol), *R*: gas constant (8.314 J/K·mol), and *T*: absolute temperature (K).

GRESP: soil respiration rate (mmol CO_2 or O_2/kg/min), *T*: temperature (°C), *M*: water content (% dry weight), a_1: percent water content (dry weight basis) at half field capacity (%), a_2: percent water content at half maximum retentive capacity (%), a_3: maximum respiration rate at 10 °C (mmol CO_2 or O_2/kg/min), and a_4: Q_{10} coefficient.

BRESP: soil respiration rate (mmol CO_2 or O_2/kg/min), *T*, *M*, a_1 and a_2 are the same as in the *GRESP* equation, a_3: scaling factor, a_4: Q_{10}-related fitting parameter, and a_5: lower limit of respiration (mmol CO_2 or O_2/kg/min), and $a_6 = \frac{1}{(\text{upper limit of respiration}) - a_5}$.

FRESP: soil respiration rate (mmol CO_2 or O_2 /kg /min), *T*, *M*, a_1, a_2 and a_3 are the same as in the *GRESP* equation, a_4: fitting parameter linking Q_{10} to water content, a_5: lower limit of the Q_{10} coefficient, $A_4 = \frac{1}{a_6 + a_4^{(M-10)}} + a_5$, and $a_6 = \frac{1}{upper\ limit\ for\ Q_{10}} - a_5$.

3.3.3 Modification of the GRESP model

In this study, the Michaelis-Menten equation and Q_{10} temperature coefficient were considered in combination to address the coupled effects of soil temperature and unfrozen water content on soil respiration activity in the thawing biotreated site soils, both below and above 0 °C. The Michaelis-Menten equation with the water content term combined with the Q_{10} temperature coefficient is presented in Eq. 3.3, where *RESP* is the soil respiration rate (mmol CO_2 or O_2/kg/min), R_{max} is the soil respiration at 0 °C (mmol CO_2 or O_2/kg/min), K_m is the unfrozen water content (%) at which soil respiration is half of R_{max}, M is the water content (% dry weight), T is temperature (°C), and Q_{10} is a temperature-sensitivity coefficient (dimensionless). The Q_{10} temperature relationship has been frequently employed to correct soil respiration rates in consideration of temperature effects (Chen et al., 2010; Kukumägi et al., 2017; Makita et al., 2018). The combination of the Michaelis-Menten equation (water content effect, and/or substrate and oxygen availability) and the Arrhenius equation or Q_{10} relationship (temperature effect) as a mathematical expression for soil respiration was similarly suggested in GRESP-based models (Bunnell et al., 1977; Carlyle and Than, 1988; Schlentner and Cleve, 1985), and in the DAMM models (Davidson et al., 2012; Tucker, 2014).

$$RESP = \frac{R_{max} \times M}{K_m + M} \times Q_{10}^{\left(\frac{T-10}{10}\right)} \quad (3.3)$$

The original form of the Michaelis-Menten term in Eq. 3.3 was further modified to accurately address changes in soil respiration activity at sub-zero temperatures (-5 to 0 °C) in partially frozen and frozen site soils. The modification of the Michaelis-Menten term was validated using the multiple statistical evaluators mentioned above (R^2, RMSE, and MARE). The adjusted Michaelis-Menten term was then incorporated into the original GRESP model framework, producing a new modified GRESP model for the site soils that extends to -5 °C in its applicable temperature range and is designated in this study as the URESP model.

Several assumptions were made to simplify the model development for the engineered soil bioremediation system. The pilot-scale soil tank was continuously supplied by compressed air to promote aerobic bioremediation and oxygen-limiting conditions were thus discounted. The hydrocarbon-contaminated soils were amended with nutrients, therefore soil nutrients were not deficient for enhancing hydrocarbon biodegradation. Hydrocarbon-degrading bacteria were active

in the site soils (Chang et al., 2011). Petroleum hydrocarbon compounds (substrates, as total petroleum hydrocarbons: TPH) were bioavailable to hydrocarbon-degrading bacteria. If petroleum hydrocarbons were to become limited due to the end of bioremediation, the RQ-values of the soil respiration activity would deviate significantly from the range specific to hydrocarbon utilization (based on the stoichiometry of hydrocarbon biodegradation). The model calibration is based on the direct observation of the relationship between unfrozen water content and respiration activity from the pilot-scale bioremediation experiment under seasonal freeze-thaw conditions (Chang et al., 2011).

3.3.4 Application of the URESP model

3.3.4.1 Data acquisition through a TEMP/W simulation for predicted *T* and *M*

The seasonal soil thawing regime applied to the pilot-scale soil tank in Chang et al. (2011) was simulated using a TEMP/W model to obtain predicted *T* and *M* input data. TEMP/W is a finite-element-based numerical soil thermal simulation software (GEO-SLOPE International Ltd., Calgary, Canada). The transient soil thermal analysis simulated the 90-day seasonal thawing of the site soils from -5 to 15 °C at the site-specific thawing rates above in 60 time steps (i.e., 1.5-day intervals over 90 days). A two-dimensional (2D) finite-element computational mesh representing a vertical cross-section of the pilot-scale soil tank (1 m in length × 0.2 m in soil depth) was generated to have 125 elements (4 cm × 4 cm) and 156 nodes. As input soil data for the TEMP/W simulation (Table D.4 and Fig. D.1), the thermal conductivity and volumetric heat capacity of the sandy, non-compacted contaminated site soils were assumed to be in the ranges of 15 to 40 kJ/day·m·°C and 1500 to 1830 kJ/m^3·°C, respectively, depending on the thermal phase of the soils (frozen and unfrozen) (Ghuman and Lal, 1985; Instanes, 2016). The soil freeze-thaw characteristic curve (SFCC) specific to the site soils, which was derived from Chang et al. (2011), was also used as input soil data. The SFCC is generally expressed a function of soil temperature and unfrozen water content (or unfrozen water content vs. water potential) (Ma et al., 2017; Spaans and Baker, 1996). It depends on soil physical and chemical properties, and thus varies from fine-grained to coarse-grained soils (Ma et al., 2017). The SFCC was introduced into the TEMP/W model (called unfrozen water content function in TEMP/W) to predict volumetric unfrozen water content (GEO-SLOPE, 2014). The 2D-domain of the frozen soil in the tank formed in the TEMP/W model was

then subjected to external ambient temperatures rising from -5 to 15 °C for the seasonal thawing scenario. The detailed input parameters are presented in supplementary Table D.4 and Fig. D.1.

The TEMP/W simulation of soil thawing generated predicted *T* and *M* data for each element of the 2D domain of the soils. These predicted *T* and *M* data were used as input data for the URESP model to produce calculated soil respiration data (CO_2 production and O_2 consumption rates) for each element of the soil cross-section (Fig. 3.2). The distributions of the simulated *T* and *M*, along with the calculated soil respiration data, were visualized using Surfer 13 (Golden Software Inc., Colorado, USA).

3.3.4.2 RQ-value calculation and validation

As shown in Fig. 3.2, the URESP model was employed to generate two sets of calculated RQ values (molar ratio between CO_2 production and O_2 consumption), generated using the two different sets of input data: measured and predicted *T* and *M*. The *measured T* and *M* data obtained from Chang et al. (2011) (Section 3.3.1) were used as input data in the URESP model for the low temperature regime to produce calculated soil respiration data (mmol CO_2 or O_2/kg/min). For comparison, the *predicted T* and *M* data obtained from the TEMP/W simulation of Chang et al. (2011)'s soil tank experiment (Section 3.3.4.1) were used in the same URESP equation to calculate corresponding respiration rates for the thawing soils. RQ values were then calculated using Eq. 4 for each of these sets of URESP-computed soil respiration data. The two calculated sets of RQ values derived from the new soil respiration model were validated by comparing them to experimental RQ values obtained from the *measured soil respiration data* in Chang et al. (2011) (Fig. 3.2).

$$RQ = \frac{[CO_2]\ produced}{[O_2]\ consumed} \quad \text{..... (3.4)}$$

3.4 Results and discussion

3.4.1 Coupled effects of soil temperature and unfrozen water content on soil respiration

The increasing trend in soil respiration activity (CO_2 production rate) observed *above* 0 °C (after soil thawing) is well fitted to the Arrhenius relationship, as shown in Fig. 3.3B (R^2 = 0.94

above 0 °C). As anticipated, this observation is explained by the temperature dependency of soil respiration when water content in the treated soils stabilizes after thawing completely. However, below 0 °C, a slightly inferior fit is observed between the measured respiration rates and those computed from the Arrhenius equation, suggesting that the temperature dependency alone cannot explain soil respiration activity in the biologically treated hydrocarbon-contaminated soils at sub-zero temperatures (Tilston et al., 2010). The percent error of 57% between the measured and computed soil respiration rates was significant under the sub-zero temperature regime (Fig. 3.3A). This deviation was observable between -5 and 0 °C, as the soils were in the deeply frozen and partially frozen phases. More specifically, the Pearson coefficient for the correlation between the CO_2 production rate and water content was 0.82 below 0 °C, which was significantly higher than the Pearson coefficient of 0.22 above 0 °C (Fig. 3.3; Table 3.2). The CO_2 production rate increased with increasing unfrozen water content during the soil phase change from frozen to partially frozen, to thawed. This strong correlation in this study supports the assertion that changing unfrozen water content becomes a practical indicator for variable soil respiration activity during soil phase changes. Although other underlying soil factors such as soil temperature, water potential, and soil properties regulate the fate of unfrozen water content and influence microbial activity in partially frozen and frozen soils, focusing on water content may be especially practical. Time-varying water content and soil temperature data from continuous soil monitoring during seasonal shifts are often available through large-scale bioremediation experiments for petroleum hydrocarbon-contaminated soils (Kim et al., 2018; McWatters et al., 2016). The observable relationship between unfrozen water content and soil respiration, even at larger scales, can be useful in developing soil respiration models extended to sub-zero temperature regimes.

As shown in the combined profiles of soil temperature, unfrozen water content and soil respiration (Fig. 3.3A), the soil phase change that occurs just below 0 °C associated with the melting point of the soils (from frozen to unfrozen, and *vice versa*) has a critical influence on soil respiration activity. This study suggests that the abrupt increase in unfrozen water content just below 0 °C during soil thawing (or the rapid decline during soil freezing) may approximate the effective seasonal onset and shutdown of soil respiration in biologically enhanced petroleum-contaminated soils. This likely relates to previous observations in the literature of the sub-zero activation and inactivation of microbial respiration activity in the annual profiles of CO_2 and O_2 soil gas concentrations (Rike et al., 2003; Rike et al., 2005). The abrupt change in soil water content

in cold climates has been designated as an inflection point (or a critical point) in several previous studies (Mikan et al., 2002; Tilston et al., 2010; Tucker, 2014) and was observed in biologically active petroleum-contaminated site soils (Chang et al., 2011; Kim et al., 2018; Rike et al., 2003; Rike et al., 2005) and in pristine soils (Du et al., 2013; Elberling and Brandt, 2003; Gaumont-Guay et al., 2006; Liu et al., 2016; Panikov et al., 2006; Tilston et al., 2010; Wang et al., 2014).

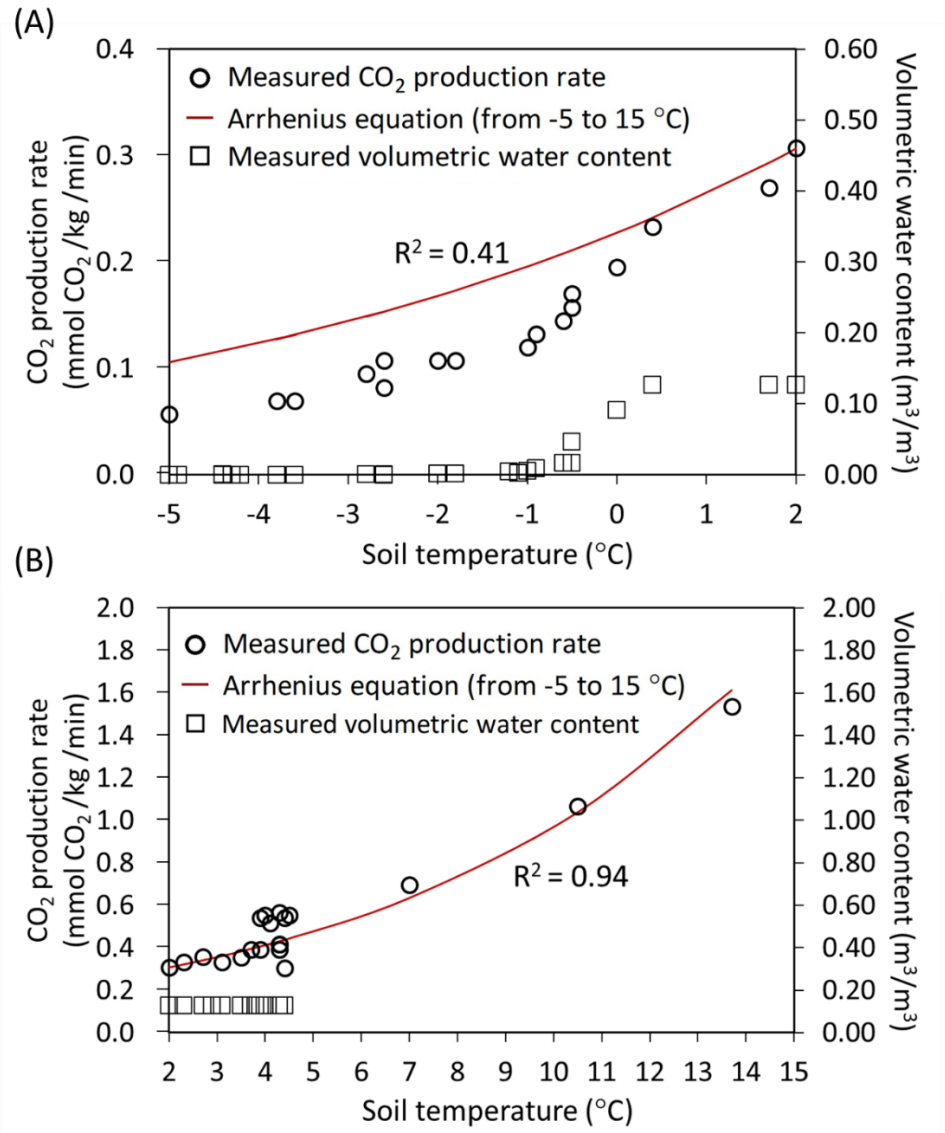

Figure 3.3. The combined profiles of measured soil temperature, volumetric unfrozen water content and soil respiration (CO_2 production rate) as the soil temperature increased from −5 to 15 °C, along with the soil respiration computed by the Arrhenius equation. The data are shown for two temperature ranges: during thawing from −5 to 2 °C (A) and after thawing from 2 to 15 °C (B).

Table 3.2. Correlation of soil respiration rates with soil temperature and water content, using the datasets obtained from Chang et al., (2011).

		Correlation coefficient (Pearson r)	
		-5 to 0 °C	0 to 15 °C
Detectable water content	CO_2 production rate	0.82	0.22
	O_2 consumption rate	0.69	0.21
Soil temperature	CO_2 production rate	0.94	0.96
	O_2 consumption rate	0.76	0.94

These laboratory and field observations of soil respiration during thermal phase changes may relate to the interactive effects of soil temperature and unfrozen water availability in partially frozen and frozen soils. Seasonal shifts in indigenous microbial community compositions in biostimulated petroleum hydrocarbon-contaminated soils, including the emergence of hydrocarbon-degrading bacteria, were indicated in the previous laboratory and field experiments carried out under seasonal freeze-thaw conditions (Chang et al, 2011; Kim et al., 2018). Hydrocarbon biodegradation was significantly enhanced in the biostimulated, petroleum hydrocarbon-contaminated soils under seasonal freeze-thaw conditions. At the same time, the presence of unfrozen water, which is a prerequisite for extending soil bioremediation under freezing conditions, was detected in the bioremediation experiments (Chang et al., 2011; Karppinen et al., 2017a; Karppinen et al., 2017b; Kim et al., 2018). Temperature-dependent soil respiration models that do not consider unfrozen water availability may not fully represent seasonal changes in soil respiration activity and sub-zero respiration activity associated with residual unfrozen water (often undetectable with monitoring probes). Unfrozen water sustains indigenous winter microbial communities that are metabolically active at sub-zero temperatures in biostimulated, petroleum-contaminated, cold-climate soils.

3.4.2 GRESP-based models with temperature and water content

The applicability to sub-zero temperatures of the existing GRESP, BRESP, and FRESP models, which are functions of soil temperature and unfrozen water content (Table 3.1), was assessed using the CO_2 respiration rates measured during soil thawing (between -5 and 15 °C). As shown in Fig. 3.4, the three selected soil respiration models did not accurately approximate the variable CO_2 respiration rates observed during the phase change from frozen, to partially frozen, to unfrozen. The statistical curve-fitting parameters considered in this study (R^2, RMSE, and MARE) indicated that those models poorly fit the measured respiration rates (Fig. 3.4 and supplementary Table D.2). Although the BRESP equation produced a relatively better fit among the models considered in this study (R^2: 0.80), the BRESP model did not describe the abrupt increase in soil respiration activity associated with the onset of increasing unfrozen water content just below 0 °C (Fig. 3.4B and 3.4C). In this study, it appeared that the GRESP, BRESP, and FRESP models without modifications may not be applicable to the biologically enhanced cold-site soils at sub-zero temperatures. The unfrozen water content in the frozen soils (below 0.016 m^3/m^3) is much lower than in previous GRESP-based modelling studies for unfrozen soils (above 0 °C) and corresponds to extremely low or undetectable respiration rates in the existing respiration models (Fig. 3.4B).

Measurable soil respiration activity was observed in this study in the partially frozen phase of the soils (between -2 and 0 °C), when unfrozen bulk water and frozen water most likely coexisted. Below -2 °C, unfrozen water was undetectable and the soils were deeply frozen. However, sub-zero CO_2 production was still detectable and likely extended below -2 °C to the frozen phase of the site soils (Chang et al., 2011). Extremely scarce unfrozen water in cryoenvironments, which is likely present as detectable or undetectable films in soil microenvironments, potentially allows sub-zero microbial respiration activity to occur in deeply frozen soils (Chang et al., 2018; Deming, 2002; Mykytczuk et al., 2012; Öquist et al., 2009; Panikov et al., 2006; Rivkina et al., 2000; Tilston et al., 2010; Tucker, 2014). It has been reported that fundamental soil characteristics (soil types, mineral compositions, surface areas, pore networks, solute concentrations, clay content, organic matter, and/or hydrocarbon contamination, and so on) may influence the formation of complex soil microsites, the soil freezing point depression, and residual unfrozen liquid water retention below 0 °C (Drotz et al., 2009; Wen et al., 2012). For petroleum hydrocarbon-contaminated soils, the significant biodegradation of petroleum

hydrocarbons was reported in freezing and frozen contaminated sandy and clayey soils treated by nutrient supplies and/or other soil amendments (humate and biochar) (Chang et al., 2011; Karppinen et al., 2017a; Karppinen et al., 2017b; Kim et al., 2018). Alternative soil respiration models are thus necessary to represent soil respiration in biotreated petroleum hydrocarbon-contaminated soils below 0 °C.

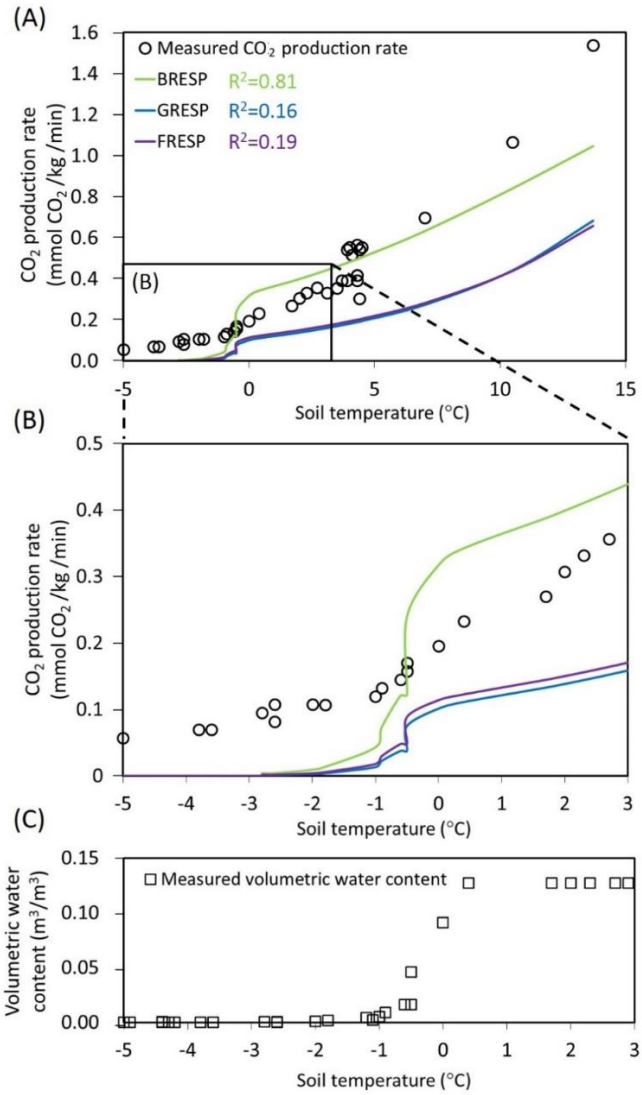

Figure 3.4. Evaluation of several existing soil respiration models using respiration data measured during thawing in biologically enhanced petroleum-contaminated soils: (A) the computed CO_2 production rates obtained from the GRESP-based models with the measured CO_2 production rates from Chang et al. (2011), (B) the same plot shown on a smaller scale to highlight the soil thawing phase, and (C) the corresponding water content profile from Chang et al. (2011).

3.4.3 GRESP model modification: unfrozen water content consideration

In this study, the Michaelis-Menten equation, which is a substrate-dependent microbial kinetic expression (Menten and Michaelis, 1913), was considered to address extended respiration activity in the biologically enhanced petroleum-contaminated soils under the sub-zero temperature regime. It is generally assumed that hydrocarbons and nutrients in frozen water may not be available to bacteria, and the availability of dissolved substrates (e.g., petroleum hydrocarbons) and supplied nutrients increases with increasing unfrozen water content during soil thawing, and *vice versa* during freezing (Clein and Schimel, 1995; Mikan et al., 2002; Öquist et al., 2009; Tilston et al., 2010; Tucker, 2014). As experimentally observed, this assumption may be valid when expressing soil respiration activity as a function of unfrozen water content during phase changes in cold-climate soil microenvironments (Sections 3.4.1 and 3.4.2). Some hydrocarbons and nutrients may be concentrated in residual unfrozen water films due to exclusion from ice, thus sustaining sub-zero respiration activity in the deeply frozen soils (below -2 °C). The original Michaelis-Menten equation alone cannot be used to model the detectable soil respiration below -2 °C in the deeply frozen soil, when unfrozen bulk water was mostly undetectable by the FDR-based probes used in the study (Chang et al., 2011) but with the possible presence of residual unfrozen water films with accumulated ice-excluded solutes (Konrad and McCammon, 1990; Panday and Corapcioglu, 1991). This is because the undetectable unfrozen water content (*M* of zero in Eq. 3.3) makes the respiration term also equal to zero. Therefore, a site-specific sub-zero respiration term R_{bg} (mmol CO_2 or O_2/kg/min) was introduced for the deeply frozen cold-climate soils (below the effective endpoint of -2 °C for measurable unfrozen water) and incorporated into the Michaelis-Menten equation for this study, as shown in Eq. 3.5. R_{bg} is an empirical term determined using respiration activity measured below 0 °C in the soils.

$$RESP = \frac{R_{max} \times M}{K_m + M} + R_{bg} \quad \text{..... (3.5)}$$

Fig. 3.5 shows that the modified Michaelis-Menten equation (Eq. 3.5) addresses the observable relationship between CO_2 production and unfrozen water content during the soil phase change, including at sub-zero temperatures (R^2 = 0.91). As the soil thawed, the CO_2 production rate increased with increasing unfrozen water content (increasing nutrient availability), which was well approximated by the modified Michaelis-Menten relationship. The O_2 consumption rate showed a similar pattern, which was also reasonably approximated using the modified Michaelis-

Menten equation ($R^2 = 0.69$), considering the low sensitivity of the oxygen sensor (Supplementary Fig. D.2). The term ($\frac{R_{max} \times M}{K_m + M}$) in the Michaelis-Menten expression is valid only during soil thermal phase changes (partially frozen soils) when unfrozen water content is most strongly correlated with microbial respiration activity and when the temporal variation in unfrozen water content, including the inflection point, becomes the predominant influencing factor for dissolved substrate availability and diffusion to bacteria (nutrients and hydrocarbons). When residual unfrozen liquid water in deeply frozen soils was extremely limited and stable, or even undetectable, sub-zero respiration activity was expressed in this study by the new empirical term, R_{bg}. The assumption is that with decreasing unfrozen water content, substrate availability (e.g., light hydrocarbons) decreases in the dissolved/aqueous phase, where active hydrocarbon-degrading bacteria are present during soil phase transitions (partially frozen soil). Change in detectable unfrozen water content is therefore directly related to change in dissolved substrate availability to hydrocarbon degraders. Less soluble or non-aqueous substrates (heavy hydrocarbons) at the interfaces of liquid water and other phases (e.g., soil particles) could interact directly with hydrocarbon-degrading bacteria through physical contact and adhesion, for potential hydrocarbon uptake by bacteria (Abbasnezhad et al., 2011). However, microbial uptake mechanisms for hydrocarbons in aqueous and non-aqueous phases within the microsites of partially frozen and frozen soil, where unfrozen water and ice coexist, have not been established through modelling studies. The underlying role of water potential in controlling substrate diffusion/availability, and the corresponding microbial activity, were not included in the model framework in suggested this study.

Mechanistic modelling using detailed soil parameters that control soil temperature, unfrozen water content, water potential, and corresponding microbial respiration responses, supported by systematic experimental studies, may improve our understanding of underlying microbial uptake mechanisms for hydrocarbons in frozen microsites and may expand the applicability of the GRESP-based model toward a diversity of biologically enhanced petroleum-contaminated soils in cold climates. Nonetheless, the current model, which is based on the strong observable correlation between temporal changes in unfrozen water content and soil respiration activity, suggests that change in detectable unfrozen water during seasonal soil phase transitions may be a practical indicator for tracking soil respiration activity. This is of particular interest in the context of extending bioremediation of petroleum-contaminated soils to seasonal freeze-thaw

conditions and may help approximate the seasonal activation an inactivation of measurably efficient bioremediation.

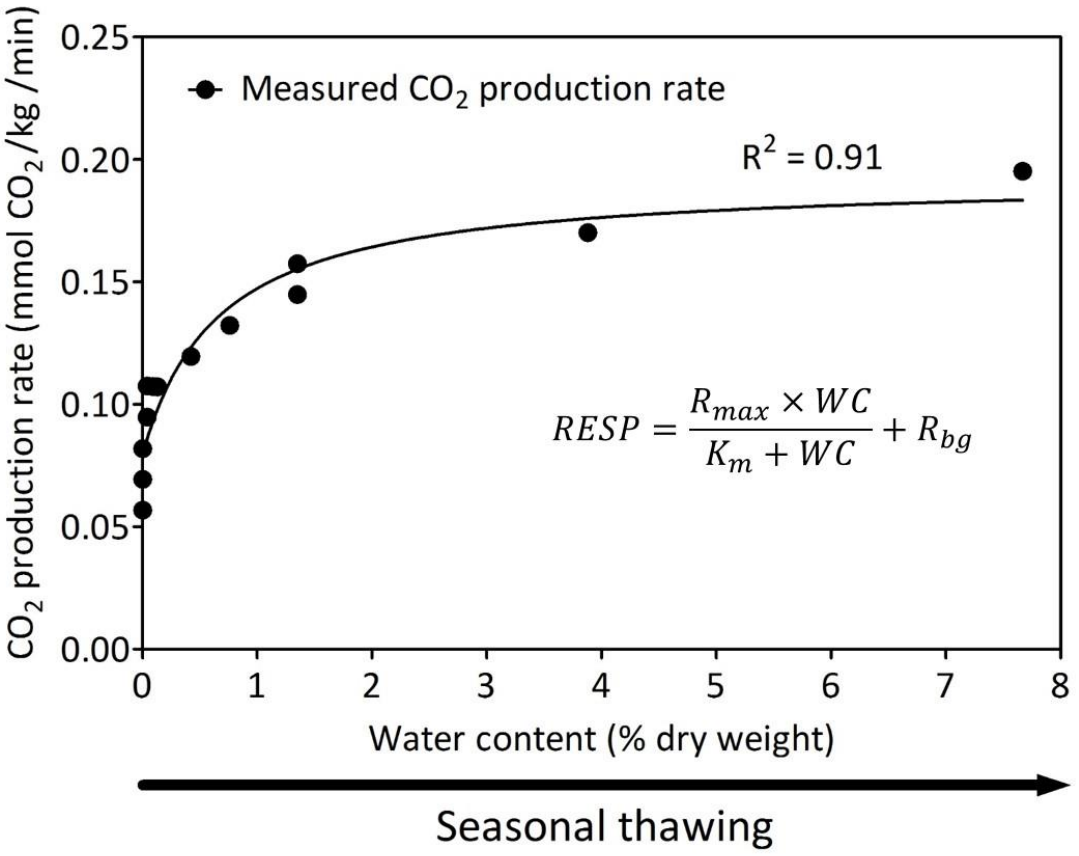

Figure 3.5. The Michaelis-Menten equation modified with sub-zero respiration activity term R_{bg} to address changes in CO_2 production rates as a function of unfrozen water content in unsaturated, nutrient-amended, petroleum hydrocarbon-contaminated sub-Arctic soils.

3.4.4 GRESP modification: URESP model framework

The new GRESP-based respiration model, tentatively named the URESP model as per the nomenclature of other published GRESP-based models, was constructed by incorporating the modified Michaelis-Menten equation and the conventional Q_{10} temperature relationship into the original GRESP model to produce Eq. 3.6, where *URESP* is the soil respiration rate (mmol CO_2 or O_2/kg/min). The U in URESP implies the critical role of *unfrozen* water content at sub-zero temperatures, which is the key feature of the new GRESP-based model. The URESP equation

consists of (1) the GRESP equation, (2) the modified Michaelis-Menten equation with R_{bg} and an empirical fitting coefficient, F, and (3) the Q_{10} equation for temperature sensitivity. The detailed terms of the original GRESP model are presented in Table 3.1, and the Michaelis-Menten equation with R_{bg} is presented in Section 3.4.3.

$$URESP = GRESP + \left(\frac{R_{max} \times M}{K_m + M} + R_{bg}\right)^F \times Q_{10}^{\frac{T-10}{10}} \quad \text{..... (3.6)}$$

Figure 3.6 shows the roles of the three major components of the URESP model using the pre-verified input parameters (a_1 to a_4, R_{max}, K_m, and R_{bg} in Table 3.3 and F in Fig. 3.7), as well as the improved fit of the URESP model across both the negative and positive temperatures of the studied range. The GRESP term acts as base framework for expressing the overall respiration trend when unfrozen water content is measurable. The modified Michaelis-Menten equation addresses sub-zero respiration activity as a function of unfrozen water content. The Q_{10} term in the URESP model accounts for the temperature sensitivity of soil respiration.

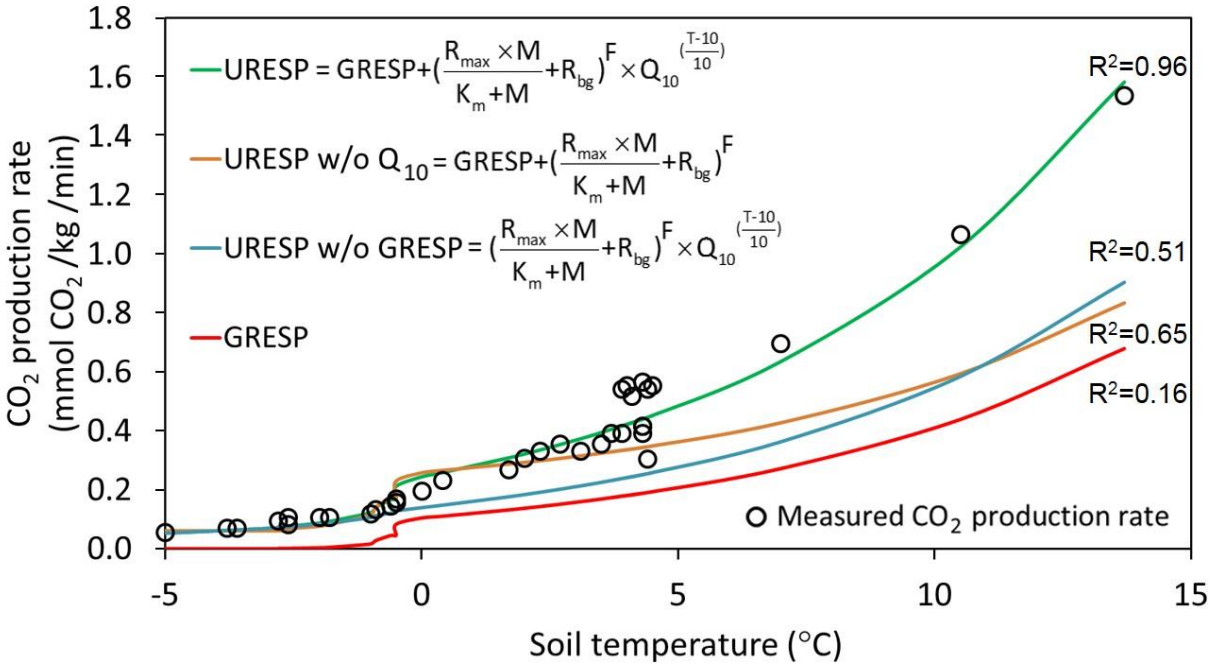

Figure 3.6. The roles of the major components built into of the URESP model.

Table 3.3. Input parameters for the URESP model.

Parameter	CO_2 production rate	O_2 consumption rate
a_1	5.29%	5.29%
a_2	15.68%	15.68%
a_3	1.032 mmol CO_2/kg/min	1.254 mmol O_2/kg/min
a_4	3.9	6.6
R_{max}	0.113 mmol CO_2/kg/min	0.089 mmol O_2/kg/min
K_m	0.664%	0.437%
R_{bg}	0.080 mmol CO_2/kg/min	0.017 mmol O_2/kg/min

Figure 3.7 shows the sensitivity analysis with changing *F*-values used to produce the best-fitting URESP model for the measured respiration data. With the given input parameters (a_1 to a_4, R_{max}, K_m, and R_{bg} in Table 3.3), the URESP model with an *F*-value of 0.36 produced the best fit for the measured soil CO_2 data between -5 and 15 °C (R^2 = 0.96, RMSE = 0.058, and MARE = 0.132 in Fig. 3.7 and supplementary Table D.3). The URESP model with the optimized *F*-value successfully expresses changes in CO_2 production rate during freeze-thaw as it is influenced by variable soil temperature and unfrozen water (Fig. 3.7). The abrupt change in CO_2 production just below 0 °C during soil thawing was approximated. Similarly, the sensitivity analysis determined that the URESP equation with an *F*-value of 0.19 produced the best fit for the variable oxygen consumption rates measured during soil thawing (R^2 = 0.92, RMSE = 0.114, and MARE = 0.724 in Fig. 3.7 and supplementary Table D.3).

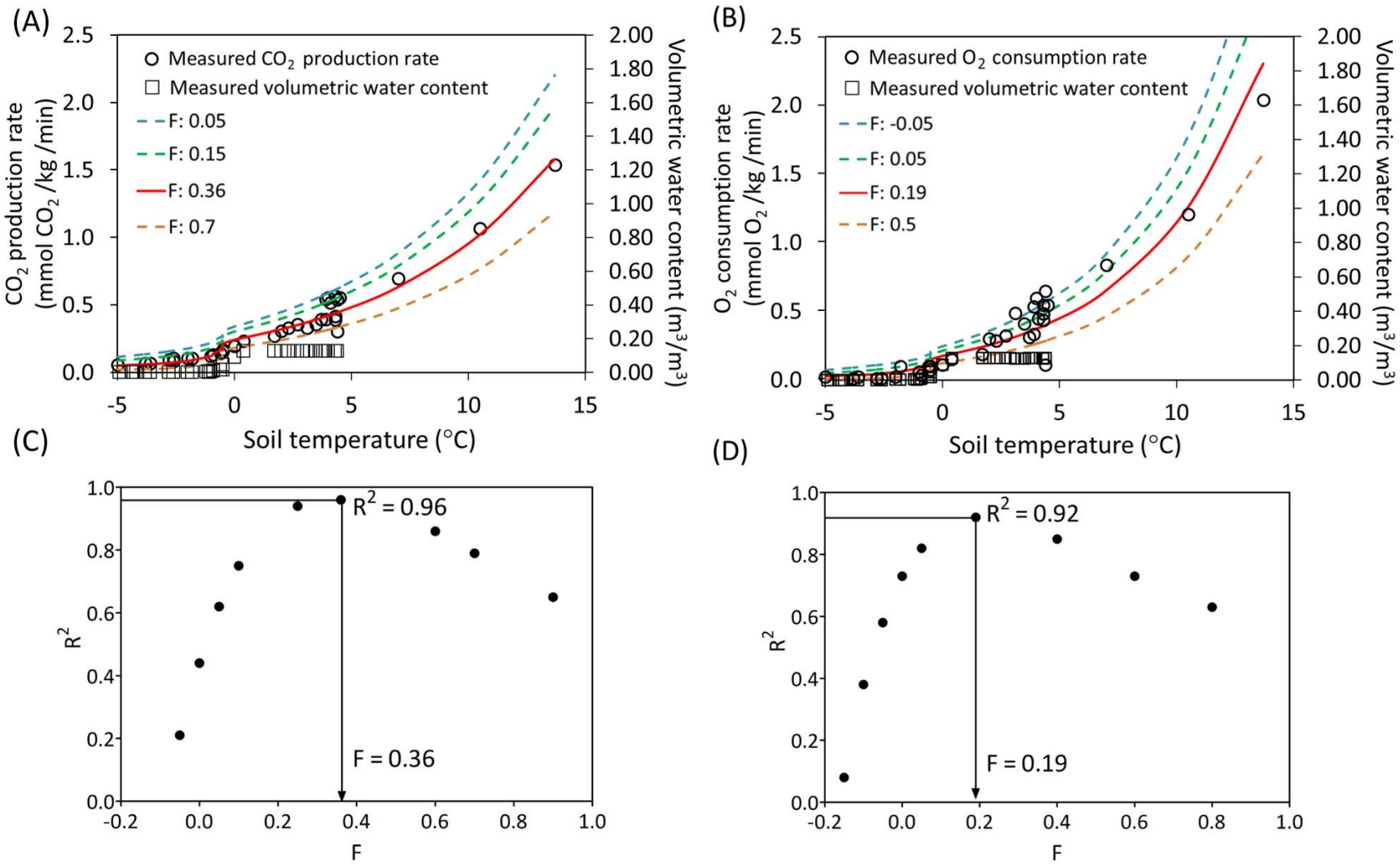

Figure 3.7. The outputs of the URESP model in the sensitivity analysis used to determine fitting constant *F*. Other inputs to the URESP model (a_1 to a_4; R_{max}, K_m, and R_{bg}) are shown in Table 3.3.

The validated framework of the URESP model suggested in this study reasonably approximates soil respiration activity extended to sub-zero temperatures in the biostimulated soils. It simultaneously reflects the abrupt change in soil respiration activity just below 0 °C related to the inflection point of unfrozen water content and the temperature-dependent respiration activity above 0 °C. The *F*-value is likely site-specific and is used in the sensitivity analyses to adjust the overall curvature of the soil respiration curves. The fitting coefficient allows the model to be adjusted for obscured effects and unconsidered influencing factors. It should be noted that for the measured soil data used in this study, oxygen availability was not a limiting factor for soil respiration activity and hydrocarbon biodegradation because the soil tank was continuously supplied with air (Chang et al., 2011).

The URESP model is valid as a simplified module for expressing variable soil respiration activity correlated with temporal change in unfrozen water content in biologically enhanced petroleum hydrocarbon-contaminated soils under seasonal freeze-thaw conditions. Unfrozen water content can be used as an effective soil thermal property parameter, along with soil temperature and respiration data, to connect the URESP model to the numerical thermal modelling tool (TEMP/W) run using the SFCC to predict soil temperature (*T*) and unfrozen water content (*M*) in soils subjected to same seasonal freeze-thaw scenario. This connection allows the high-throughput generation of predictive time-variable input data (*T* and *M*) that can transferred to the soil respiration model for corresponding predictive soil respiration data. This is further explained in the next section (Section 3.4.5).

3.4.5 Application of URESP model for RQ calculations

3.4.5.1 Input data acquisition: measured vs. predicted *T* and *M*

Using the URESP model, which outputs CO_2 and O_2 soil respiration data, requires the input of soil temperature and volumetric water content data. This study initially used the *measured T* and *M* from the soils subjected to seasonal thawing in Chang et al. (2011) as input data (Sections 3.4.3 and 3.4.4). However, in practice, soil monitoring at remote cold-climate sites is very challenging and prohibitively expensive, especially during seasonal freeze-thaw periods and long winters (Filler et al., 2015; McCarthy et al., 2004; McWatters et al., 2016; Snape et al., 2008a). Valid predicted *T* and *M* datasets would therefore be useful as input data for the URESP model.

Using the numerical TEMP/W soil thermal model, the same seasonal soil thawing scenario from -5 to 15 °C was applied to a simulation of the pilot-scale soil tank. The measured and predicted data (*T* and *M*) were compared. Upon validation of the predicted soil data, the URESP model as a function of *T* and *M* was employed to generate two different sets of computed soil respiration data (CO_2 and O_2) using the measured and predicted input datasets (*T* and *M*). Accordingly, two different sets of calculated RQ values were then produced using the sets of URESP-computed soil respiration data. As presented in the flow chart of the study protocol (Fig. 3.2), the performance of the URESP model was revalidated by comparing the calculated and experimental RQ values for the soil tank experiment. Fig. 3.8 illustrates the pilot-scale soil tank (Fig. 3.8A), the computational mesh of the soil tank (Fig. 3.8B), the *T* and *M* data predicted using TEMP/W (Figs. 3.8C and 3.8D), and the corresponding URESP-computed CO_2 and O_2 data (Figs. 3.8E and 3.8F). The spatial distributions of *T* and *M* in the soil tank predicted by TEMP/W are visualized as two-dimensional snapshots of the partially frozen soils on Day 30 of the soil thawing experiment. Corresponding spatial distributions of CO_2 and O_2 respiration rates were also generated using the URESP model.

As shown in Fig. 3.9A, the TEMP/W-predicted *T* and *M* for the soil tank experiment are generally in good agreement with the measured data over the entire 90-day soil thawing period. In the TEMP/W simulation, 156 data points for *T* and *M* were generated per time step for each node in the soil tank mesh, producing a total of 9360 data points over the entire 90-day simulation period (156 data points per time step for total 60 time steps). The simulated data points shown for each day in Fig. 3.9A are therefore averages of the 156 data points for *T* and *M*. However, the measured data for each sampling day are averages of only three measuring points (surface, middle and bottom soil layers) (Chang et al., 2011). As shown in Fig. 3.9B, the two respiration curves produced by the URESP model are almost identical using the measured *T* and *M* input dataset from Chang et al. (2011) and the TEMP/W-simulated *T* and *M* dataset, suggesting the URESP model in combination with soil thermal modelling was well calibrated to the thawing soils for the range of -5 to 15 °C. In this study, the acquisition of validated, predicted input data (*T* and *M*) through the TEMP/W model was feasible and the simulated data was usable in the downstream application of the URESP model to calculate corresponding RQ values.

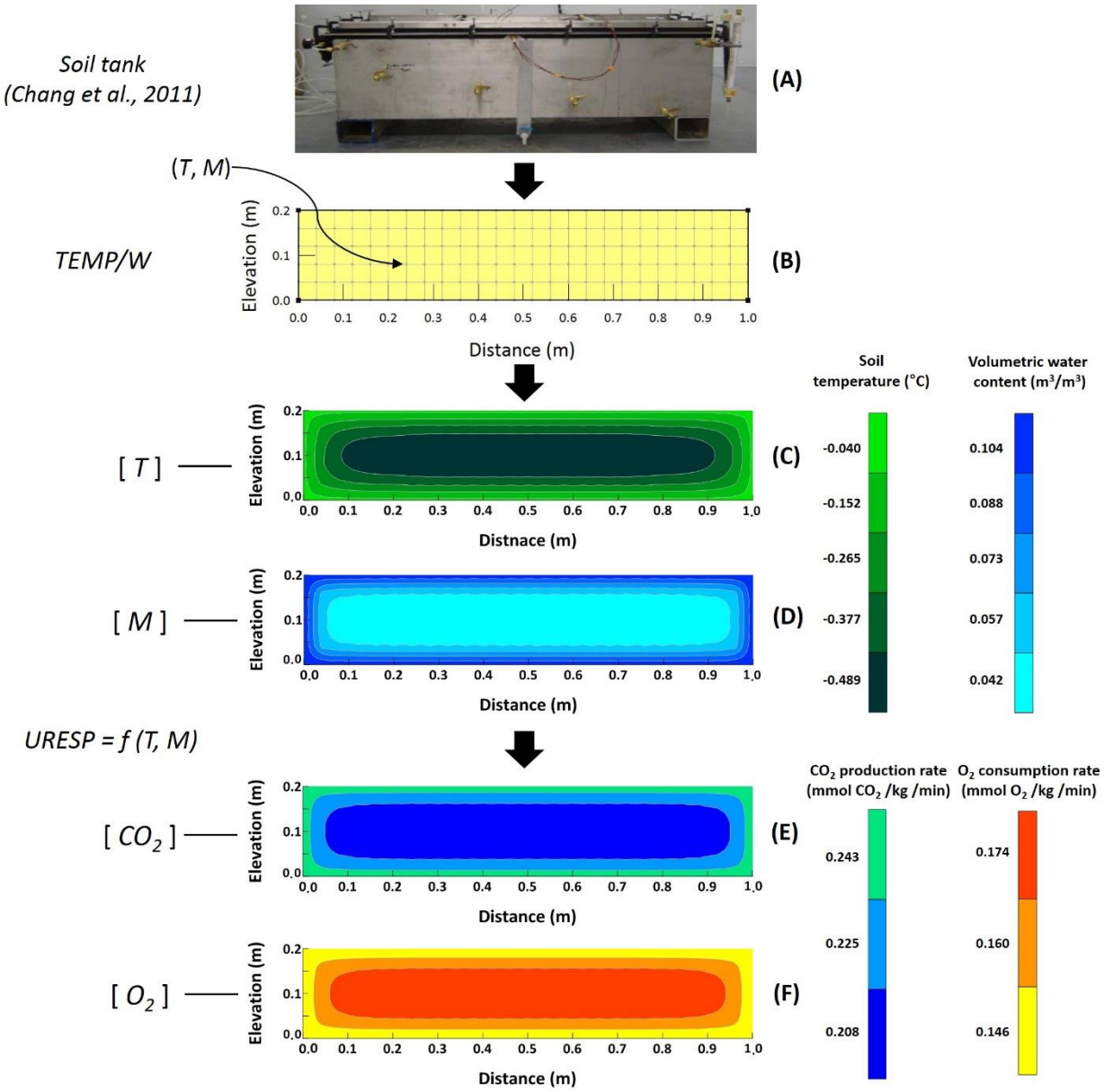

Figure 3.8. The pilot-scale soil tank used for hydrocarbon biodegradation at sub-zero temperatures (A), the computational mesh of the soil tank (B), the TEMP/W-predicted spatial distributions of soil temperature (C) and unfrozen water content (D), and the corresponding URESP-computed distributions of CO_2 production (E) and O_2 consumption (F) rates.

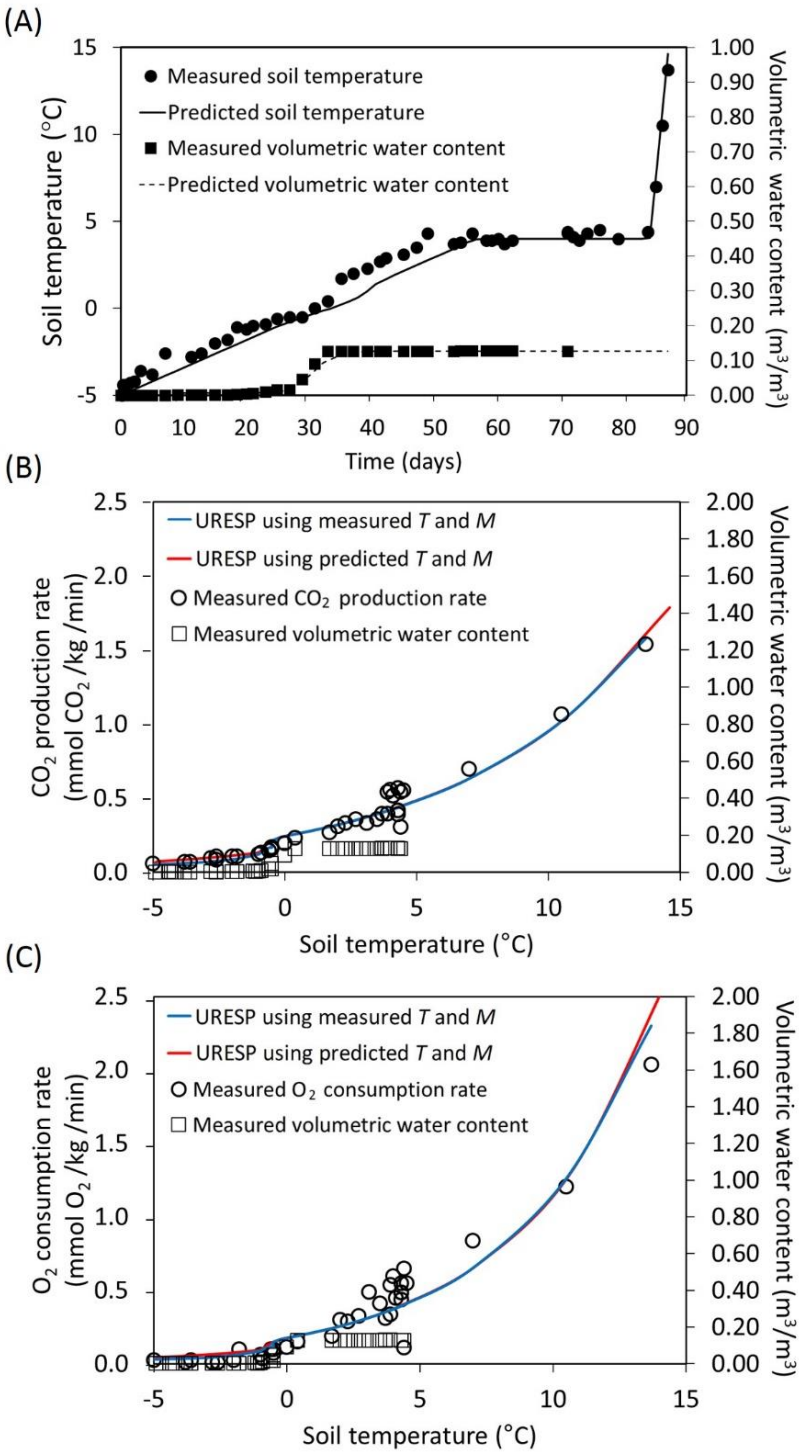

Figure 3.9. The TEMP/W-predicted soil temperature (*T*) and unfrozen water content (*M*) for the contaminated soils in the pilot-scale soil tank subjected to simulated thawing from −5 to 15 °C (A), and the URESP-computed CO_2 production (B) and O_2 consumption (C) rates with the measured and predicted *T* and *M* as input data.

3.4.5.2 URESP model for RQ approximation: URESP-derived vs. experimental RQ values

URESP-derived RQ values were obtained using the CO_2 and O_2 respiration data computed by the URESP model. As described in Chang and Ghoshal (2014), linear regression analyses were performed for RQ plots of the molar CO_2 production and O_2 consumption rates, from both the measured and simulated *T* and *M* input datasets, to produce averaged overall RQ values of 0.698 and 0.695, respectively (Fig. 3.10). These URESP-derived RQ values are within the theoretical RQ range of 0.63–0.80 for petroleum hydrocarbon degradation under aerobic conditions. They also generally matched the experimental RQ value of 0.690 for thawing phase derived directly from measured respiration data from the pilot-scale soil tank experiment. The variation in the URESP-derived RQ values each day was negligible and likely due to the homogeneity of the soil system used for thermal modelling and URESP analyses, and variation in the experimental RQ values did not largely influence the overall linearity of the RQ plot (Fig. 3.10). RQ values generally reflect the types of substrates being utilized for microbial respiration in soils. However, other direct chemistry measurements (e.g., total petroleum hydrocarbons; TPH) are generally necessary to confirm the occurrence of hydrocarbon biodegradation (functional substrate utilization) (Aspray et al., 2008; Chang and Ghoshal, 2014; Lamy et al., 2013; Sanscartier et al., 2011). Nevertheless, this study showed that the RQ assessment using the URESP model is feasible with both the measured and simulated *T* and *M* input data and may suggest the hydrocarbon specificity of substrate utilization.

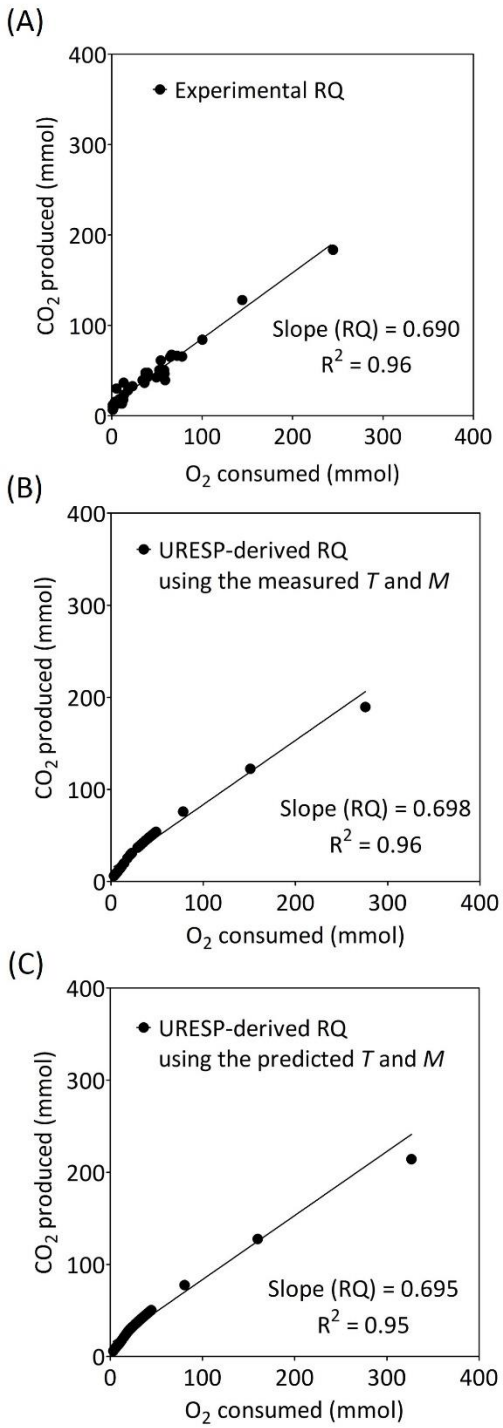

Figure 3.10. The experimental and URESP-derived RQ values. The experimental RQ values were calculated from CO_2-O_2 data measured from the pilot-scale soil bioremediation experiment in Chang et al. (2011) (A), and the URESP-derived RQ values were calculated using the measured (B) and simulated (C) *T* and *M* data.

3.5 Conclusions

The existing GRESP-based soil respiration models (GRESP, BRESP and FRESP) considered in this study do not account for the coupled effects of soil temperature and unfrozen water content on soil respiration activity near and below 0 °C. The inflection point in unfrozen water content just below 0 °C during soil thawing correlates to the onset of soil respiration activity. The observable relationship between unfrozen water content and respiration activity during soil phase change (increasing soil respiration with increasing unfrozen water content) was a practical rationale for modifying the GRESP soil respiration model and make it applicable to the sub-zero temperature regimes (down to -5 °C) of the aerobic soil bioremediation system. To express the observed relationship between unfrozen water and respiration, a modified Michaelis-Menten expression with sub-zero respiration term R_{bg}, combined with the Q_{10} temperature-sensitivity term, was incorporated into the original GRESP model to produce a new GRESP-based model called the URESP model. Sensitivity analyses for the URESP model were used to generate the best fits to CO_2 production and O_2 consumption rates measured previously in the biostimulated, petroleum hydrocarbon-contaminated, sub-Arctic soils between -5 and 15 °C (R^2 = 0.96 for CO_2 data and R^2 = 0.92 for O_2 data). The URESP model approximates sub-zero respiration activity, changes in respiration activity near 0 °C, and ramping respiration above 0 °C.

Using the numerical TEMP/W soil thermal model, a large dataset of predicted soil temperatures and water contents was generated. Using both the measured and predicted input data (T and M), the URESP model successfully generated CO_2 production and O_2 consumption rates. These URESP-computed soil respiration datasets were then used to determine RQ values and assess the substrate dependency of the soil respiration. The URESP-derived RQ values (0.695 to 0.698) were within the theoretical range for hydrocarbon degradation under aerobic conditions, and were generally in good agreement with the experimental RQ value (0.69) from the soil tank bioremediation experiment (Chang et al., 2011). Using these multiple approaches, it appears that the URESP model was effective for approximating the variable soil respiration rates in the simplified engineered soil bioremediation system between -5 and 15 °C. The model also allows semi-quantitative RQ assessments to be performed for the characterization of respiration activity.

Further research is necessary to improve the model (URESP module linked with soil thermal modelling) and incorporate freezing characteristics associated with soil types, soil

properties, soil treatment conditions, and environmental and climate conditions. Nonetheless, the URESP model expands the potential applicability of the GRESP model to sub-zero temperature regimes in simplified soil bioremediation systems and connects it to conventional high-throughput numerical soil thermal analysis.

3.6 Acknowledgements

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3.7 References

Abbasnezhad, H., Gray, M., Foght, J.M., 2011. Influence of adhesion on aerobic biodegradation and bioremediation of liquid hydrocarbons. Appl. Microbiol. Biotechnol. 92, 653-675.

Aislabie, J., Saul, D.J., Foght, J.M., 2006. Bioremediation of hydrocarbon-contaminated polar soils. Extremophiles 10, 171-179.

Arrhenius, S., 1898. The effect of constant influences upon physiological relationships. Scandinav. Archiv f. Physiol. 8, 367-415.

Aspray, T., Gluszek, A., Carvalho, D., 2008. Effect of nitrogen amendment on respiration and respiratory quotient (RQ) in three hydrocarbon contaminated soils of different type. Chemosphere 72, 947-951.

Bunnell, F., Tait, D., Flanagan, P., Van Clever, K., 1977. Microbial respiration and substrate weight loss—I: A general model of the influences of abiotic variables. Soil Biol. Biochem. 9, 33-40.

Carlyle, J.t., Than, U.B., 1988. Abiotic controls of soil respiration beneath an eighteen-year-old Pinus radiata stand in south-eastern Australia. J. Ecol. 76, 654-662.

Carson, J.K., Gonzalez-Quiñones, V., Murphy, D.V., Hinz, C., Shaw, J.A., Gleeson, D.B., 2010. Low pore connectivity increases bacterial diversity in soil. Appl. Environ. Microbiol. 76, 3936-3942.

CCME, 2001. Reference Method for the Canada – Wide Standard for Petroleum Hydrocarbons in Soil – Tier 1 Method. Canadian Council of Ministers of the Environment (CCME), Winnipeg, MB, Canada.

Chang, W., Akbari, A., David, C., Ghoshal, S., 2018. Selective biostimulation of cold- and salt-tolerant hydrocarbon-degrading Dietzia maris in petroleum-contaminated sub-Arctic soils with high salinity. J. Chem. Technol. Biotechnol. 93, 294-304.

Chang, W., Dyen, M., Spagnuolo, L., Simon, P., Whyte, L., Ghoshal, S., 2010. Biodegradation of semi- and non-volatile petroleum hydrocarbons in aged, contaminated soils from a sub-Arctic site: Laboratory pilot-scale experiments at site temperatures. Chemosphere 80, 319-326.

Chang, W., Ghoshal, S., 2014. Respiratory quotients as a useful indicator of the enhancement of petroleum hydrocarbon biodegradation in field-aged contaminated soils in cold climates. Cold Reg. Sci. Technol. 106, 110-119.

Chang, W., Klemm, S., Beaulieu, C., Hawari, J., Whyte, L., Ghoshal, S., 2011. Petroleum hydrocarbon biodegradation under seasonal freeze−thaw soil temperature regimes in contaminated soils from a sub-Arctic Site. Environ. Sci. Technol. 45, 1061-1066.

Chen, B., Liu, S., Ge, J., Chu, J., 2010. Annual and seasonal variations of Q10 soil respiration in the sub-alpine forests of the Eastern Qinghai-Tibet Plateau, China. Soil Biol. Biochem. 42,

1735-1742.

Clein, J.S., Schimel, J.P., 1995. Microbial activity of tundra and taiga soils at sub-zero temperatures. Soil Biol. Biochem. 27, 1231-1234.

Davidson, E.A., Samanta, S., Caramori, S.S., Savage, K., 2012. The Dual Arrhenius and Michaelis–Menten kinetics model for decomposition of soil organic matter at hourly to seasonal time scales. Global Change Biol. 18, 371-384.

Del Grosso, S., Parton, W., Mosier, A., Holland, E., Pendall, E., Schimel, D., Ojima, D., 2005. Modeling soil CO2 emissions from ecosystems. Biogeochemistry 73, 71-91.

Deming, J.W., 2002. Psychrophiles and polar regions. Curr. Opin. Microbiol. 5, 301-309.

Dilly, O., Nii-Annang, S., Franke, G., Fischer, T., Buegger, F., Zyakun, A., 2011. Resilience of microbial respiration, respiratory quotient and stable isotope characteristics to soil hydrocarbon addition. Soil Biol. Biochem. 43, 1808-1811.

Drotz, S.H., Sparrman, T., Nilsson, M.B., Schleucher, J., Öquist, M.G., 2010. Both catabolic and anabolic heterotrophic microbial activity proceed in frozen soils. Proc. Natl. Acad. Sci. U.S.A. 107, 21046-21051.

Drotz, S.H., Tilston, E.L., Sparrman, T., Schleucher, J., Nilsson, M., Öquist, M.G., 2009. Contributions of matric and osmotic potentials to the unfrozen water content of frozen soils. Geoderma 148, 392-398.

Du, E., Zhou, Z., Li, P., Jiang, L., Hu, X., Fang, J., 2013. Winter soil respiration during soil-freezing process in a boreal forest in Northeast China. J. Plant Ecol. 6, 349-357.

Ehlers, L.J., Luthy, R.G., 2003. Peer Reviewed: Contaminant Bioavailability in Soil and Sediment. Environ. Sci. Technol. 37, 295A-302A.

Elberling, B., Brandt, K.K., 2003. Uncoupling of microbial CO2 production and release in frozen soil and its implications for field studies of arctic C cycling. Soil Biol. Biochem. 35, 263-272.

Filler, D., Kennicutt, M.C., Snape, I., Sweet, S.T., Klein, A.G., 2015. Arctic and Antarctic spills. In: Fingas, M. (Ed.), Handbook of Oil Spill Science and Technology. John Wiley & Sons, Inc., Hoboken, pp. 495-512.

Flattery, P., Fealy, R., Fealy, R.M., Lanigan, G., Green, S., 2018. Simulation of soil carbon efflux from an arable soil using the ECOSSE model: Need for an improved model evaluation framework? Sci. Total Environ. 622, 1241-1249.

Gaumont-Guay, D., Black, T.A., Griffis, T.J., Barr, A.G., Jassal, R.S., Nesic, Z., 2006. Interpreting the dependence of soil respiration on soil temperature and water content in a boreal aspen stand. Agric. For. Meteorol. 140, 220-235.

Krahn, J., 2014. Thermal modeling with TEMP/W: An engineering methodology. GEO-SLOPE International, Ltd., Calgary, Canada.

Ghoshal, S., Luthy, R.G., 1998. Biodegradation kinetics of naphthalene in nonaqueous phase liquid-water mixed batch systems: Comparison of model predictions and experimental results. Biotechnol. Bioeng. 57, 356-366.

Ghuman, B., Lal, R., 1985. Thermal conductivity, thermal diffusivity, and thermal capacity of some Nigerian soils. Soil Sci. 139, 74-80.

Gomez, F., Sartaj, M., 2013. Field scale ex-situ bioremediation of petroleum contaminated soil under cold climate conditions. Int. Biodeterior. Biodegradation 85, 375-382.

Instanes, A., 2016. Incorporating climate warming scenarios in coastal permafrost engineering design–Case studies from Svalbard and northwest Russia. Cold Reg. Sci. Technol. 131, 76-87.

Karppinen, E.M., Siciliano, S.D., Stewart, K.J., 2017a. Application method and biochar type affect petroleum hydrocarbon degradation in northern landfarms. J. Environ. Qual. 46, 751-759.

Karppinen, E.M., Stewart, K.J., Farrell, R.E., Siciliano, S.D., 2017b. Petroleum hydrocarbon remediation in frozen soil using a meat and bonemeal biochar plus fertilizer. Chemosphere 173, 330-339.

Kim, J., Lee, A.H., Chang, W., 2018. Enhanced bioremediation of nutrient-amended, petroleum hydrocarbon-contaminated soils over a cold-climate winter: The rate and extent of hydrocarbon biodegradation and microbial response in a pilot-scale biopile subjected to natural seasonal freeze-thaw temperatures. Sci. Total Environ. 612, 903-913.

Konrad, J.M., McCammon, A., 1990. Solute partitioning in freezing soils. Can. Geotech. J. 27, 726-736.

Kukumägi, M., Ostonen, I., Uri, V., Helmisaari, H.S., Kanal, A., Kull, O., Lõhmus, K., 2017. Variation of soil respiration and its components in hemiboreal Norway spruce stands of different ages. Plant Soil 414, 265-280.

Lamy, E., Tran, T.C., Mottelet, S., Pauss, A., Schoefs, O., 2013. Relationships of respiratory quotient to microbial biomass and hydrocarbon contaminant degradation during soil bioremediation. Int. Biodeterior. Biodegradation 83, 85-91.

Liu, B., Mou, C., Yan, G., Xu, L., Jiang, S., Xing, Y., Han, S., Yu, J., Wang, Q., 2016. Annual soil CO2 efflux in a cold temperate forest in northeastern China: effects of winter snowpack and artificial nitrogen deposition. Sci. Rep. 6, 1-9.

Lloyd, J., Taylor, J., 1994. On the temperature dependence of soil respiration. Funct. Ecol. 8, 315-323.

Luo, Y., Zhou, X., 2006. Modeling Synthesis and Analysis, Soil respiration and the environment. Academic press, San Diego, pp. 215-246.

Ma, T., Wei, C., Xia, X., Zhou, J., Chen, P., 2017. Soil Freezing and Soil Water Retention Characteristics: Connection and Solute Effects. J. Perform. Constr. Fac. 31, D4015001-1-8.

Makita, N., Kosugi, Y., Sakabe, A., Kanazawa, A., Ohkubo, S., Tani, M., 2018. Seasonal and diurnal patterns of soil respiration in an evergreen coniferous forest: Evidence from six years of observation with automatic chambers. PloS One 13, 1-16.

Margesin, R., Gander, S., Zacke, G., Gounot, A.M., Schinner, F., 2003. Hydrocarbon degradation and enzyme activities of cold-adapted bacteria and yeasts. Extremophiles 7, 451-458.

Margesin, R., Schinner, F., 1999. Biological decontamination of oil spills in cold environments. J. Chem. Technol. Biotechnol. 74, 381-389.

Margesin, R., Schinner, F., 2001. Bioremediation (natural attenuation and biostimulation) of diesel-oil-contaminated soil in an alpine glacier skiing area. Appl. Environ. Microbiol. 67, 3127-3133.

McCarthy, K., Walker, L., Vigoren, L., Bartel, J., 2004. Remediation of spilled petroleum hydrocarbons by in situ landfarming at an arctic site. Cold Reg. Sci. Technol. 40, 31-39.

McWatters, R., Wilkins, D., Spedding, T., Hince, G., Raymond, B., Lagerewskij, G., Terry, D., Wise, L., Snape, I., 2016. On site remediation of a fuel spill and soil reuse in Antarctica. Sci. Total Environ. 571, 963-973.

Menten, L., Michaelis, M., 1913. Die kinetik der invertinwirkung. Biochem. Z. 49, 333-369.

Mikan, C.J., Schimel, J.P., Doyle, A.P., 2002. Temperature controls of microbial respiration in arctic tundra soils above and below freezing. Soil Biol. Biochem. 34, 1785-1795.

Moinet, G.Y., Hunt, J.E., Kirschbaum, M.U., Morcom, C.P., Midwood, A.J., Millard, P., 2018 The temperature sensitivity of soil organic matter decomposition is constrained by microbial access to substrates. Soil Biol. Biochem. 116, 333-339.

Mykytczuk, N.C., Wilhelm, R.C., Whyte, L.G., 2012. Planococcus halocryophilus sp. nov., an extreme sub-zero species from high Arctic permafrost. Int. J. Syst. Evol. Microbiol. 62, 1937-1944.

Olsson, P.Q., Sturm, M., Racine, C.H., Romanovsky, V., Liston, G.E., 2003. Five Stages of the Alaskan Arctic Cold Season with Ecosystem Implications. Arct. Antarct. Alp. Res. 35, 74-81.

Öquist, M.G., Sparrman, T., Klemedtsson, L., Drotz, S.H., Grip, H., Schleucher, J., Nilsson, M., 2009. Water availability controls microbial temperature responses in frozen soil CO2 production. Global Change Biol. 15, 2715-2722.

Or, D., Smets, B.F., Wraith, J.M., Dechesne, A., Friedman, S.P., 2007. Physical constraints affecting bacterial habitats and activity in unsaturated porous media – a review. Adv. Water Resour. 30, 1505-1527.

Panday, S., Corapcioglu, M.Y., 1991. Solute rejection in freezing soils. Adv. Water Resour. 27, 99-108.

Panikov, N., Flanagan, P., Oechel, W., Mastepanov, M., Christensen, T., 2006. Microbial activity

in soils frozen to below -39 °C. Soil Biol. Biochem. 38, 785-794.

Qi, Y., Xu, M., Wu, J., 2002. Temperature sensitivity of soil respiration and its effects on ecosystem carbon budget: nonlinearity begets surprises. Ecol. Modell. 153, 131-142.

Ramaswami, A., Ghoshal, S., Luthy, R.G., 1997. Mass Transfer and Bioavailability of PAH Compounds in Coal Tar NAPL−Slurry Systems. 2. Experimental Evaluations. Environ. Sci. Technol. 31, 2268-2276.

Raymond-Bouchard, I., Whyte, L.G., 2017. From Transcriptomes to Metatranscriptomes: Cold Adaptation and Active Metabolisms of Psychrophiles from Cold Environments. In: Margesin, R., Schinner, F., Marx, J.C., Gerday, C., (Eds), Psychrophiles: From Biodiversity to Biotechnology. Springer International Publishing, Cham, pp. 437-457.

Rike, A.G., Haugen, K.B., Børresen, M., Engene, B., Kolstad, P., 2003. In situ biodegradation of petroleum hydrocarbons in frozen arctic soils. Cold Reg. Sci. Technol. 37, 97-120.

Rike, A.G., Haugen, K.B., Engene, B., 2005. In situ biodegradation of hydrocarbons in arctic soil at sub-zero temperatures—field monitoring and theoretical simulation of the microbial activation temperature at a Spitsbergen contaminated site. Cold Reg. Sci. Technol. 41, 189-209.

Rivkina, E., Friedmann, E., McKay, C., Gilichinsky, D., 2000. Metabolic activity of permafrost bacteria below the freezing point. Appl. Environ. Microbiol. 66, 3230-3233.

Sánchez-Cañete, E.P., Barron-Gafford, G.A., Chorover, J., 2018. A considerable fraction of soil-respired CO2 is not emitted directly to the atmosphere. Sci. Rep. 8, 2-11.

Sanscartier, D., Laing, T., Reimer, K., Zeeb, B., 2009. Bioremediation of weathered petroleum hydrocarbon soil contamination in the Canadian High Arctic: Laboratory and field studies. Chemosphere 77, 1121-1126.

Sanscartier, D., Reimer, K., Zeeb, B., Koch, I., 2011. The effect of temperature and aeration rate on bioremediation of diesel-contaminated soil in solid-phase bench-scale bioreactors. Soil Sediment Contam. 20, 353-369.

Schaefer, K., Jafarov, E., 2016. A parameterization of respiration in frozen soils based on substrate availability. Biogeosciences 13, 1991-2001.

Schlentner, R.E., Cleve, K.V., 1985. Relationships between CO2 evolution from soil, substrate temperature, and substrate moisture in four mature forest types in interior Alaska. Can. J. Forest Res. 15, 97-106.

Siciliano, S.D., Schafer, A.N., Forgeron, M.A., Snape, I., 2008. Hydrocarbon contamination increases the liquid water content of frozen Antarctic soils. Environ. Sci. Technol. 42, 8324-8329.

Snape, I., Acomb, L., Barnes, D.L., Bainbridge, S., Eno, R., Filler, D.M., lato, N., Poland, J.S., Raymond, T.C., Rayner, J.L., Riddle, M.J., Rike, A.G., Rutter, A., Schafer, A.N., Siciliano, S.D., Walworth, J.L., 2008a. Contamination, regulation, and remediation: an introduction

to bioremediation of petroleum hydrocarbons in cold regions. In: Filler, D.M., Snape, I., Barnes, D.L., (Eds), Bioremediation of Petroleum Hydrocarbons in Cold Regions. Cambridge University Press, Cambridge, pp. 1-37.

Snape, I., Ferguson, S., Reynolds, M.C., Walworth, J.L., 2008b. Treatability studies: microcosms, mesocosms, and field trials. In: Filler, D.M., Snape, I., Barnes, D.L., (Eds), Bioremediation of Petroleum Hydrocarbons in Cold Regions. Cambridge University Press, Cambridge, pp. 125-153.

Sobek, S., Gudasz, C., Koehler, B., Tranvik, L.J., Bastviken, D., Morales-Pineda, M., 2017. Temperature dependence of apparent respiratory quotients and oxygen penetration depth in contrasting lake sediments. J. Geophys. Res. Biogeosci. 122, 3076-3087.

Spaans, E.J.A., Baker, J.M., 1996. The Soil Freezing Characteristic: Its Measurement and Similarity to the Soil Moisture Characteristic. Soil Sci. Soc. Am. J. 60, 13-19.

Steinweg, J.M., Dukes, J.S., Wallenstein, M.D., 2012. Modeling the effects of temperature and moisture on soil enzyme activity: Linking laboratory assays to continuous field data. Soil Biol. Biochem. 55, 85-92.

Suzuki, S., 2004. Verification of freezing point depression method for measuring matric potential of soil water. Soil Sci. Plant Nutr. 50, 1277-1280.

Tilston, E., Sparrman, T., Öquist, M., 2010. Unfrozen water content moderates temperature dependence of sub-zero microbial respiration. Soil Biol. Biochem. 42, 1396-1407.

Tucker, C., 2014. Reduction of air-and liquid water-filled soil pore space with freezing explains high temperature sensitivity of soil respiration below 0 °C. Soil Biol. Biochem. 78, 90-96.

Wang, G., Jagadamma, S., Mayes, M.A., Schadt, C.W., Steinweg, J.M., Gu, L., Post, W.M., 2015. Microbial dormancy improves development and experimental validation of ecosystem model. ISME J. 9, 226-237.

Wang, Y., Liu, H., Chung, H., Yu, L., Mi, Z., Geng, Y., Jing, X., Wang, S., Zeng, H., Cao, G., 2014. Non-growing-season soil respiration is controlled by freezing and thawing processes in the summer monsoon-dominated Tibetan alpine grassland. Global Biogeochem. Cy. 28, 1081-1095.

Wen, Z., Ma, W., Feng, W., Deng, Y., Wang, D., Fan, Z., Zhou, C., 2012. Experimental study on unfrozen water content and soil matric potential of Qinghai-Tibetan silty clay. Environ. Earth Sci. 66, 1467-1476.

Whyte, L., Labbé, D., Goalen, B., Buchko, J., Nahir, M., Billowits, M., Greer, C. In-situ bioremediation of hydrocarbon contaminated soils in the High Arctic. In: Nahi, M., Biggar, K., Cotta, G., editors. Third Biennal Workshop on Assessment and Remediation of Contaminated Sites in Arctic and Cold Climates (ARCSACC). Edmonton, Canada, 2003, pp. 245-256.

Wilson, J.M., Griffin, D.M., 1975. Water potential and the respiration of microorganisms in the soil. Soil Biol. Biochem. 7, 199-204.

Yoshikawa, K., Overduin, P.P., 2005. Comparing unfrozen water content measurements of frozen soil using recently developed commercial sensors. Cold Reg. Sci. Technol. 42, 250-256.

CONNECTING TEXT: CHAPTER 3 AND 4

In Chapter 2, we found that enhanced petroleum hydrocarbon biodegradation in the field occurred during seasonal freezing and thawing when unfrozen water retention was high. In Chapter 3, we estimated biodegradation activity related to unfrozen water content in thawing biotreated soil. The remaining research questions were (1) how to optimally control unfrozen water and (2) what are the mechanisms for extended biodegradation in freezing and frozen soils. Chapter 4 answers these questions by demonstrating how to manipulate unfrozen water content using soil treatment and how to link soil freezing properties to enhanced biodegradation during the freezing and frozen phases.

CHAPTER 4

MANIPULATION OF UNFROZEN WATER RETENTION FOR ENHANCING PETROLEUM HYDROCARBON BIODEGRADATION IN SEASONALLY FREEZING AND FROZEN SOIL

Submitted to

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This chapter is prepared to be submitted to *Environmental Science & Technology* as Kim, J., Chang, W., Lee, A.H., 2020. *Manipulation of unfrozen water retention for enhancing petroleum hydrocarbon biodegradation in seasonally freezing and frozen soil*. Dr. Chang (supervisor) provided the funding for this study and suggested the experimental design concept, including technical details and interpretation methods. Dr. Lee assisted with the culture-independent microbial analysis and the operation of the 3D PCR platform. Jihun Kim prepared and finalized the experimental setup and design and conducted the physicochemical and culture-dependent microbial analyses. Jihun Kim completed the interpretation of the physicochemical and microbial data, including microbial community and *alkB1* gene data, under Dr. Chang's supervision. Jihun Kim completed the manuscript draft and prepared the figures and tables.

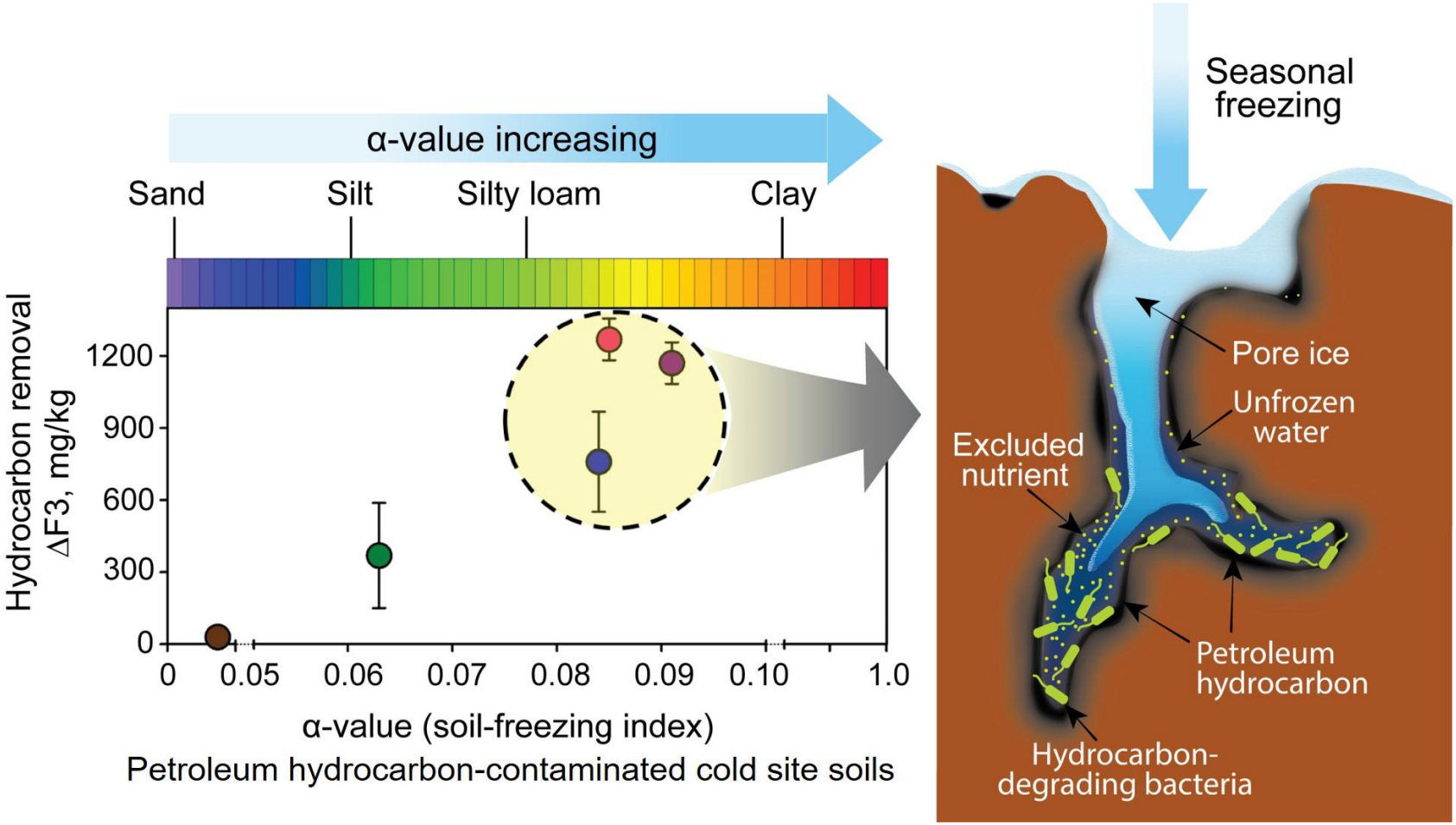

Figure 4.1. Graphical abstract of Chapter 4.

4.1 Abstract

Manipulating unfrozen water in frozen contaminated soil for prolonged bioremediation in cold climates remains unformulated. This freezing-induced biodegradation experiment shows how nutrient and zeolite amendments affect unfrozen water retention and hydrocarbon biodegradation in petroleum-contaminated site soils undergoing seasonal freezing. During soil freezing at a site-specific rate (4 to −10 °C; −0.2 °C/d), the effect of nutrients was predominant during early freezing (4 to −5 °C), alleviating the abrupt soil-freezing stress near the freezing-point depressions, elevating alkB1 gene-harboring populations, and significantly enhancing hydrocarbon biodegradation. The effect of unfrozen water retention, which increased with added zeolite surface area, was critical in extending hydrocarbon biodegradation to the subsequent frozen phase (−5 to −10 °C). A series of soil-freezing characteristic curves (SFCC) with empirical α-values (soil-freezing index) were constructed for the tested soils and shown alongside representative curves for clays to sands, indicating correlations between α-values and nutrient concentrations (soil electrical conductivity), zeolite addition (surface area), and hydrocarbon biodegradation. Heavier hydrocarbons (F3: C16-C34) notably biodegraded in all treated soils (22–37% removal), as confirmed by biomarker-based hydrocarbon analyses (17α(H),21β(H)-hopane), whereas lighter hydrocarbons were unavailable in frozen water. Below 0 °C, finer-grained soils (high α-values) can be biostimulated more readily than coarser-grained soils (low α-values).

4.2 Introduction

Petroleum contamination has amplified in cold regions, including the Arctic and Antarctic, as industrial and human activities have intensified (Snape et al., 2008). The remediation of petroleum hydrocarbon-contaminated sites in cold climates is challenging and often prohibitively expensive, especially at polar and sub-polar sites, due to low temperatures, low biomass, remote locations, complicated field logistics, and the environmental sensitivity of ecosystems, including fragile frozen soils (McDonald and Knox, 2014; Siciliano et al., 2008).

Bioremediation of petroleum hydrocarbon-contaminated soils in cold climates has been frequently considered to stimulate the metabolic activity of indigenous cold-adapted hydrocarbon-degrading bacteria by supplying nutrients (e.g., nitrogen- and phosphorus-based fertilizer)

(Aislabie et al., 2006; Camenzuli and Freidman, 2015; Margesin and Schinner, 1997; Walworth et al., 2007; Whyte et al., 2001). The majority of cold-region bioremediation studies target short summers (2-4 months) to leverage elevated microbial activity and better field accessibility (McCarthy et al., 2004; Whyte et al., 2001). However, a comparatively limited effort has been made to assess the feasibility of extending hydrocarbon biodegradation in seasonally freezing and deeply frozen soils subjected to variable in situ freeze-thaw conditions (Chang et al., 2011).

Seasonal soil freezing *in situ* is considered a slow process. For example, natural seasonal soil freezing rates can be as slow as −0.1 °C/d except in the uppermost ~5–10 cm of topsoil (Aislabie et al., 2004; Chang et al., 2011; Kim et al., 2018; Konrad and McCammon, 1990; Olsson et al., 2003), which is influenced by daily and periodic temperature fluctuations and sunlight depending on local climatic, geographical, geological, and environmental conditions (Aislabie et al., 2004; Henry, 2007). When soil freezes at such slow rates, solutes in unfrozen water tend to be rejected from the ice (Baker and Osterkamp, 1989; Konrad and McCammon, 1990; Marion, 1995; Panday and Corapcioglu, 1991), thus increasing the salinity of unfrozen water (Baker and Osterkamp, 1989; Konrad and McCammon, 1990), decreasing the freezing point of soils (Bing and Ma, 2011; Han et al., 2018; Wu et al., 2015), and preserving unfrozen water (Marion, 1995). The duration of the partially frozen soil phase, in which unfrozen water and ice coexist, can be prolonged from a few weeks to months, partly as a result of the zero-curtain effect that holds soil temperatures near freezing during seasonal shifts in cold climates (Olsson et al., 2003). The soils eventually enter the deeply frozen phase, when unfrozen water content becomes limited at a threshold level that is minimal but vital for cold-adapted microbial populations to survive at extreme subzero temperatures (Drotz et al., 2010; Öquist et al., 2009; Panikov et al., 2006; Rivkina et al., 2000). The freezing rate also influences microbial survivability associated with physiological acclimation and osmoregulation of microbial cells (Jefferies et al., 2010; Margesin, 2003; Mazur, 1984; Or et al., 2007; Schimel et al., 2007). Substantial salt-tolerance (5–10% NaCl) is reported for cold-adapted bacteria isolated from polar and sub-polar habitats such as the active layer of freezing soils, permafrost, sea ice, and even petroleum-contaminated sub-Arctic soils (Chang et al., 2018; Deming, 2002; Mykytczuk et al., 2013; Mykytczuk et al., 2012). Laboratory and field bioremediation experiments conducted using petroleum-contaminated cold-climate soils freezing at natural *in situ* seasonal rates (near −0.1 °C/d) indicate the emergence of winter microbial populations in partially frozen and deeply frozen soils (Chang et al., 2011; Kim et al., 2018).

Conventional laboratory studies conducted using rapid repeated freeze-thaw cycles are intended to mimic diurnal, periodic, and drastic fluctuation effects (e.g., ±20 °C per day) and use rates that are much faster than seasonal rates (Børresen et al., 2007; Eriksson et al., 2001; Liu et al., 2016; Morley et al., 1983; Schimel and Mikan, 2005; Walker et al., 2006). Applying soil freezing rates that are well controlled and site-representative, in combination with a sampling strategy based on the soil thermal phase, is crucial for studying seasonally freezing and thawing soil environments, as noted and similarly discussed in prior work (Henry, 2007).

Previous bioremediation experiments indicate the presence of unfrozen water is critical for extending hydrocarbon biodegradation in frozen contaminated soil (Harvey et al., 2012; Karppinen et al., 2019; Karppinen et al., 2017a; Karppinen et al., 2017b; Siciliano et al., 2008). Yet, the relationship between the manipulation of unfrozen water retention via controllable soil factors and the enhancement of petroleum hydrocarbon biodegradation is not understood. Unfrozen water content, influenced by a variety of factors, gradually decreases as soils undergo seasonal freezing, before stabilizing at a minimum threshold in deeply frozen soil (Kim et al., 2018). The present freezing-induced experiment focuses on how to manipulate unfrozen water retention using *externally* controllable soil factors (nutrient solutes and zeolite for added surface area) to enhance hydrocarbon biodegradation activity in seasonally freezing and frozen contaminated soil from a cold site.

4.3 Materials and methods

4.3.1 Site soil

The site soil was shipped from an outdoor soil remediation facility in Saskatoon, Saskatchewan (PINTER & Associates, Ltd.). Soil properties, local climatic classification, and measured air and soil temperatures at the site where the soils were treated are available in the authors' field study (Kim et al., 2018). Briefly, on-site ambient air and soil temperatures fluctuated between −30 and −10 °C over the winter and the soil is classified as fine-grained (Unified Soil Classification System), clay loam (United States Department of Agriculture), and deficient in inorganic nitrogen (N) species. It is contaminated with F2 (C10-C16; semi-volatiles) and F3 (C16-C34; non-volatiles) hydrocarbons and has negligible F1 (C6-C10; volatiles) and F4 (>C34)

concentrations. The initial F2 and F3 concentrations were 2858 ± 285.7 and 3441 ± 141.9 mg/kg, respectively.

4.3.2 Microcosms subjected to seasonal freezing

Soil microcosms containing 100–200 g of wet site soils were aseptically prepared using sterilized 500-mL amber glass jars. For each tested soil treatment, the microcosms were divided into several sets: a sacrificial soil-sampling set and two subsets of monitoring microcosms, one for measuring soil temperature and volumetric water content and the other to measure CO_2 soil gas. The monitoring microcosms provided real-time data for soil temperature, unfrozen water content, and CO_2 soil gas, and allowed for the soil-sampling microcosms to be analyzed during the distinct freezing and frozen phases. Within each set, the microcosms were further divided into smaller sets: treated soil, positive control (untreated soil), and negative control (sterilized soil) microcosms, in triplicate for the soil-sampling microcosms and in duplicate for the monitoring microcosms (Table E.2, Supporting Information). All microcosm sets were simultaneously prepared and run for 150 d in a temperature-programmable incubator (MIR-254-PA, Panasonic®).

The programmed temperature regime was devised to simulate site-representative, seasonal freezing and frozen phases based on an *in situ* soil temperature dataset for the same soils (Kim et al., 2018). Specifically, it was designed to induce a naturally slow decrease in soil temperature from 4 to −10 °C at −0.2 °C/d until Day 71 before holding it at −10 °C until Day 150. The soil-sampling microcosms were sacrificed at precise times: (1) as the soils were experiencing the *freezing* phase at Day 42, when unfrozen water content varied among the microcosms and soil temperatures were approximately −5 or −6 °C depending on the soil treatment, and (2) when the soils were distinctly in the *frozen* phase at Day 142, when soil temperatures had reached −10 °C and detectable unfrozen water content had stabilized.

4.3.3 Monitoring microcosms and soil freezing characteristics

A real-time monitoring probe for soil temperature and water content was inserted into each microcosm of one subset of the monitoring microcosms, consisting of a surface-mounted thermistor for soil temperature and a frequency domain reflectometry (FDR) sensor for volumetric

water content (5TM VWC + Temp, Decagon Devices, Pullman, USA) (Chang et al., 2011; Kim et al., 2018). All probes were connected to two data loggers (EM50, Decagon Devices, Pullman, USA). The other subset of monitoring microcosms was connected to a soil gas collection system designed for continuous soil-gas monitoring, as similarly described elsewhere (Chang and Ghoshal, 2014). For each of the various treated and untreated site soil microcosms, a soil freezing characteristic curve (SFCC) was generated. Using nonlinear curve-fitting, the SFCCs were each used to express an empirical function relating soil temperature and volumetric water content (Andersland and Ladanyi, 2003). Frozen soil parameters were determined, including freezing-point depression, soil-phase transition rate, and residual water content. The SFCC is expressed as the empirical relationship between soil temperature and corresponding unfrozen water content:

$$\theta = \alpha T^{\beta} \quad \text{..... (4.1)}$$

where θ is the measured volumetric unfrozen water content (m^3/m^3), T is the absolute value of the negative soil temperature, and α and β are fitting constants (Andersland and Ladanyi, 2003). The empirical α-value is dependent on soil type and represents soil freezing characteristics, and was determined by fitting the experimental data (θ and T) to the SFCC equation (Andersland and Ladanyi, 2003).

4.3.4 Soil treatment

Three soil treatments were studied using the soil-sampling and monitoring microcosms: (1) an NPK fertilizer amendment only, (2) NPK with 4% peat moss (w/w), and (3) NPK with 5% zeolite (w/w). An inorganic NPK fertilizer (Plant Prod®; 20% total N to 20% P_2O_5 to 20% K_2O) was applied to all treated microcosms to serve as a baseline for the effect of background nutrient elements, which were deficient in the site soils (especially nitrogen).

The soil microcosms that received NPK only were treated with a pre-optimized dose of 49 mg N/kg and considered as low-applied-nutrient microcosms (*NPK only*). A higher dosage of NPK fertilizer, 249 mg N/kg, was applied to the set treated with peat moss, serving as high-applied-nutrient microcosms (*NPK + 4% peat moss*). The 4% peat moss amendment provided additional porous organic carbon (Table E.4, Supporting Information). Finally, the set that received the additional zeolite had a total nitrogen concentration of 49 mg N/kg, the same as the

NPK only set, to test the effect of increased soil surface area provided by the zeolite (*NPK + 5% zeolite*). All treated and untreated soil microcosms received additional water to equalize the initial water contents at 18.3 % (w/w) and were similarly treated throughout the microcosm preparation process, which involved a one-time soil treatment and homogenization. Detailed information about the soil treatment is available in Supporting Information, Section E.4.3 (Figures E.1 to E.3; Tables E.3 to E.5), including soil nutrient concentrations (C, N, P, K), soil amendment data (compositions, effective particle sizes and surface areas), dosages of the soil amendments (peat moss and zeolites) for optimizing water holding capacity, and soil pH.

4.3.5 Petroleum hydrocarbon analyses

The detailed procedures for petroleum hydrocarbon extraction, post-processing (sodium sulfate-based moisture trapping, silica gel clean-up and N_2 gas blow down), and gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) analyses, along with the quality assurance and quality control (QA/QC) programs for the analytical equipment, protocols, and hydrocarbon extractability, are extensively described in the authors' prior studies (Chang et al., 2011; Kim et al., 2018). Briefly, petroleum hydrocarbon analyses were conducted using GC-FID coupled with GC-MS based on the CWS PHC Tier 1 Method (CCME, 2001) (Agilent 7890A, AccuTOF GCV 4G Mass Spectrometer). Wet soil samples (~10 g) were obtained from the treated and untreated soil microcosms at the start (Day 0), during the freezing phase (Day 42), and during the frozen phase (Day 145), then loaded into an automatic Soxhlet extractor for hydrocarbon extraction (Gerhardt Soxtherm, Germany). O-terphenyl was spiked into every soil sample as a surrogate and over 85% extractability was maintained. The diagnostic ratios for hydrocarbon biodegradation were quantified using a conservative biomarker, 17α(H),21β(H)-hopane, to confirm the occurrence of hydrocarbon biodegradation over abiotic losses (Prince et al., 1994; Wang et al., 2016). Two-way ANOVA integrated with Bonferroni post-tests was conducted for the hydrocarbon data using GraphPad Prism 5 (GraphPad Software, USA).

4.3.6 Microbial analyses

The viability of hydrocarbon-degrading bacteria, microbial community compositions, and *alkB1* gene copy numbers were also analyzed for each soil sample on Days 0, 42, and 145. The microbial analyses are described in detail in the authors' previous study (Kim et al., 2018). Briefly, genomic DNA from the homogenized 0.25-g soil samples was extracted using DNeasy PowerSoil Kits (Qiagen, USA). The concentration and purity of DNA extracts were determined by BioDrop μLITE UV-Vis spectrophotometry (Montreal Biotech, Canada) using A260/280 and A260/230 ratios. A 3D digital polymerase chain reaction (PCR) system (QuantStudio™, Life Technologies) was used to quantify *alkB1* gene copy numbers, as described in the authors' study(Kim et al., 2018). The digital PCR analyses were conducted for a total of 81 DNA samples extracted from the treated and untreated soil microcosms. The TaqMan® assay (Thermo Fisher Scientific Canada) was used to target the *alkB1* gene with a forward primer (5'- ATC TGG GCG CGT TGG GAT TTG AGC G-3'), a reverse primer (5'-CGC ATG GTG ATC GCT GTG CCG CTG C-3'), and a FAM™-dye labelled probe (5'-ACT CCG GAA GAT CCG GCG A-3'), as described by others (Whyte et al., 2002).

A total of nine gDNA samples were submitted to RTL Genomics (Lubbock, Texas) for *Illumina* MiSeq analyses of 16S rRNA V4 amplicons (with 515F/806R primer) based on paired-end sequencing (Kennedy et al., 2014). The high-throughput sequencing data obtained were submitted to the BioProject archive under Accession No. PRJNA587573 at the National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/bioproject/). The relative abundances of operational taxonomic units (OTUs) were calculated for the treated and untreated soil samples and sorted using a sequence similarity cut-off of 97%. Sequence data were processed using a high-throughput metagenome sequencing and all analyses were conducted in R and phyloseq packages (McMurdie and Holmes, 2013; Team, 2013). Principal coordinate analysis (PCoA) was conducted using the weighted UniFrac distances of equal numbers of bacterial sequences (n = 2,546). Heat maps showing the top 20 bacterial genera were generated as described elsewhere (Kim et al., 2018).

4.4 Results

4.4.1 Shifts in soil freezing characteristics linked to unfrozen water retention

SFCCs have not been reported for petroleum-contaminated cold-climate soils treated for biostimulation. Figure 4.2 shows shifts in the SFCCs of the contaminated site soils connected to increased unfrozen water retention due to the soil treatments, as compared to the SFCC of the untreated site soil (*Control*). The overall trend in the SFCCs shows unfrozen water was retained most in *NPK + 5% zeolite*, followed by *NPK only* and *NPK + 4% peat moss*. The untreated control soils retained the least unfrozen water throughout freezing.

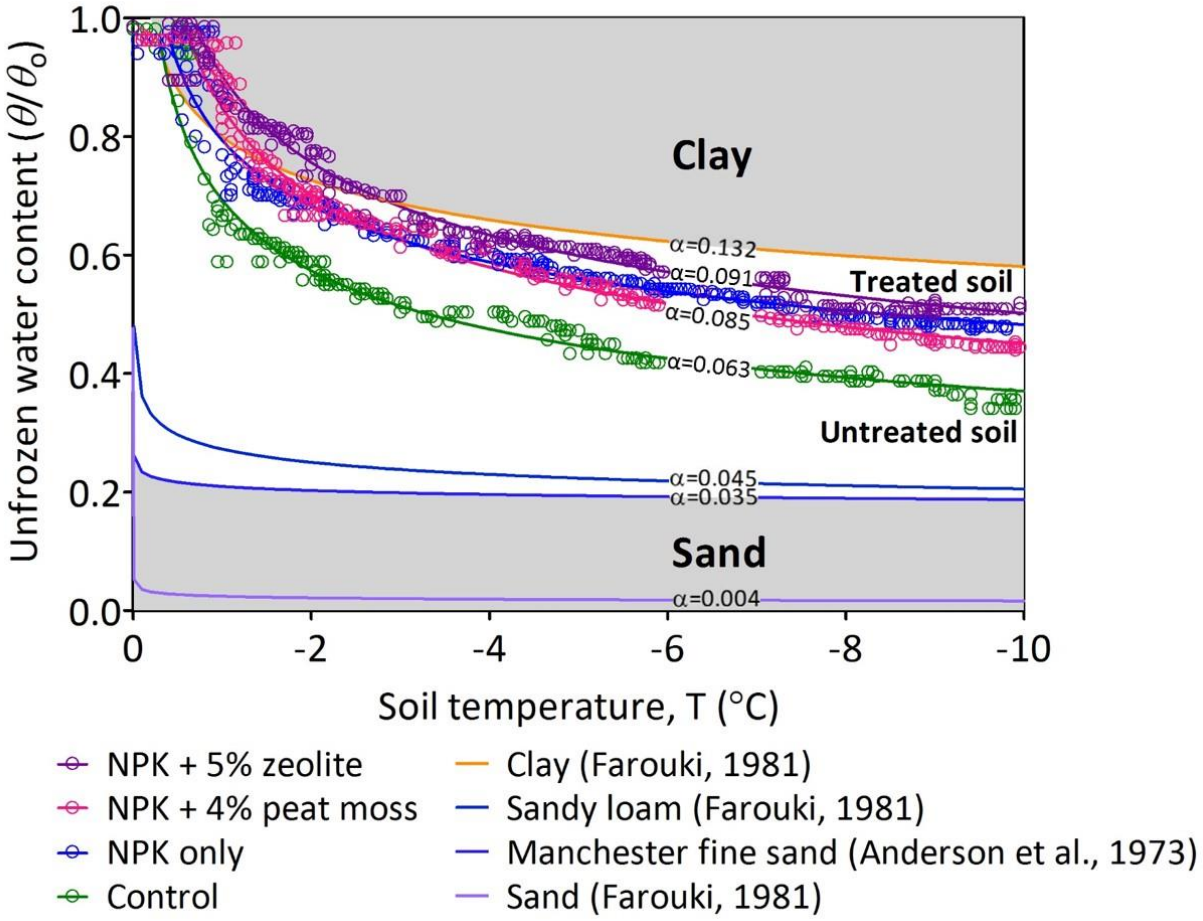

Figure 4.2. SFCCs of the treated and untreated site soils, along with those of reference soils from clays to sands (Anderson et al., 1973; Farouki, 1981).

The untreated site soil (*Control*), which is clay loam, had an α-value of 0.063 and exhibited significantly higher unfrozen water retention than the reference sands, which had low α-values below 0.045 (Fig. 4.2). The soils amended with water-based inorganic nutrients (N, P, and K) and carbon, *NPK only* and *NPK + 4% peat moss,* had similar α-values of 0.084 and 0.085, respectively. *NPK + 5% zeolite* exhibited the highest α-value of 0.094. The clay-sized zeolite (D_{10} ≈ 1.9 μm) in *NPK + 5% zeolite* increased the surface area of the treated soils by 30% to 6.1 m^2/g compared to the control soil (4.7 m^2/g), thus producing an α-value closer to those of clayey soils, which are typically above 0.1. The most significant unfrozen water retention was therefore observed in *NPK + 5% zeolite*. Additional investigation of the relationship between surface area, α-values, and unfrozen water retention, using 0, 5, and 10% zeolite amendments (w/w), showed higher zeolite doses increased the surface area (4.7, 6.1, 6.9 $m^{2/}g$, respectively) and α-value of the site soil (0.063, 0.072, 0.083, respectively), ultimately increasing unfrozen water retention (normalized VWC of 0.48, 0.58, 0.63, respectively) (Table S4.8). The α-value closely correlated with residual unfrozen water (r = 0.97). SFCCs with α-values can be used to represent shifts in soil freezing characteristics linked to the manipulation of unfrozen water retention via soil biostimulation treatments.

4.4.2 Enhanced hydrocarbon biodegradation in the freezing and frozen soil phases

Figure 4.3 shows the significant biodegradation of F3 hydrocarbons in the soils during the freezing phase (by Day 42) and the subsequent frozen phase (by Day 142). Overall decreases in F3 hydrocarbon concentrations were similar for *NPK + 4% peat moss* and *NPK + 5% zeolite*, at 37 and 34%, respectively, and slightly lower for *NPK only* (22%). The non-amended *Control* and sterilized soils exhibited F3 removals of 11 and 2%, respectively (Table E.9, Supporting Information). The decreases in F3 hydrocarbons, branched alkanes (represented by phytane; $C_{20}H_{42}$), and individual alkanes (C16-C26) were statistically significant in the treated soils (two-way ANOVA plus Bonferroni post-hoc, $p<0.01$ for Fig. 4.3; and Fig. E.8 and Table E.9 Supporting Information). The biomarker-based hydrocarbon analyses using 17α(H),21β(H)-hopane support the conclusion that hydrocarbon removal in the freezing and frozen soils is due to enhanced biodegradation over abiotic loss. No degradation in F2 hydrocarbons occurred (Table E.9, Supporting Information).

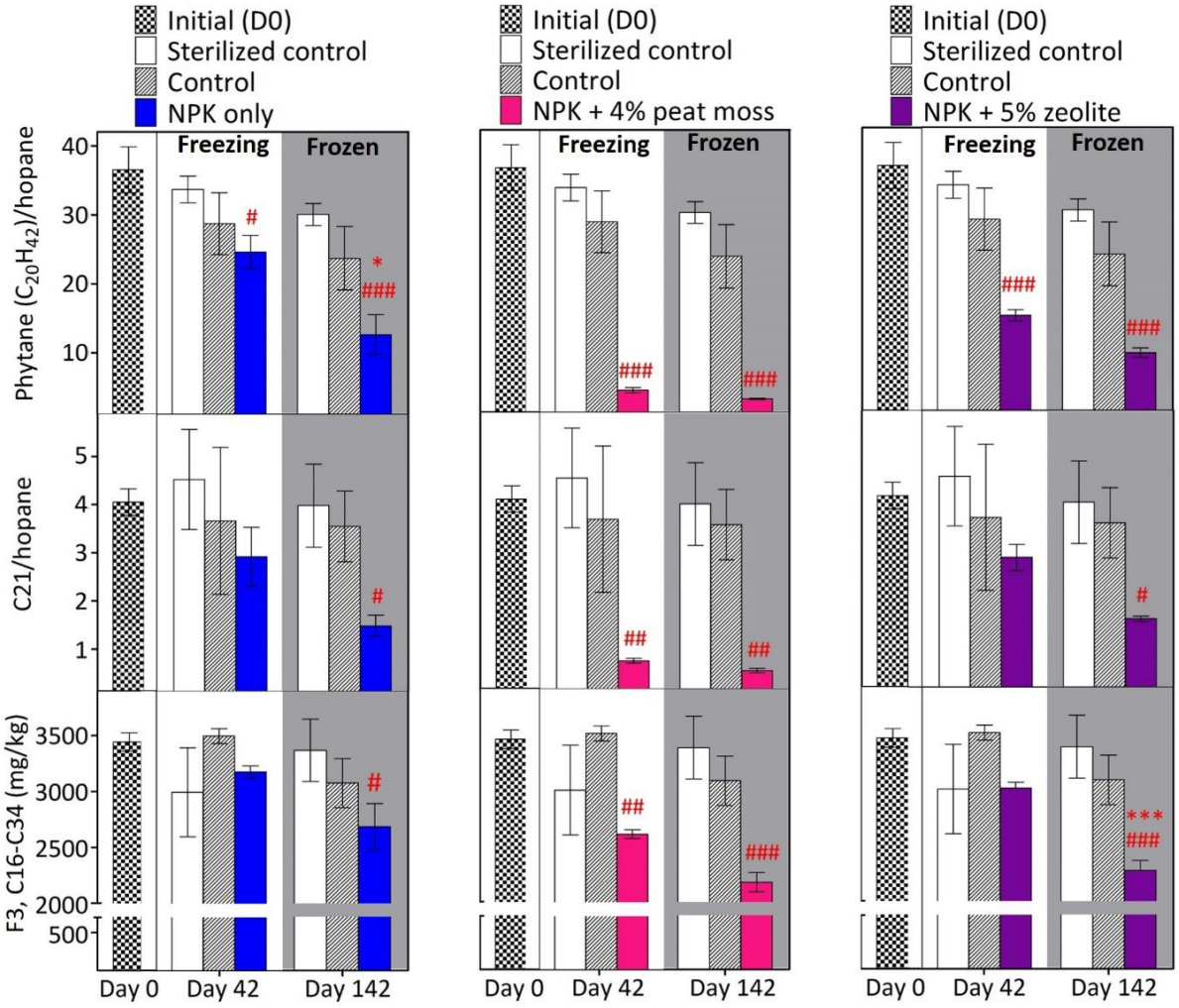

Figure 4.3. Concentrations of bulk F3 hydrocarbons in the freezing and frozen soil phases and of representative branched and alkane hydrocarbons over that of the conservative biomarker. The soils were collected at −5 °C on Day 42 and at −10 °C on Day 142 ($p < 0.05$: #, *; $p < 0.01$: ##, **; and $p < 0.001$: ###, ***, where # indicates a comparison with the initial concentration at Day 0 and * indicates a comparison with the concentration at Day 42).

The F3 hydrocarbon data also indicate the individual effects of the nutrient and zeolite amendments on hydrocarbon biodegradation are dependent on the soil freezing phase. The *NPK + 4% peat moss* received the highest nutrient dose, and hydrocarbon biodegradation was enhanced most in the earlier freezing phase (24% F3 removal) than in the subsequent frozen phase (14% F3 removal). Conversely, hydrocarbon biodegradation in *NPK + 5% zeolite*, which had the largest surface area, was enhanced less in the freezing phase (13% F3 removal) than in the frozen phase (20% F3 removal).

4.4.3 Increased abundance of hydrocarbon degraders during seasonal freezing

The microbial aspect of this study focused on the relationship between hydrocarbon degrader abundance and F3 hydrocarbon removal during the seasonal freezing. The *alkB1* gene copy numbers correlated with the F3 hydrocarbon removal data. Previous field data for the same site soils, obtained *in situ* under seasonal freezing conditions, were also plotted with the laboratory data for comparison. In that field study, the pilot-scale biopile (3 tons) was similarly treated with nutrients and humate (carbon source) and the soil-phase change began near −1 °C; detectable unfrozen water content in partially frozen soil was prolonged to around −6 °C (Kim et al., 2018). As a result, the biostimulation increased the abundance of *alkB1* gene copy numbers by one or two orders of magnitude during seasonal soil freezing and an increase in F3 removal was observed in both the laboratory and field experiments (Fig. 4.4). This laboratory study confirmed that seasonal freezing stress is not an inhibiting factor for enhancing microbial activity for F3 hydrocarbon biodegradation in site soils amended with zeolite and/or nutrients. The increases in viable hydrocarbon-degrading microbial populations and CO_2 soil gas production during seasonal soil freezing are in good agreement with the *alkB1* gene and F3 hydrocarbon data ($r = 0.74$ for CO_2 production vs. F3 removal; Fig. E.11, Supporting Information).

The soil treatment increased the relative abundance of hydrocarbon-degrading genera, as indicated by the high-throughput 16S rRNA gene sequencing analysis (Fig. E.9, Supporting Information). Among the top 20 genera detected in the freezing and frozen phases, 12 are relatives of hydrocarbon degraders and labelled with black circles in Fig. E.9 (*Acidovorax*, *Variovorax*, *Massilia*, *Pseudomonas*, *Pseudoxanthomonas*, *Luteimonas*, *Sphingomonas*, *Sphingobium*, *Anthrobacter*, *Nocardioides*, *Mycobacterium*, and *Rhodococcus*). The PCoA analyses combined

with microbial composition analyses consistently indicate the soil treatment altered the relative abundance of hydrocarbon-degrading genera in the seasonal freezing phase (4 to −5 °C). However, the microbial composition was stable in the subsequent frozen phase (−5 to −10 °C) (Figs. E.9 to E.13, Supporting Information). Both culture-independent and -dependent analyses suggest microbial selection and the significant enhancement of hydrocarbon biodegradation during seasonal freezing.

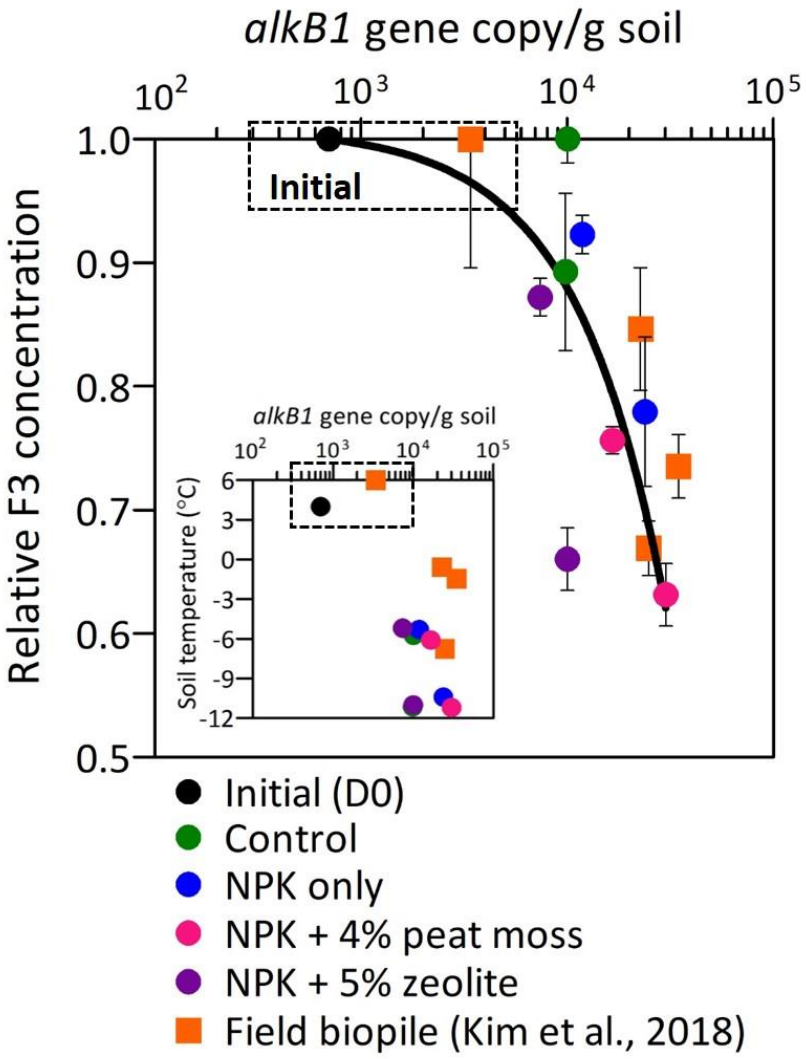

Figure 4.4. Increased *alkB1* gene copy numbers and corresponding relative F3 concentrations in the laboratory and field studies during seasonal freezing. Relative F3 concentration: $[F3]_t/[F3]_o$, t = 0, 42, 142.

4.4.4 Sequential roles of nutrients and unfrozen water in seasonal freezing and frozen soils

Throughout the seasonal freezing of the site soils in this study, the critical roles of nutrients and unfrozen water retention in hydrocarbon biodegradation were sequentially observed, in the freezing phase and in the frozen phase. As shown in Fig. 4.5, the nutrient amendments (NPK and peat moss) depressed the freezing point of the soils in proportion to nutrient concentrations, as demonstrated by the inverse relationship between the electrical conductivity (EC) and freezing-point depression of the treated soils near −1 °C (correlation r = −0.95, R^2 = 0.92; Fig. 4). Near freezing, detailed soil-freezing parameters such as the soil-phase transition rate (STR) and abruptly frozen water near freezing (θ_{FPD}) provide further evidence that the nutrient amendments altered the characteristics of the soils during the early phase of soil freezing (Tables E.6 to E.7; Figs. E.4 to E.6; Supporting Information). The supply of nutrients is positively correlated with F3 hydrocarbon biodegradation between 4 and −5 °C, when the soil-phase change occurred (r = 0.94; R^2 = 0.88; Fig. 4.5). However, in the subsequent deeply frozen phase (−5 to −10 °C), the role of residual unfrozen water became critical for F3 biodegradation (r = 0.67; R^2 = 0.82; Fig. 4.5; Fig. E.7, Supporting Information). The increase in soil surface area due to the zeolite amendment increased the retention residual unfrozen water (Table E.8, Supporting Information).

4.4.5 The α-value as a soil-freezing index for hydrocarbon biodegradation below 0 °C

The range of datasets for zeolite and nutrient addition, residual unfrozen water content, and F3 removal aligned with the α-values (the empirical soil-freezing index of the SFCC equation) (Fig. 4.6). As zeolite content and nutrient concentrations increased, residual unfrozen water content and F3 removal under seasonal freezing conditions also increased, and this correlated with increasing α-values (Fig. 4.6). As the soil surface area and EC increased, the near-freezing soil parameters STR and θ_{FPD} decreased. The removal of branched hydrocarbons, represented by phytane/hopane, increased in freezing and frozen phases, also in correlation with the increasing α-values. A variety of the α-values for soils ranging from clays to sands are available in the literature (Andersland and Ladanyi, 2003), including for biostimulated petroleum-contaminated *sandy* cold-climate soils (NPK) under similar seasonal freezing conditions (-0.1 °C/d) (Chang et al., 2011).

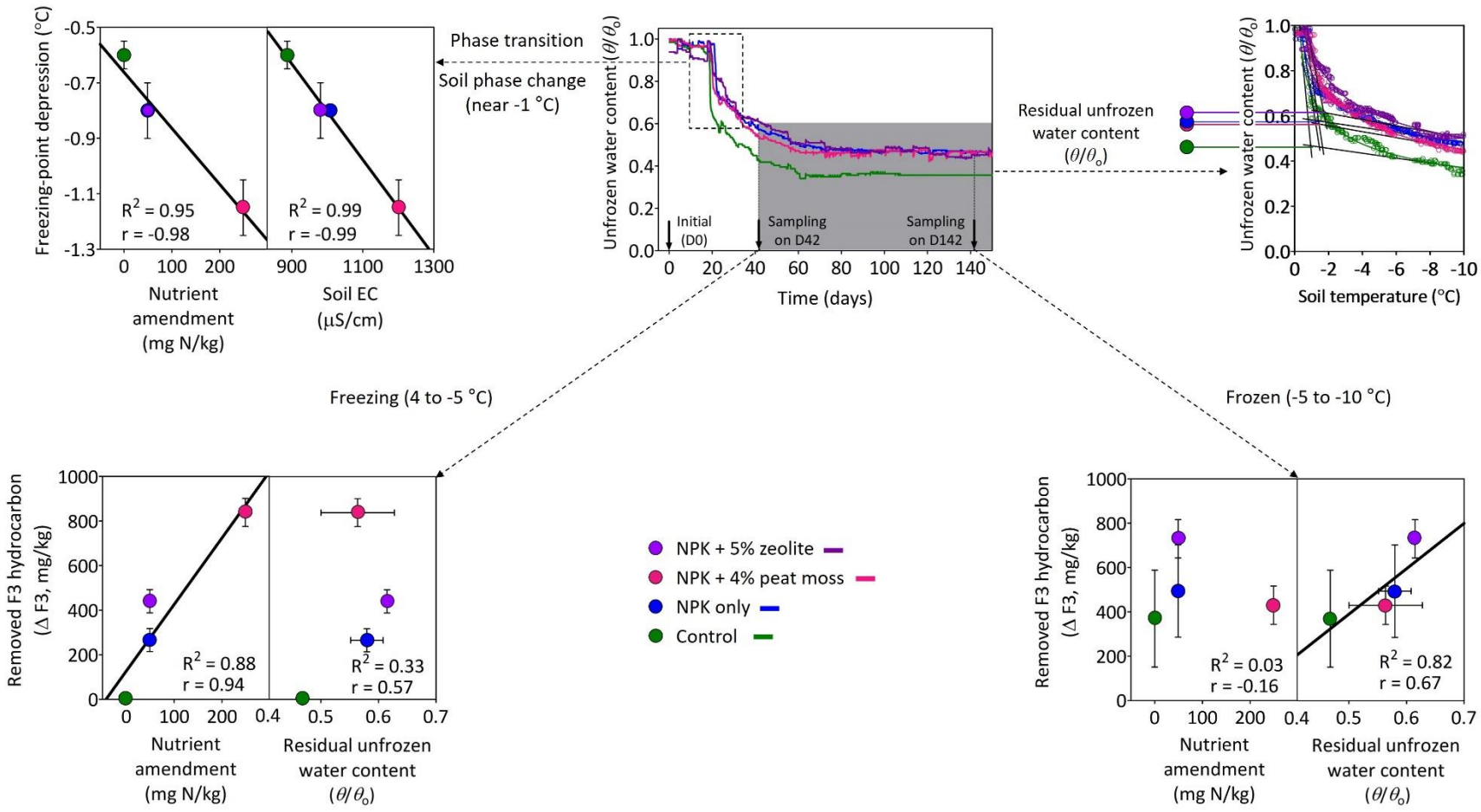

Figure 4.5. The sequential roles of nutrients and retained unfrozen water in enhancing hydrocarbon degradation in the early and deeply frozen phases of seasonal freezing in the site soils.

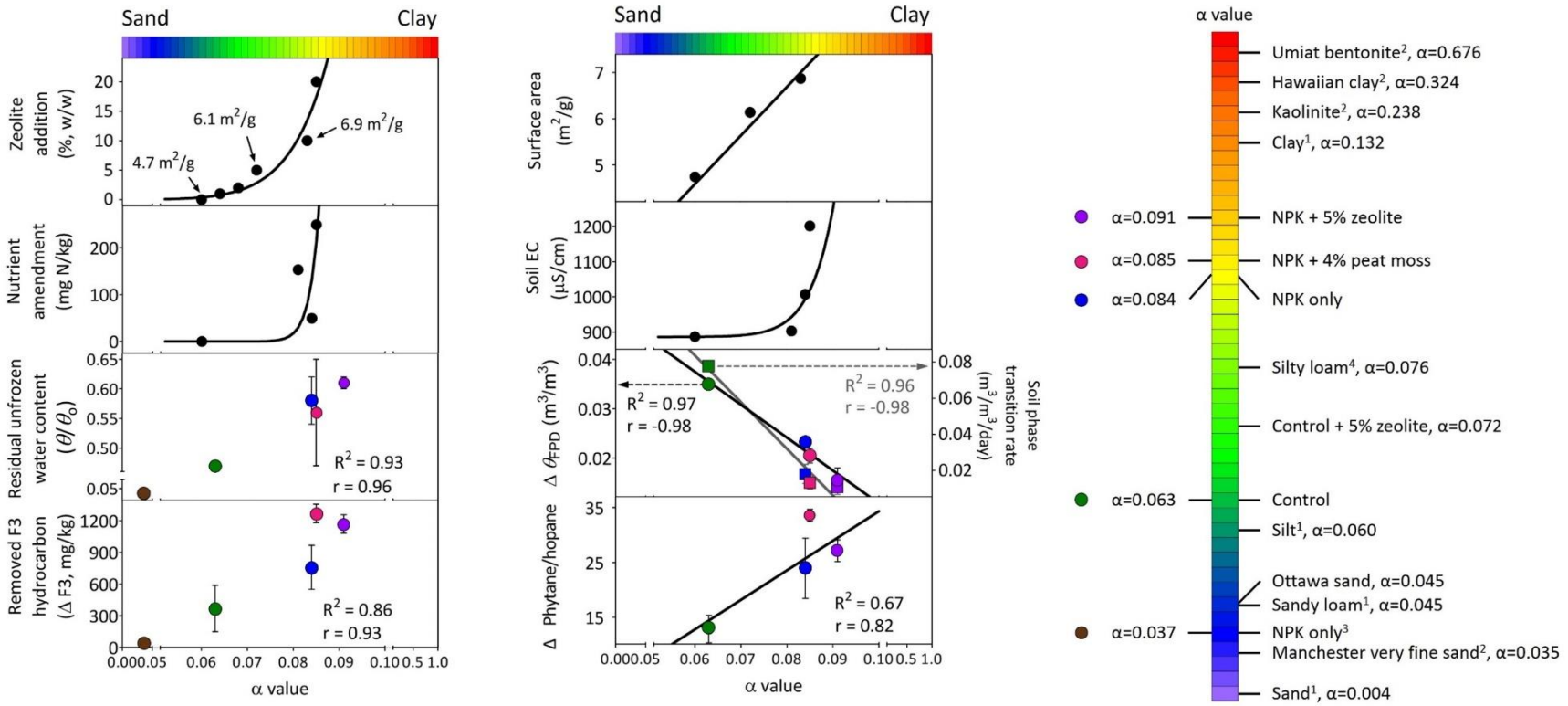

Figure 4.6. The α-values of the treated and untreated soils related to hydrocarbon removal (ΔF3 and Δphytane/hopane) and other soil parameters (added zeolite surface area, nutrients, soil EC, residual unfrozen water content, soil phase transition rate, and abruptly frozen water near freezing, θ_{FPD}).

These latter values were compared to the α-values obtained from this study (Fig. 4.6), where the sandy contaminated soil exhibited a lower α-value of 0.037 that corresponded to much lower hydrocarbon removal than noted for the clayey site soils from this study (Fig. 4.6); this indicates hydrocarbon biodegradation is notably enhanced in soils with α-values approaching 0.1 (Fig. 4.6). In this study, higher α-values in the range of 0.08–0.1 were indicative of greater hydrocarbon biodegradation during seasonal freezing. Clayey soils appear to be favorable over sandy soils for hydrocarbon biodegradation below 0 °C.

4.5 Discussion

This study identified the critical roles of nutrients and residual unfrozen water in hydrocarbon biodegradation in unsaturated, field-aged, petroleum-contaminated, cold-climate soils subjected to site-representative seasonal freezing from 4 to −10 °C. By delineating the *seasonally freezing phase* (4 to −5 °C) and *subsequent frozen (−5 to −10* °C*) phase,* the effects of nutrients and unfrozen water retention were sequentially observed. Nutrients are crucial in the seasonal freezing phase, when the soil-phase change occurred and bulk unfrozen water decreased relatively rapidly. Nutrients induce osmotic pressure that regulates the early stage of seasonal freezing in contaminated soil (e.g., freezing-point depression), while stimulating biodegradation activity. The seasonal freezing phase (*partially frozen soil*), characterized by pockets of nutrient-concentrated residual unfrozen water driving microbial selection and biodegradation enhancement to occur, appears to play a vital role in setting up the extension of hydrocarbon biodegradation to the subsequent frozen phase.

However, unfrozen water retention becomes the most critical factor in extending hydrocarbon biodegradation to the deeply frozen phase, when detectable unfrozen water content stabilizes at a minimum and matric water potential is an internal driver for unfrozen water retention (Drotz et al., 2009). This study suggests soil nutrients and surface area (or osmotic and matric water potential) can be externally controlled via soil treatments designed to extend biodegradation activity in seasonally freezing contaminated soils. Nutrient-induced osmotic pressure has been regarded as a negative factor for oligotrophic microbial activity in cold-climate soils, and the highest nutrient dose used in this study (249 mg N/kg) falls within the upper range of N doses

previously published in the context of hydrocarbon biodegradation in cold-climate soils at low temperatures (Braddock et al., 1997; Chang and Ghoshal, 2014; Walworth et al., 2007). However, the high N concentration in *freezing* contaminated clayey site soils in this study enhanced hydrocarbon biodegradation, reflecting the potential adaptation of indigenous freezing-tolerant hydrocarbon degraders to nutrient-induced osmotic pressure. Cold- and salt-tolerant hydrocarbon-degrading bacteria (5% NaCl , w/v, 10 °C) have been isolated from *non-saline*, petroleum-contaminated, sub-Arctic soils, inferring the potential connection between salt tolerance and microbial survivors in contaminated cold-climate soils (Chang et al., 2018). Yet, it has not been extensively demonstrated that freezing-tolerant hydrocarbon degraders are capable of resisting higher osmotic stress, perhaps in association with concentrated nutrients in residual unfrozen water as a result of solute exclusion from ice during seasonally freezing.

Another deviation from the general principles of bioremediation above 0 °C (including low positive temperatures) observed in subzero biodegradation in this study was the preferential degradation of high-molecular-weight (HMW) hydrocarbons (F3: C16-C34) over low-molecular-weight (LMW) hydrocarbons (F2: C10-C16), which was also observed in the field bioremediation experiment conducted over the winter at a cold site using the same site soil (Saskatchewan, Canada) (Kim et al., 2018). The lighter F2 hydrocarbons were likely associated with frozen water, making them physically inaccessible to hydrocarbon degraders in unfrozen microsites. The heavier F3 hydrocarbons presumably interacted with salt-concentrated unfrozen water films on and within soil aggregates, making them accessible to freezing-tolerant hydrocarbon degraders. The previous field study conducted by the authors of this study indicated that the onset of F2 biodegradation occurred during thawing, suggesting a seasonal variation in the bioaccessibility of different hydrocarbon fractions in contaminated cold-climate soils (Kim et al., 2018).

The abundance of unfrozen microbial niches associated with increased surface area is critical for extending biodegradation activity in seasonally freezing contaminated soil. Above 0 °C, coarse-grained soils are generally favorable for bioremediation compared to fine-grained soils, mainly due to microbial enhancement under the aerobic conditions maintained by the rapid diffusion of oxygen and nutrients. However, bioremediation below 0 °C can be enhanced more readily in finer-grained, nutrient-amended soils with high α-values (0.08–0.091 in this study) than in coarser-grained, nutrient-amended soils with low α-values (0.07–0.037). The α-value approach,

which provides important insight into the unfrozen microsites that support biodegradation in frozen contaminated soil, suggests conventional bioremediation knowledge cannot be directly applied below 0 °C. Creating unfrozen nutrient-rich microbial habitats *before seasonal freezing* is critical for extending hydrocarbon biodegradation activity to the winter in cold climates. Whereas controlling unfrozen water content in contaminated soil remains unformulated for prolonging soil bioremediation during freezing, this study proposes how the α-value can be used as soil-freezing index for optimizing surface area, nutrient concentrations, unfrozen water retention, and soil-freezing characteristics for extended petroleum-hydrocarbon biodegradation in seasonal freezing and frozen contaminated soils.

4.6 Implication and conclusion

Previous studies indicated that the presence of unfrozen water is a key factor for microbial survival in contaminated freezing and frozen soil environments. However, this present study aimed to go beyond observing the natural resilience of microorganisms in frozen soil habitats, and instead posed the following questions:

- *Can we manipulate unfrozen water retention to meaningfully extend petroleum hydrocarbon biodegradation in contaminated soil to seasonally freezing and frozen conditions*?
- *Can we do so by manipulating common soil factors? If so, does unfrozen water retention correlate to hydrocarbon biodegradation below 0 °C?*

These questions have remained unanswered and few studies have focused on them. We do not know how to treat contaminated soils that undergo seasonally freezing to effectively leverage the natural resilience of hydrocarbon-degrading bacteria in freezing and frozen soil environments. These are crucial questions for the advancement of bioremediation concepts and strategies adapted to contaminated sites in vast cold-climate regions, including sub-polar and polar areas in Canada, the U.S., Northern Europe, China, Argentina, New Zealand, and so on. We currently face the challenge of remediating numerous contaminated sites in cold climates where ecosystems are environmentally sensitive, where field-accessibility is limited, and where costs for long-term

remediation are prohibitive. Conventional remediation practices have tended to rely only on short active summer treatment seasons.

This new study extensively shows that *unfrozen water can be manipulated using externally controllable soil factors to extend biodegradation of petroleum hydrocarbons in seasonally freezing and frozen contaminated soil*. Soil nutrients and surface area were adjusted via soil amendments (nutrients and zeolite) to control unfrozen water retention during freezing. We successfully linked enhanced petroleum hydrocarbon biodegradation observed during seasonal phase changes in the biologically treated soils to the soil-freezing characteristics, which were represented by a soil-freezing index (the α-value) applicable to all soils, from clays to sands.

This study specifically suggests that the norms and knowledge of conventional bioremediation, established above 0 °C, cannot be applied to bioremediation below 0 °C. For example, fine-grained soils (clays) are favorable over coarse-grained soils for bioremediation below 0 °C, which is not the case above 0 °C. Creating unfrozen microbial niches in frozen contaminated soils is more important for extending microbial activity to sub-zero conditions. Heavier hydrocarbons (higher molecular weight; F3: C16-C34) biodegraded favorably during the freezing and frozen soil phases, compared to lighter hydrocarbons (F2: C10-C16), due to variations in the bioaccessibility of hydrocarbons below 0 °C.

This study also reveals that the roles of nutrients and unfrozen water retention emerge *sequentially* in response to seasonal freezing. During early seasonal freezing, before the deeply frozen soil phase, unfrozen water and pore ice coexist in soils, which is important for the selection of seasonal microbial compositions and increasing the abundance of freezing-tolerant hydrocarbon-degrading populations. The effects of added nutrients governed the seasonal freezing phase (4 to -5 °C; seasonal transition), whereas the effects of greater unfrozen water retention from added soil surface area were critical in extending biodegradation in the subsequent frozen phase (-5 to -10 °C; deeply frozen).

This study newly suggests that the *α-value* soil-freezing index can be used to inform the biological treatment and management of petroleum hydrocarbon-contaminated soils in cold climates. It suggests that the *α-value* can be used to identify contaminated soils in which it is feasible to extend hydrocarbon biodegradation to seasonally freezing and frozen conditions. We

found that the soil-freezing index is greatly correlated to nutrient concentrations, soil surface area, residual unfrozen water retention, other soil-freezing parameters, and hydrocarbon biodegradation below 0 °C. This study makes the link between the soil-freezing index and bioremediation feasibility below 0 °C, which is an influential finding for researchers and practitioners in the field of bioremediation of petroleum-contaminated soils in cold climates. *In practice, petroleum hydrocarbon-contaminated soils can be treated with nutrients and zeolites (or other additives) to adjust their α-values to within the range of 0.08-0.1 in time for the seasonal transition period, before freezing*. A greater number of unfrozen microbial niches enriched with nutrients are created by the expanded surface area in the treated soils to prepare for the partially frozen and frozen phases. This study contributes to the development of freezing-tolerant bioremediation strategies for cold-climate sites with long winters, and takes a crucial step beyond conventional approaches that focus on short summer seasons.

4.7 Acknowledgement

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4.8 References

Aislabie, J., Saul, D.J., Foght, J.M., 2006. Bioremediation of hydrocarbon-contaminated polar soils. Extremophiles 10, 171-179.

Aislabie, J.M., Balks, M.R., Foght, J.M., Waterhouse, E.J., 2004. Hydrocarbon spills on Antarctic soils: Effects and management. Environ. Sci. Technol. 38, 1265-1274.

Andersland, O.B., Ladanyi, B. 2003. Frozen ground engineering: John Wiley & Sons,

Anderson, D.M., Tice, A.R., McKim, H.L. The unfrozen water and the apparent specific heat capacity of frozen soils. Second International Conference on Permafrost, Yakutsk, USSR. North American contribution, 1973, pp. 289-295.

Børresen, M., Barnes, D., Rike, A., 2007. Repeated freeze–thaw cycles and their effects on mineralization of hexadecane and phenanthrene in cold climate soils. Cold Reg. Sci. Technol. 49, 215-225.

Baker, G., Osterkamp, T., 1989. Salt redistribution during freezing of saline sand columns at constant rates. Water Resources Research 25, 1825-1831.

Bing, H., Ma, W., 2011. Laboratory investigation of the freezing point of saline soil. Cold Reg. Sci. Technol. 67, 79-88.

Braddock, J.F., Ruth, M.L., Catterall, P.H., Walworth, J.L., McCarthy, K.A., 1997. Enhancement and inhibition of microbial activity in hydrocarbon-contaminated arctic soils: implications for nutrient-amended bioremediation. Environ. Sci. Technol. 31, 2078-2084.

Camenzuli, D., Freidman, B.L., 2015. On-site and in situ remediation technologies applicable to petroleum hydrocarbon contaminated sites in the Antarctic and Arctic. Polar Res. 34, 1-19.

CCME. Reference Method for the Canada - Wide Standard for Petroleum Hydrocarbons in Soil – Tier 1 Method, Canadian Council of Ministers of the Environment, Winnipeg, MB, Canada, 2001.

Chang, W., Akbari, A., David, C., Ghoshal, S., 2018. Selective biostimulation of cold- and salt-tolerant hydrocarbon-degrading Dietzia maris in petroleum-contaminated sub-Arctic soils with high salinity. J. Chem. Technol. Biotechnol. 93, 294-304.

Chang, W., Ghoshal, S., 2014. Respiratory quotients as a useful indicator of the enhancement of petroleum hydrocarbon biodegradation in field-aged contaminated soils in cold climates. Cold Reg. Sci. Technol. 106, 110-119.

Chang, W., Klemm, S., Beaulieu, C., Hawari, J., Whyte, L., Ghoshal, S., 2011. Petroleum hydrocarbon biodegradation under seasonal freeze−thaw soil temperature regimes in contaminated soils from a sub-Arctic Site. Environ. Sci. Technol. 45, 1061-1066.

Deming, J.W., 2002. Psychrophiles and polar regions. Curr. Opin. Microbiol. 5, 301-309.

Drotz, S.H., Tilston, E.L., Sparrman, T., Schleucher, J., Nilsson, M., Öquist, M.G., 2009.

Contributions of matric and osmotic potentials to the unfrozen water content of frozen soils. Geoderma 148, 392-398.

Drotz, S.H., Sparrman, T., Nilsson, M., Schleucher, J., Öquist, M.G., 2010. Both catabolic and anabolic heterotrophic microbial activity proceed in frozen soils. PNAS 49, 21046-21051.

Eriksson, M., Ka, J.-O., Mohn, W.W., 2001. Effects of low temperature and freeze-thaw cycles on hydrocarbon biodegradation in Arctic tundra soil. Appl. Environ. Microbiol. 67, 5107-5112.

Farouki, O.T. Thermal properties of soils. U.S. Army Corps of Engineers, Cold Regions Research and Engineering Laboratory, 1981.

Goetz, J.D., Price, J.S., 2015. Role of morphological structure and layering of Sphagnum and Tomenthypnum mosses on moss productivity and evaporation rates. Can. J. Soil Sci. 95, 109-124.

Grechishchev, S., Instanes, A., Sheshin, J., Pavlov, A., Grechishcheva, O., 2001. Laboratory investigation of the freezing point of oil-polluted soils. Cold Reg. Sci. Technol. 32, 183-189.

Han, Y., Wang, Q., Kong, Y., Cheng, S., Wang, J., Zhang, X., Wang, N., 2018. Experiments on the initial freezing point of dispersive saline soil. Catena 171, 681-690.

Harvey, A.N., Snape, I., Siciliano, S.D., 2012. Changes in liquid water alter nutrient bioavailability and gas diffusion in frozen antarctic soils contaminated with petroleum hydrocarbons. Environ. Toxicol. Chem. 31, 395-401.

Henry, H.A., 2007. Soil freeze–thaw cycle experiments: trends, methodological weaknesses and suggested improvements. Soil Biol. Biochem. 39, 977-986.

Jefferies, R.L., Walker, N.A., Edwards, K.A., Dainty, J., 2010. Is the decline of soil microbial biomass in late winter coupled to changes in the physical state of cold soils? Soil Biology and Biochemistry 42, 129-135.

Karppinen, E.M., Mamet, S.D., Stewart, K.J., Siciliano, S.D., 2019. The Charosphere Promotes Mineralization of 13C-Phenanthrene by Psychrotrophic Microorganisms in Greenland Soils. J. Environ. Qual.

Karppinen, E.M., Siciliano, S.D., Stewart, K.J., 2017a. Application method and biochar type affect petroleum hydrocarbon degradation in northern landfarms. J. Environ. Qual. 46, 751-759.

Karppinen, E.M., Stewart, K.J., Farrell, R.E., Siciliano, S.D., 2017b. Petroleum hydrocarbon remediation in frozen soil using a meat and bonemeal biochar plus fertilizer. Chemosphere 173, 330-339.

Kennedy, K., Hall, M.W., Lynch, M.D., Moreno-Hagelsieb, G., Neufeld, J.D., 2014. Evaluating bias of Illumina-based bacterial 16S rRNA gene profiles. Appl. Environ. Microbiol. 80, 5717-5722.

Kim, J., Lee, A.H., Chang, W., 2018. Enhanced bioremediation of nutrient-amended, petroleum

hydrocarbon-contaminated soils over a cold-climate winter: The rate and extent of hydrocarbon biodegradation and microbial response in a pilot-scale biopile subjected to natural seasonal freeze-thaw temperatures. Sci. Total Environ. 612, 903-913.

Konrad, J., McCammon, A., 1990. Solute partitioning in freezing soils. Can. Geotech. J. 27, 726-736.

Lee, S.-J., Park, J.H., Ahn, Y.-T., Chung, J.W., 2015. Comparison of heavy metal adsorption by peat moss and peat moss-derived biochar produced under different carbonization conditions. Water, Air, & Soil Pollution 226, 9.

Liu, P., Zha, T., Jia, X., Wang, B., Guo, X., Zhang, Y., Wu, B., Yang, Q., Peltola, H., 2016. Diurnal Freeze-Thaw Cycles Modify Winter Soil Respiration in a Desert Shrub-Land Ecosystem. Forests 7, 161.

Margesin, R., Feller, G., Gerday, C., Russell, N.J., 2003. Cold-Adapted Microorganisms: Adaptation Strategies and Biotechnological Potential, Encyclopedia of Environmental Microbiology. John Wiley & Sons, Inc. .

Margesin, R., Schinner, F., 1997. Bioremediation of diesel-oil-contaminated alpine soils at low temperatures. Appl. Microbiol. Biotechnol. 47, 462-468.

Marion, G.M. Freeze-Thaw Processes and Soil Chemistry. COLD REGIONS RESEARCH AND ENGINEERING LAB HANOVER NH, 1995.

Mazur, P., 1984. Freezing of living cells: mechanisms and implications. Am. J. Physiol. Cell Physiol. 247, C125-C142.

McCarthy, K., Walker, L., Vigoren, L., Bartel, J., 2004. Remediation of spilled petroleum hydrocarbons by in situ landfarming at an arctic site. Cold Reg. Sci. Technol. 40, 31-39.

McDonald, R., Knox, O.G. Cold region bioremediation of hydrocarbon contaminated soils: do we know enough? ACS Publications, 2014.

McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS one 8, e61217.

Morley, C., Trofymow, J., Coleman, D.C., Cambardella, C., 1983. Effects of freeze-thaw stress on bacterial populations in soil microcosms. Microbial Ecology 9, 329-340.

Mykytczuk, N.C., Foote, S.J., Omelon, C.R., Southam, G., Greer, C.W., Whyte, L.G., 2013. Bacterial growth at− 15 C; molecular insights from the permafrost bacterium Planococcus halocryophilus Or1. The ISME journal 7, 1211.

Mykytczuk, N.C., Wilhelm, R.C., Whyte, L.G., 2012. Planococcus halocryophilus sp. nov., an extreme sub-zero species from high Arctic permafrost. Int. J. Syst. Evol. Microbiol. 62, 1937-1944.

Olsson, P.Q., Sturm, M., Racine, C.H., Romanovsky, V., Liston, G.E., 2003. Five stages of the Alaskan Arctic cold season with ecosystem implications. Arct. Antarct. Alp. Res. 35, 74-

81.

Or, D., Smets, B.F., Wraith, J., Dechesne, A., Friedman, S., 2007. Physical constraints affecting bacterial habitats and activity in unsaturated porous media–a review. Adv. Water Resour. 30, 1505-1527.

Öquist, M.G., Sparrman, T., Klemedtsson, L., Drotz, S.H., Grip, H., Schleucher, J., Nilsson, M., 2009. Water availability controls microbial temperature responses in frozen soil CO_2 production. Global Change Biology 15, 2715-2722.

Panday, S., Corapcioglu, M.Y., 1991. Solute rejection in freezing soils. Adv. Water Resour. 27, 99-108.

Panikov, N., Flanagan, P., Oechel, W., Mastepanov, M., Christensen, T., 2006. Microbial activity in soils frozen to below -39 °C. Soil Biol. Biochem. 38, 785-794.

Paul, B., Dynes, J.J., Chang, W., 2017. Modified zeolite adsorbents for the remediation of potash brine-impacted groundwater: Built-in dual functions for desalination and pH neutralization. Desalination 419, 141-151.

Prince, R.C., Elmendorf, D.L., Lute, J.R., Hsu, C.S., Haith, C.E., Senius, J.D., Dechert, G.J., Douglas, G.S., Butler, E.L., 1994. 17α(H), 21β(H)-hopane as a conserved internal marker for estimating the biodegradation of crude oil. Environ. Sci. Technol. 28, 142-145.

Ren, J., Vanapalli, S.K., Han, Z., 2017. Soil freezing process and different expressions for the soil-freezing characteristic curve. Sci. Cold. Arid. Reg. 9, 221-228.

Rivkina, E.M., Friedmann, E.I., Mckay, C.P., Gilichinsky, D.A., 2000. Metabolic Activity of Permafrost Bacteria below the Freezing Point. Appl. Environ. Microbiol. 66, 3230-3233.

Schimel, J., Balser, T.C., Wallenstein, M., 2007. Microbial stress-response physiology and its implications for ecosystem function. Ecology 88, 1386-1394.

Schimel, J.P., Mikan, C., 2005. Changing microbial substrate use in Arctic tundra soils through a freeze-thaw cycle. Soil Biology and Biochemistry 37, 1411-1418.

Siciliano, S.D., Schafer, A.N., Forgeron, M.A., Snape, I., 2008. Hydrocarbon contamination increases the liquid water content of frozen Antarctic soils. Environ. Sci. Technol. 42, 8324-8329.

Snape, I., Acomb, L., Barnes, D.L., Bainbridge, S., Eno, R., Filler, D.M., Plato, N., Poland, J.S., Raymond, T.C., Rayner, J.L., Riddle, M.J., Rike, A.G., Rutter, A., Schafer, A.N., Siciliano, S.D., Walworth, J.L., 2008. Contamination, regulation, and remediation: an introduction to bioremediation of petroleum hydrocarbons in cold regions. In: Filler, D. M., Snape, I., Barnes, D. L., (Eds), Bioremediation of Petroleum Hydrocarbons in Cold Regions. Cambridge University Press, Cambridge, pp. 1-37.

Team, R.C., 2013. R: A language and environment for statistical computing.

Walker, V.K., Palmer, G.R., Voordouw, G., 2006. Freeze-Thaw Tolerance and Clues to the Winter

Survival of a Soil Community. Applied and Environmental Microbiology 72, 1784-1792.

Walworth, J., Pond, A., Snape, I., Rayner, J., Ferguson, S., Harvey, P., 2007. Nitrogen requirements for maximizing petroleum bioremediation in a sub-Antarctic soil. Cold Regions Science and Technology 48, 84-91.

Wang, Z., Yang, C., Yang, Z., Brown, C.E., Hollebone, B.P., Stout, S.A., 2016. Petroleum biomarker fingerprinting for oil spill characterization and source identification, Standard Handbook Oil Spill Environmental Forensics. Elsevier, pp. 131-254.

Whyte, L., Goalen, B., Hawari, J., Labbé, D., Greer, C., Nahir, M., 2001. Bioremediation treatability assessment of hydrocarbon-contaminated soils from Eureka, Nunavut. Cold Reg. Sci. Technol. 32, 121-132.

Whyte, L., Schultz, A., Van Beilen, J., Luz, A., Pellizari, V., Labbé, D., Greer, C., 2002. Prevalence of alkane monooxygenase genes in Arctic and Antarctic hydrocarbon-contaminated and pristine soils. FEMS Microbiol. Ecol. 41, 141-150.

Wu, M., Tan, X., Huang, J., Wu, J., Jansson, P.-E., 2015. Solute and water effects on soil freezing characteristics based on laboratory experiments. Cold Reg. Sci. Technol. 115, 22-29.

Zhang, L., Ma, W., Yang, C., 2015. Pore water pressure changes of supercooling and ice nucleation stages during freezing point testing. Geotech. Lett. 5, 39-42.

CHAPTER 5

CONCLUSION

5.1 Summary of findings

The overall goal of this thesis was to extensively investigate enhanced petroleum hydrocarbon biodegradation in seasonally freezing and frozen soils in cold climates with laboratory, field, and modelling investigations (Section 1.7 Research objectives and thesis organization). First, enhanced hydrocarbon biodegradation during seasonal freezing and thawing using pilot-scale on-site biopiles was investigated (Obj. 1, Chapter 2: *Field experiment*). Second, soil respiration in petroleum hydrocarbon-contaminated soils below 0 °C was predicted using a new soil respiration model developed based on soil temperature and unfrozen water content and focusing on the soil phase change at near 0 °C (Obj. 2, Chapter 3: *Modelling study*). Last, the manipulation of unfrozen water content by soil treatment and the role of nutrients and unfrozen water content were demonstrated with respect to enhanced hydrocarbon biodegradation in freezing and frozen soils (Obj. 3, Chapter 4: *Laboratory study*).

Chapter 2 (Obj. 1) described a pilot-scale biopile field experiment for hydrocarbon-contaminated fine-grained soils from winter to summer at a cold-climate site. The rate and extent of hydrocarbon biodegradation and microbial community responses corresponding to on-site soil phase changes (from unfrozen to partially frozen, deeply frozen, and thawed) under natural seasonal freeze-thaw conditions were determined. Treated and untreated (control) biopiles were constructed (~3,500 kg each) on an open outdoor surface at a remediation facility in Saskatoon, Canada. The treated biopile received pre-determined N-P-K-based nutrient and humate amendments before seasonal freezing. Real-time field monitoring indicated that the ambient air temperature, soil temperature, and volumetric unfrozen water content reached around −30 °C, −10 °C, and 0.046 ± 0.018 m^3/m^3, respectively, in the treated biopile in mid-January. The soil CO_2 production and O_2 consumption and detectable unfrozen water in the treated biopile were greater than in the untreated biopile. The total removal percentages for F2 (C10–C16), F3 (C16–C34), and TPH in the treated biopile were 58, 57, and 58%, respectively, of which 26, 39, and 33% were removed during seasonal freezing and early thawing from November to early March. F3 degradation occurred during freezing, while F2 hydrocarbons were primarily removed during thawing. Biomarker-based hydrocarbon analyses confirmed enhanced biodegradation in the treated biopile during freezing. The soil treatment increased the first-order rate constants for F2, F3, and TPH degradation by a factor of 2 to 7 compared to the untreated biopile. The seasonality

and soil treatment resulted in bacterial community shifts with *Arthrobacter*, *Rhodococcus*, and *Polaromonas* being relatively more abundant during freezing than in summer. Increased *alkB*1 gene copy numbers in the treated biopile (except during the deeply frozen phase) suggest that enhanced hydrocarbon degraders adapted to freezing soil microenvironments with limited unfrozen water, especially in the partially thawed phase during early thawing.

Chapter 3 (Obj. 2) modified the GRESP model, a function of soil temperature (*T*) and unfrozen water content (*M*), to cover the frozen, partially frozen, and unfrozen phases of successfully bioremediated, hydrocarbon-contaminated, sandy subarctic soils. The Michaelis-Menten equation was modified to express the observable change in unfrozen water content near 0 °C, which is related to soil respiration activity during soil phase changes and at temperatures below the effective endpoint of detectable unfrozen water at −2 °C. The modified Michaelis-Menten equation was further combined with a Q_{10} temperature term and was then incorporated into the GRESP equation to produce a new URESP model for the engineered soil bioremediation system at sub-zero temperatures. The URESP model was applied to published input data measured from the biostimulated site soils of a pilot-scale soil tank experiment conducted between −5 and 15 °C. The model fit well with the experimental data for CO_2 production ($R^2 = 0.96$) and O_2 consumption ($R^2 = 0.92$). A numerical soil thermal model (TEMP/W model) of the thawing biotreated soils in the tank was also used in this study to produce valid alternative (predictive) input *T* and *M* data for the URESP model. The URESP-derived respiration quotients (RQ: 0.695 to 0.698), or the ratios of CO_2 production to O_2 consumption, aligned with the experimental RQ values from the soil tank experiment (0.69) and fell within the theoretical RQ range for aerobic hydrocarbon degradation (0.63–0.80). The URESP model combined with the TEMP/W simulation approximated changes in soil respiration during thawing and characterized the computed soil respiration outputs as related to hydrocarbon utilization based on their RQ values.

Chapter 4 (Obj. 3) assessed how soil amendments (nutrients and zeolite) manipulate unfrozen water retention, which was then linked to hydrocarbon biodegradation and microbial responses at freezing (4 to −5 °C) and frozen (−5 to −10 °C) seasonal soil phases. The SFCC of the treated soils was connected to increased unfrozen water retention and retarded soil freezing at the phase change due to the addition of nutrients, peat moss, and zeolite when compared to the SFCC of untreated site soil (control). The sequential roles of nutrients and unfrozen water content

at seasonal freezing and frozen phases were addressed with enhanced biodegradation. Nutrients had a more significant effect at the freezing phase by decreasing the freezing-point depression, shifting microbial communities, and stimulating biodegradation activity. During soil freezing, biostimulation increased the abundance of *alkB1* gene copy numbers and the relative abundance of hydrocarbon-degrading genera with increasing F3 removal. The unfrozen water content (unfrozen microbial habitats) subsequently became critical in extending hydrocarbon biodegradation at the frozen phases by increasing surface area, which correlated with extended biodegradation. Non-volatile hydrocarbons were significantly removed (F3: > C16, 22–37% removal) during the freezing and frozen phases. The cold-adapted hydrocarbon-degrading bacteria were tolerant to both osmotic stress (up to 250 mg N/kg) and sub-zero temperatures (down to −10 °C), resulting in significant hydrocarbon biodegradation. Hydrocarbon biodegradation at sub-zero temperatures was positively correlated to the α-values of conventional SFCC indexed for various soil types; higher α-values corresponded to greater F3 removal. The fine-grain soils with higher α-values (> 0.06–0.1), due to the soil amendments, favored bioremediation below 0 °C more than the coarser-grain soils with lower α-values (< 0.06).

5.2 Intellectual contributions

Many northern cold sites are located in environmentally sensitive areas that are threatened by climate change and provide socioeconomic resources for First Nations communities. The outcomes of this research provide in-depth knowledge and insight relevant to stress-tolerant bioremediation strategies for extending petroleum hydrocarbon biodegradation to seasonally freezing and frozen contaminated soil phases. The key findings and contributions of this research will be beneficial to policymakers, planners, and engineers responsible for the remediation and management of hydrocarbon-contaminated soils. This research will contribute to meeting Canada's needs today and in the future.

Chapter 2 (Obj. 1). The field study produced new field data for enhanced bioremediation of petroleum hydrocarbon-contaminated soils during the off-season in cold climates (post- and pre-summer). The significant potential for microbial enhancement and hydrocarbon removal in hydrocarbon-contaminated soils under seasonal freeze-thaw conditions during the pre- and post-

summer period in cold climates was obviously demonstrated. By stimulating indigenous cold-adapted hydrocarbon-degrading bacteria before the freezing season, Chapter 2 sheds new light on the feasibility of enhancing petroleum hydrocarbon biodegradation in biopiles during seasonal freeze-thaw periods, including under sub-zero temperature regimes. Conventional remediation practices for cold sites have so far heavily focused on summer operations. Chapter 2 indicates a sequential degradation pattern during the typical winter off-season, from non-volatile F3 hydrocarbons (>C10-C16) during the freezing season to semi-volatile F2 hydrocarbons (>C10-C16) during the thawing season. The late seasonal inactivation and early seasonal activation of hydrocarbon-degrading bacteria were indicated in the treated biopile when the soils were partially frozen at sub-zero temperatures, which coincided with the occurrence of meaningful off-season hydrocarbon degradation. The findings of Chapter 2 suggest a promising new bioremediation strategy for extending the effective duration of bioremediation at petroleum hydrocarbon-contaminated sites subjected to cold seasonal climatic conditions. Chapter 2 directly serves the overall objective, which is to indicate the potential of stress-tolerant bioremediation beyond the conventional summer seasons in the field. To the best of our knowledge, the outcomes of Chapter 2, as well as their important implications for bioremediation practices, have not been previously reported.

Chapter 3 (Obj. 2). Soil respiration data have been frequently used to track soil responses to contamination, monitor bioremediation, and inform the management of site soils. Chapter 3 focuses on developing a soil respiration model as a tool for understanding soil responses in treated petroleum-hydrocarbon contaminated soils during seasonal thermal phase changes. The new soil respiration model, called the URESP model, addresses soil respiration activity extended to the partially frozen and deeply frozen soil phases by coupling the effects of unfrozen water content and soil temperature during phase changes near 0 °C. We assessed other soil respiration models and determined that our coupling approach is useful in addressing extended soil respiration activity in sub-zero temperature regimes of cold climates. The URESP model expands the potential applicability of the GRESP model to sub-zero temperature regimes in simplified soil bioremediation systems and connects it to conventional high-throughput numerical soil thermal analysis. This model and the model development process contribute to the framework for planning, designing, and managing the remediation of hydrocarbon-contaminated soils in cold climates. Combining the developed respiration model and soil thermal models will give clues to estimate

biodegradation activity under various environmental scenarios corresponding to climate change. Chapter 3 investigates how the seasonal activation and inactivation of biodegradation activity are related to soil temperature and water content during seasonal thawing. The findings of Chapter 3 are novel and have the potential to be comprehensively utilized to further extend bioremediation practices during seasonal soil phase changes.

Chapter 4 (Obj 3). Chapter 4 extensively demonstrates that unfrozen water can be manipulated to extend biodegradation of petroleum hydrocarbons in seasonally freezing and frozen contaminated soil, using externally controllable soil factors. Soil nutrients and surface area were adjusted via soil amendments (nutrients and zeolite) to control unfrozen water retention during freezing. The roles of nutrients and unfrozen water retention emerged *sequentially* in response to seasonal freezing. During early seasonal freezing, before the deeply frozen soil phase, unfrozen water and pore ice coexist in soils, which is important for the seasonal determination of microbial composition and increasing freezing-tolerant hydrocarbon-degrading populations. The effects of added nutrients governed the seasonal freezing phase (4 to −5 °C; seasonal transition), whereas the effects of increasing unfrozen water retention by adding soil surface area were critical for extending biodegradation in the subsequent frozen phase (−5 to −10 °C; deeply frozen). Chapter 4 makes the novel suggestion of *using the α-value (soil-freezing index)* for the management and biological treatment of petroleum hydrocarbon-contaminated soils in cold climates. The α-value can be used to identify contaminated soils in which it is feasible to extend hydrocarbon biodegradation to seasonally freezing and frozen conditions. We found the soil-freezing index is strongly correlated to nutrient concentration, soil surface area, residual unfrozen water retention, other soil-freezing parameters, and hydrocarbon biodegradation below 0 °C. The link made in this study between the soil-freezing index and bioremediation below 0 °C is an important finding for researchers and practitioners in the field of bioremediation of petroleum-contaminated soils in cold climates. It contributes to the development of freezing-tolerant bioremediation strategies for cold-climate sites with long winters that go beyond conventional approaches focusing on short summer seasons.

Our new findings specifically suggest the norms and knowledge of conventional bioremediation, established above 0 °C, cannot be applied to bioremediation below 0 °C. How petroleum hydrocarbon biodegradation can be enhanced in freezing and frozen soils is

conceptualized in Fig. 5.1. By providing more surface area and nutrients, unfrozen water content can be retained and cold-adapted bacteria can remain metabolically active in the aqueous phase. This conceptual model will provide hints on how to prepare soil treatments for enhanced hydrocarbon biodegradation in freezing and frozen soils. The findings of controlled petroleum hydrocarbon biodegradation at sub-zero temperatures contribute to the directive strategy of hydrocarbon-contaminated site remediation in cold climates.

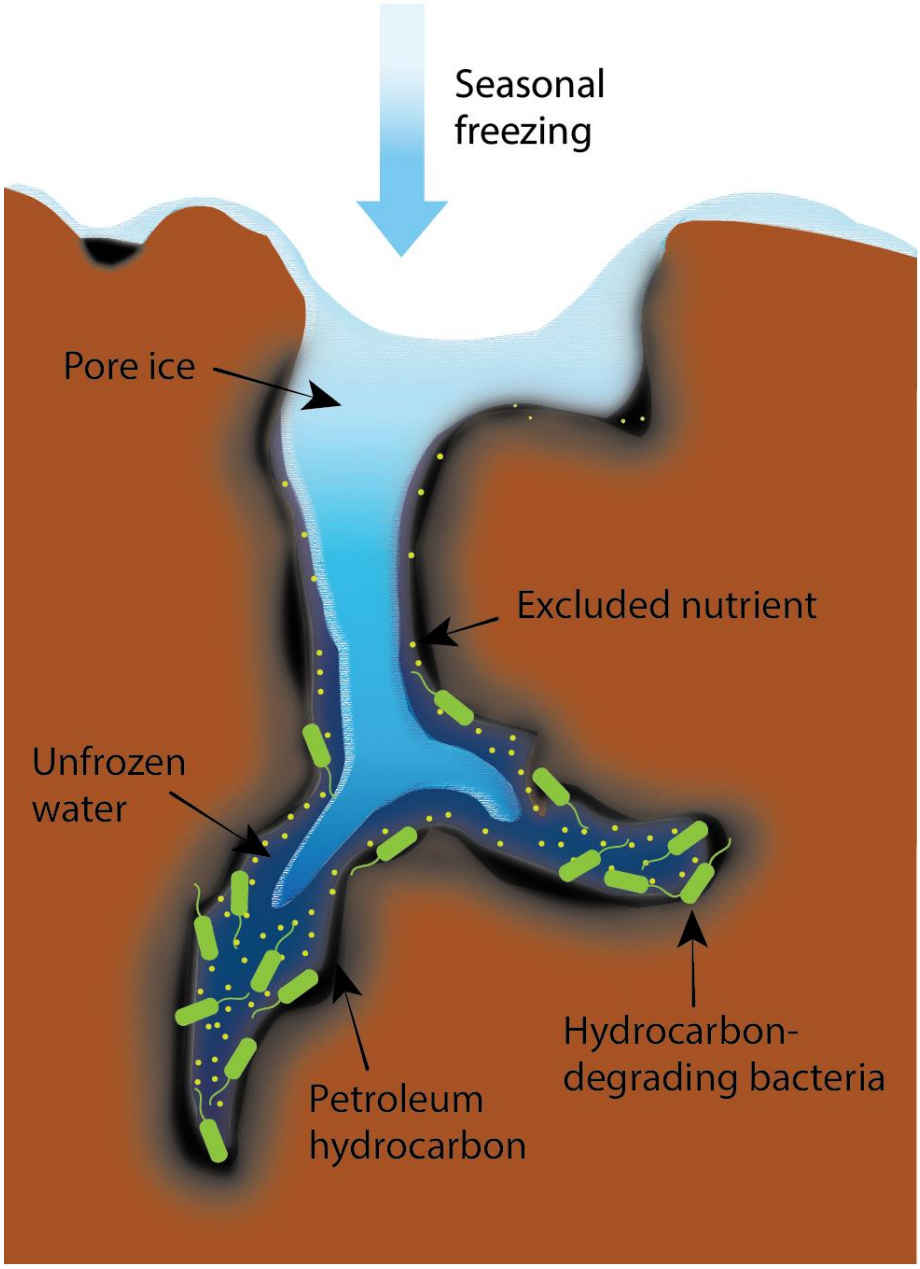

Figure 5.1. A conceptual soil niche and demonstration of how biodegradation can be enhanced in freezing and frozen soils.

5.3 Recommendation and future research

Chapter 4 stated the effects of 4% peat moss and 5% zeolite for enhanced hydrocarbon biodegradation in freezing and frozen soils. This approach may be considered for field applications. However, their dosage was based on a microcosm-scale laboratory study. Considering the massive volume of contaminated soils in the field, this dosage of peat moss and zeolite might not be practical. Before large-scale field applications, a reasonable and practical dosage of peat moss and zeolite should be determined. According to the results of Chapter 2, a humate content of only 2% resulted in significant hydrocarbon removals. Thus, a lesser dosage of peat moss and zeolite than employed in Chapter 4 may be expected in further field trials.

The soil respiration model developed in Chapter 3 requires soil temperature and water content as the key drivers of microbial activity at sub-zero temperatures. However, other key factors such as substrate (contaminant), nutrient, pH, and oxygen remain unexploited for microbial activity in frozen soils. The soil respiration model developed may be considered as a starting point for improved respiration models considering different key drivers at various conditions. The URESP model was developed by modifying the GRESP model, and new soil respiration models are expected to be generated by adding various key factors to the URESP model.

Based on the finding of preferred F3 to F2 removal in freezing and frozen soils, the combination of active bioremediation during summer treatment periods and passive bioremediation during seasonal freezing and thawing is suggested. During summer treatment periods, low molecular weight hydrocarbons (F1 and F2) can easily be volatilized using a simple tilling process. After summer, if soil treatment is performed before soil freezing starts, high molecular weight hydrocarbons (> F3) can be removed during seasonal freezing and thawing. The combination of both active (summer) and passive (winter) bioremediation strategies can be applied in cold climates throughout the year for the removal of low to high molecular weight hydrocarbons in the field. Additionally, an in-depth investigation of the mechanism of preferred hydrocarbon biodegradation is suggested in freezing and frozen soils to inform optimal field applications.

Enhanced biodegradation in freezing and frozen soils was correlated with an increasing soil-freezing index (α-value). The indicated biodegradable α-values range from 0.08 to 0.1, but the lower and upper limits of α values for extended biodegradation were not indicated. Very low α

values might not retain abundant unfrozen water content with discontinuing microbial activity in frozen soils because of the rapid decrease of unfrozen water content in sand and sandy soil at the phase change. Extreme clay minerals can generate much higher α values with small particle sizes and high surface areas ($\alpha > 0.1$), but may be ineffective for enhanced biodegradation because clay aggregates can increase the number of small pores that bacteria cannot access (i.e., decreased bioaccessibility). Thus, more datasets need to be collected in various soil types and treatments in future research on extended biodegradation.

Enhanced hydrocarbon biodegradation during seasonal freezing and thawing in the field was successfully achieved at a landfarm facility near Saskatoon. Future field trials should be performed in northern Canada where environmental conditions are more severe and extreme. Due to climate change, seasonal freezing and thawing may be more dynamic in northern Canada. The directive bioremediation strategy during seasonal freezing and thawing that resulted from this research should be applied to northern Canada so that efficient hydrocarbon-contaminated site remediation and management can be verified, with any unexpected findings providing new research questions and directions.

APPENDIX A

REMEDIATION IN CHALLENGING ENVIRONMENTS: NUMERICAL SIMULATION AND MEASUREMENT OF UNFROZEN WATER CONTENT IN SEASONALLY FREEZING PETROLEUM-CONTAMINATED SOILS IN AN OUTDOOR PILOT-SCALE BIOPILE

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This appendix A was published as Kim, J., Chang, W., 2017, Remediation in Challenging Environments: Numerical Simulation and Measurement of Unfrozen Water Content and its Relationships to Hydrocarbon Biodegradation in Freezing Petroleum-Contaminated Soils, 2017 Canadian Society for Civil Engineering (CSCE) Annual Conference and Annual General Meeting, Vancouver, May 31 – June 3. Dr. Chang supported the funding of this study and assisted the motivation of this study and experimental design. Jihun Kim performed field work such as biopile construction, site monitoring, and sampling. He also conducted laboratory analysis and data interpretation, and prepared the initial manuscript draft, the figures and tables under Dr. Chang's supervision.

A.1 Abstract

Cold-adapted bacteria are able to remain metabolically active in petroleum hydrocarbon-contaminated soils at low temperatures, and thus have been the basis for bioremediation efforts at cold regions sites. However, the ground in cold regions remains partially frozen and frozen for many months of the year, and the seasonal temperature cycles heavily affect monitoring, management and remediation practices at those sites. Recent studies have consistently reported significant microbial activity measured in partially frozen and frozen habitats, including in soils, sea ice cores and even at the interface between ice and water. Unfrozen liquid water in freezing and frozen soils is likely prerequisite for extended microbial activity. Predicting the presence and retention of unfrozen liquid water in cold site soils can be useful for planning, managing and implementing bioremediation for petroleum-contaminated soils at remote northern sites. However, available numerical simulation tools for predicting soil water content have not been extensively considered in bioremediation research for cold sites. We both simulated and measured unfrozen water content in field-aged, petroleum-contaminated, clayey soils from outdoor pilot-scale biopiles exposed to winter temperatures of Saskatoon, Saskatchewan. The simulation of unfrozen water content in the biopiles subjected to representative winter temperatures was conducted using TEMP/W. The model predicted that significant quantities of unfrozen water remain available in the biopile during the seasonal transitional and winter periods. By comparing the simulated and measured unfrozen water data for the site soils, this preliminary study conservatively predicted an abundance of unfrozen water in the site soils during the winter season.

A.2 Introduction

Petroleum hydrocarbons are one of the most frequently identified contaminants in Canada, including at northern sites, mainly due to intensified anthropogenic activities and associated impacts. Bioremediation is often considered a cost-effective and less-destructive remediation technology for petroleum-contaminated soils at cold sites (Snape et al. 2008). The potential to enhance hydrocarbon biodegradation in cold site soils by stimulating cold-adapted hydrocarbon-degrading bacteria has been frequently reported over the last ten years (Whyte et al. 2001). More recently, microbial activity extended to sub-temperatures in freezing and frozen soils has been

reported, implying that unfrozen liquid water content plays a critical role for maintaining metabolic activity of microbial survivors when temperatures are near or below the freezing-depression point of freezing and frozen soils (Panikov et al. 2006). Temperatures alone cannot explain the unique adaptations of microorganisms in freezing soils, where microbial habitats are subjected to multiple stresses. For example, Deming (2002) proposed an ecophysiological group, *eutectophiles*, to designate unique microorganisms that take advantage of soil phase changes at the water-ice interface. The microbial habitat of eutectophiles at the water-ice interface is influenced by a combination of factors including temperature, solute concentrations, and the physical state of the water.

It has been frequently observed that changes in unfrozen water availability is significantly correlated with the seasonal onset and inactivation of microbial activity in freezing and frozen soils (Chang et al. 2011). Rivkina et al. (2000) reported that microbial activity is correlated with the thickness of unfrozen water films in freezing soils. Similarly, Panikov et al. (2006) indicated that CO_2 evolution in freezing soils is related to changes in unfrozen water content. However, these prior studies are small lab-scale experiments using uncontaminated soils.

Some low-temperature studies have considered field-aged, petroleum hydrocarbon-contaminated sub-Arctic soils. Significant in-situ soil gas concentrations (CO_2 and O_2) were measured at a sub-Arctic site in Norway to estimate microbial respiration in the active and permafrost layers of oil-contaminated soils during a seasonal transition period and winter (Rike et al. 2003; 2005). Chang et al. (2011) observed a significant correlation between temporal changes in unfrozen water content and apparent microbial respiration activity (CO_2 and O_2) in soils subjected to varying site-representative freeze/thaw temperatures in an indoor, pilot-scale biodegradation experiment. However, that study was conducted in a laboratory by mimicking site temperatures using a temperature-programmable cold room.

On the other hand, the relationship between unfrozen water content and physical soil properties has been studied (Andersland and Ladanyi 2004). Empirical equations and predictive numerical soil thermal models are currently available to estimate unfrozen water content in various types of unsaturated soils (e.g., TEMP/W). Yet, a comparison of predicted unfrozen water content over time and measured unfrozen water data obtained for an outdoor biopile of field-aged petroleum-contaminated soils has rarely been reported.

The objective of this study is mainly to compare simulated unfrozen water content with measured unfrozen water content in field-aged petroleum hydrocarbon-contaminated soils during seasonal freezing in a pilot-scale outdoor biopile installed in Saskatoon, Saskatchewan (SK), Canada. This preliminary study calibrated the numerical simulation tools and associated input parameters specifc to the site soils. This study is particularly important for planinng and managing practices for contaminated site remediation at remote cold sites, where only short summers are allowed for active site monitoring and soil treatment.

A.3 Materials and methods

A.3.1 Site soil

The site soils are field-aged, petroleum hydrocarbon-contaminated clayey soils. TPH concentrations were determined using the Canada-Wide Standard for Petroleum Hydrocarbons (CWS PHC) in Soil - Tier 1 Method. Briefly, wet soil samples (10 g) were placed in a cellulose extraction thimble (Gerhardt GmbH & Co. KG, Germany) with a surrogate standard (o-terphenyl; Sigma Aldrich Canada) for estimating hydrocarbon extraction efficiencies. For hydrocarbon analyses, approximately 150 mL of a 50:50 hexane/acetone (v/v) solvent was used for hydrocarbon extraction from the soil samples by automatic Soxhlet extraction (Gerhardt Soxtherm, Germany). Extract solutions were filtered through a column containing silica gel and sodium sulfate to remove polar compounds before 20 mL of a 50:50 hexane/ dichloromethane (DCM) solvent mixture was passed through the same column. The extracts obtained after the silica gel cleanup were concentrated by a nitrogen gas blow-down concentrator. The final volumes of concentrated extracts were measured. Hydrocarbons in the concentrated extract solutions were analyzed using gas chromatography (7890A, Agilent with J&W DB-1HT capillary column) with a flame ionization detector (FID).

A.3.2 Viable hydrocarbon degraders

For microbial enumeration, 10 g of site soils were added to 95 mL of phosphate buffer solution (PBS). Serial soil dilutions (10^{-1} to 10^{-5}) were prepared after a 30-min homogenization of the soils in the PBS solution. 0.1-mL aliquots of the soil dilutions were spread onto Bushnell Haas

(BH) agar plates (0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1 g KH_2PO_4, 1 g K_2HPO_4, 1 g NH_4NO_3, 0.05 g $FeCl_3$ and 20 g agar in 1 L distilled water; Sigma Aldrich Canada). 0.1 mL of filter-sterilized diesel was added onto the plates as the sole source of carbon. Plates were incubated at 17 °C for 9 days. Colony forming units (CFU) were counted after the incubation.

A.3.3 Outdoor biopile construction

Prior to winter, a set of pilot-scale biopiles was constructed in September and October, 2015, at an outdoor soil remediation facility (PINTER & Associates) in Saskatoon. The size of the biopiles is approximately 2 m long, by 2 m wide and 1 m high. Approximately 3.2 tons of contaminated soils were used for each biopile. A drainage layer (gravel and collection pipes) was installed at the bottom of each biopile. Soil gas collection tubes were embedded in the biopile. Soil gases (CO_2 and O_2) were monitored using MX6i multi gas detectors (Industrial scientific, USA). Two sets of real-time soil temperature and water content sensors (5TM, Decagon devices, USA), along with a data logger (EM50, Decagon devices, USA), were installed in the top (at 90 cm) and bottom (at 10 cm) layers of the outdoor biopile. Soil temperature and water content in the biopile were monitored every six hours.

A.3.4 Simulation of soil temperatures and unfrozen water content

Soil temperature and unfrozen water content were simulated using TEMP/W (Geo-slope International Ltd.). An air temperature profile was constructed based on 10 years of historical air temperature data for Saskatoon (Environment Canada). The outside air temperature profile was then imported into TEMP/W to predict temperatures and water content in the soils subjected to seasonal freezing conditions. The simulated data were compared to the measured data.

A.4 Results and discussion

A.4.1 Site soil characteristics

Based on the CWS PHC Tier 1 method, the representative TPH concentration of the site soil was determined to be 5200 mg/kg. The typical hydrocarbon fractions of diesel, according to

the CCME, are F2 (semi-volatile) and F3 (non-volatile) hydrocarbons. Those fractions represent the majority of hydrocarbons detected in the site soils. Background phosphorus in the site soils was abundant, and therefore a C:N ratio for the nutrient amendment was considered, instead of a C:N:P ratio. Microcosm experiments were conducted with nutrient-amended and unamended soils, and viable hydrocarbon degraders were enumerated and compared. A C:N ratio of 100:1 was used for the site soil microcosms and the enumeration of viable hydrocarbon-degrading bacteria. As shown in Fig. A.1, significant biostimulation potential was indicated by the high population sizes of viable diesel-degrading bacteria in the nutrient-amended site soils (2.2×10^4 CFU/ g), compared to in the unamended soils (4.5×10^3 CFU/ g). The difference was statistically significant with $p < 0.05$ (t-test, GraphPad).

A.4.2 Biostimulation potential

For further adjustment of the nutrient dosage, a range of C:N ratios from 100:0.5 to 100:2 were tested. These nutrients were applied in soil microcosms with 100 g soils, while soil respiration activity (CO_2 and O_2) was monitored. As shown in Fig. A.2, the site soil readily responded to the different nutrient amendments. CO_2 production and O_2 consumption were significant greater in the nutrient-amended site soils compared to the unamended soils. Of the nutrient amendments tested, a C:N ratio of 100:0.8 exhibited the highest CO_2 production and O_2 consumption, based on the early response of the microcosm to the nutrient-amendment within 48 hours. The C:N ratio of 100:0.8 was used during the biopile setup for biostimulation.

It is important to indicate the presence of viable indigenous bacteria capable of degrading target hydrocarbon fractions (diesel fractions in this study), as well as determine favorable nutrient amendments, prior to installation of large-scale biopiles, in order to achieve successful bioremediation in practice. In particular, cold region sites have a short seasonal window for active soil treatment and field logistics are a concern. Characterizing the favourable soil conditions for stimulating indigenous hydrocarbon degraders is prerequisite. Changes in soil gas are a useful indicator, and soil gas monitoring (CO_2 production and O_2 consumption) has been widely employed to estimate microbial enhancement during bioremediation (Chang and Ghoshal 2014). Soil gases are monitored to demonstrate microbial response during biodegradation (Walworth et al. 2013; Akbari and Ghoshal 2015).

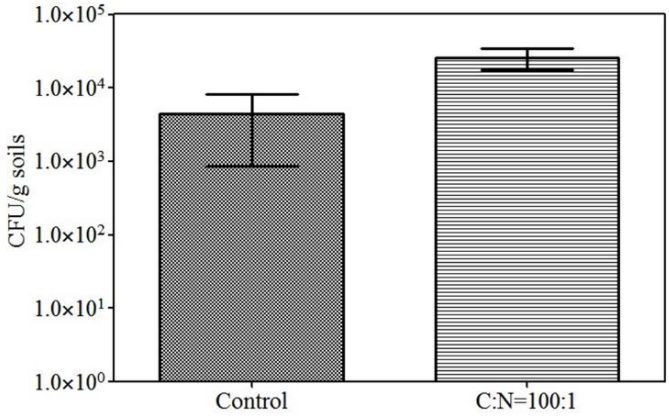

Figure A.1. The microbial enumeration of hydrocarbon-degrading bacteria from the nutrient-amended soils with a C:N ratio of 100:1 and from unamended site soils. Enumeration was performed using diesel-spiked Bushnell Hass plates at 25 °C (Error bar: standard error).

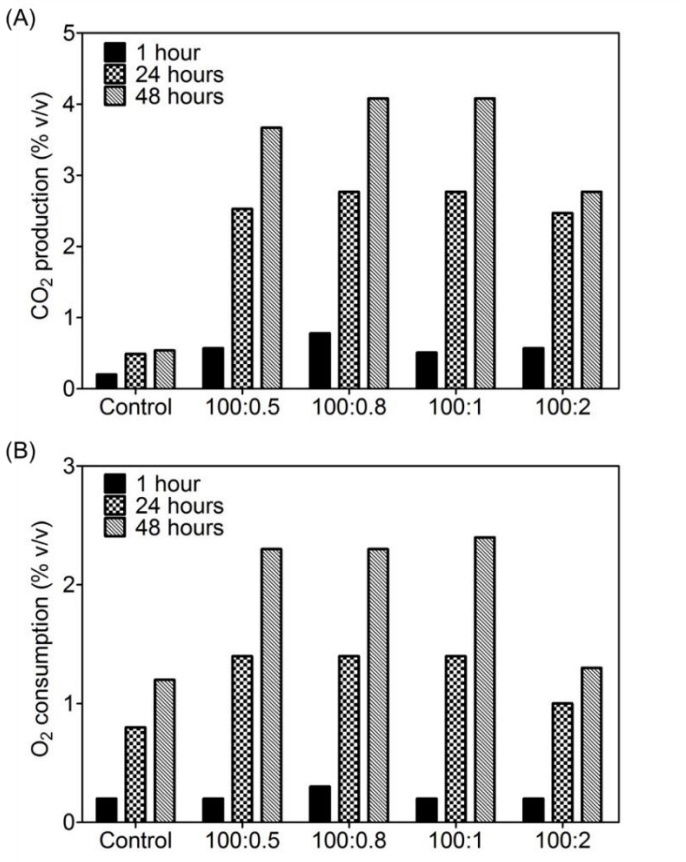

Figure A.2. Soil CO_2 production (A) and O_2 consumption (B) in the site soils in response to different C:N ratios in the nutrient amendment.

A.4.3 Simulated and measured soil temperatures and unfrozen water content

Prior to the field biopile experiment, temporal changes in the content and distribution of unfrozen water in the biopile were predicted using the historical temperatures (10-year data) and the TEMP/W model. The field biopile was directly exposed to ambient temperature conditions and snow cover, as shown in Fig. A.3. However, the simulation did not consider the effect of snow cover and wind. The highest, average and the lowest ambient temperature profiles constructed from historical data were used for the simulations, producing a range of unfrozen water content profiles as a result. In the present study, the unamended biopile was considered first for as a baseline study.

As shown in Fig. A.4, the distributions of simulated unfrozen water content in the biopile during seasonal freezing is illustrated. Significant quantities of unfrozen water could be available in the bottom layers of the biopiles until early November, implying that soil conditions could accommodate microbial populations as long as other soil parameters, such as oxygen and nutrient diffusion, do not limit microbial activity. Although free unfrozen water is limited beginning in the middle of November, small amounts of unfrozen water were still measureable in microsites in the middle of November.

Figure A.3. The outdoor pilot-scale 3.5-ton biopile of petroleum hydrocarbon-contaminated soil. Soil temperature and water content sensors were installed in the top and bottom layers of the biopile.

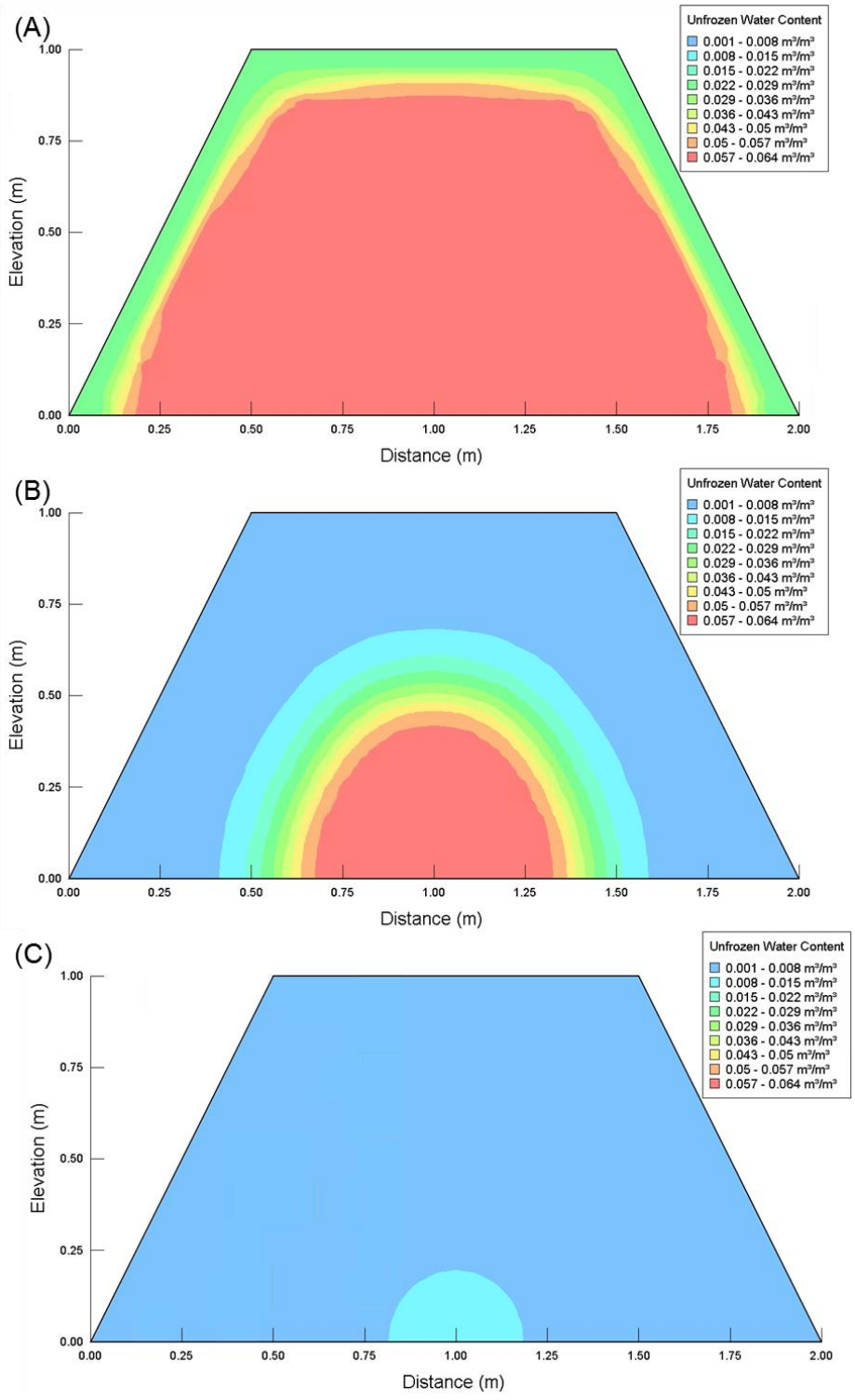

Figure A.4. Contour maps of simulated unfrozen water content distributions at the beginning of November (A), in early November (B) and in the middle of November (C).

The predicted soil temperatures, based on the highest, average and lowest ambient temperature profiles constructed using historical climate data, were generated and compared with the measured soil temperatures from the biopiles in the field. As shown in Fig. A.5, the measured soil temperatures are closer to the soil temperatures corresponding to the 10-year average soil temperature scenario. However, the measured soil temperatures were slightly higher than soil temperatures simulated using the 10-year average ambient temperatures of the site. On-site soil temperatures in the biopile (unamended) remained near 0 °C and fluctuated between 0 and -5 °C during winter. The soils were not completely frozen until January of the following year (2016). In reality, unfrozen liquid water content did not decrease during the period in which the biopile soil temperature was below 0 °C, but not lower than the typical freezing-depression point of clayey soils (around -5 °C). This result implies that the biopile soils may exhibit the metabolic activity of cold-adapted microorganisms, even though the kinetics of microbial activity are slowed due to the low temperature. In the middle of November, the simulation using 10-year average temperatures indicated the depletion of unfrozen water content. However, measured unfrozen water content from the biopile is closer to the simulated unfrozen water content that used the highest soil temperature scenario.

The on-site climate conditions (e.g., snow cover) and soil properties (e.g., clay content) seem to influence the retention of unfrozen water. The simulation of soil temperatures and unfrozen water content prior to the field biopile implementation provided indications of temporal changes in unfrozen water availability over time during a freezing period. Currently, more sensitive analyses of the effects of a variety of parameters are being conducted to produce better results. In addition, the on-site data for microbial respiration activity and hydrocarbon biodegradation in the biopiles are being obtained, which will further highlight the implications of this study's findings for planning and management of bioremediation for petroleum-contaminated soils in cold climates. The present study provides a framework for investigating the feasibility extended bioremediation during seasonal freezing through the prediction of soil temperatures and unfrozen water content in field-aged petroleum hydrocarbon-contaminated soils.

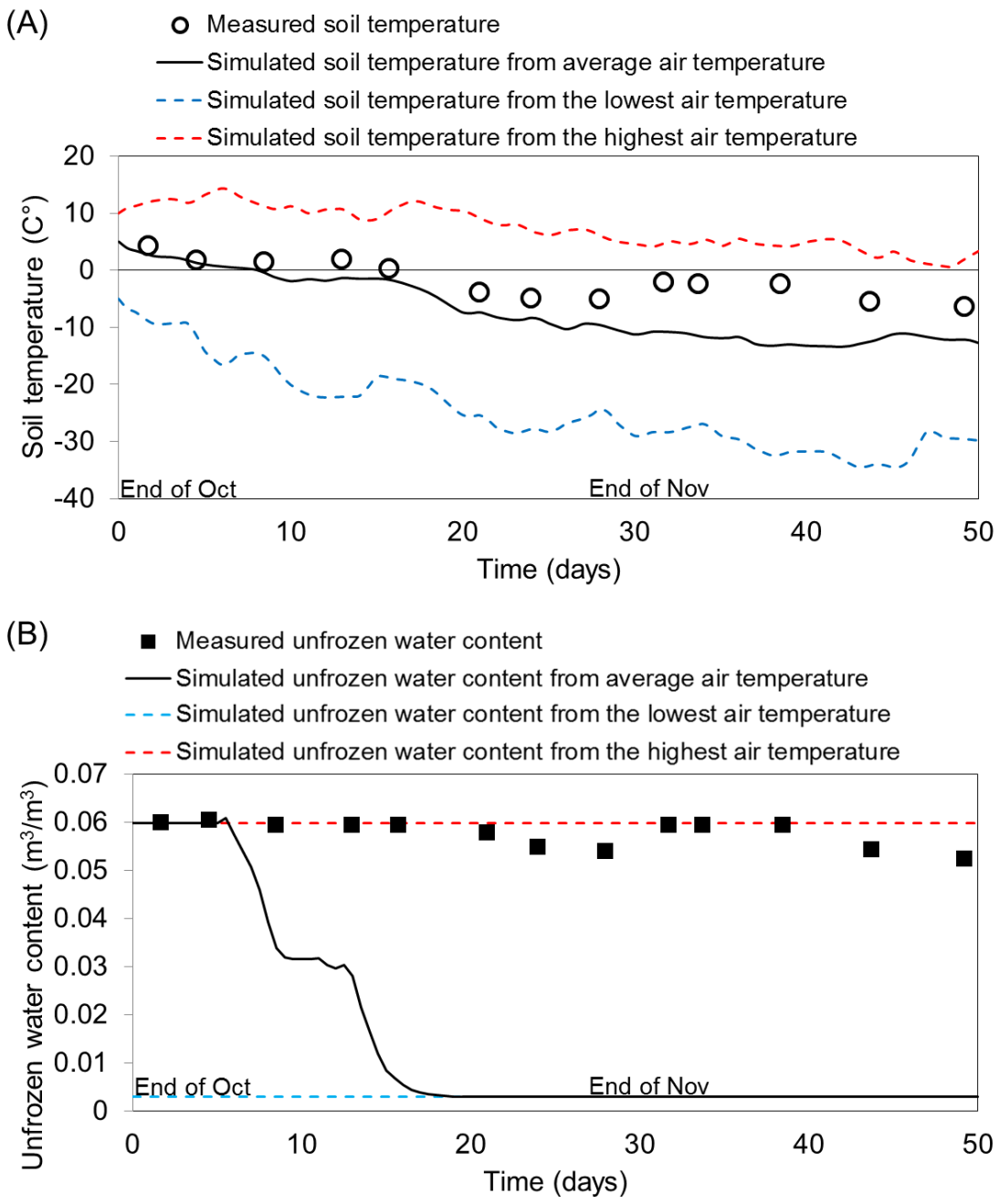

Figure A.5. The measured and simulated soil temperatures (A) and unfrozen water contents (B) at maximum, minimum and average temperatures using 10 years of historical weather data.

A.5 Conclusion and ongoing studies

This study indicated sufficient unfrozen water availability during freezing in both simulated and measured soil data. In general, existing numerical tools for predicting temporal changes in the distributions of soil temperatures and unfrozen water contents are available and applicable to contaminated soils in pilot-scale biopiles. The significant retention of unfrozen water, which is likely required for maintaining microbial activity in partially frozen and frozen soils, was observed until January. The field study has been continued to confirm the extension of on-site biodegradation activity and associated microbial response during the off-season. With the updated data for microbial activity, soil properties and on-site climate conditions, the simulation study may produce more accurate results. The current study implies the feasibility of predicting soil temperatures and unfrozen water availability using available numerical tools, which has particularly important implications for the planning and management of contaminated site remediation in remote and cold regions.

A.6 Acknowledgements

We thank Ryan Riess, Dustin Hicke and Lawrence Pinter from PINTER & Associates Ltd. and Prairie Waste Management Ltd. in Saskatoon, as well as the Natural Sciences and Engineering Research Council of Canada (NSERC; NSERC EG 488995).

A. References

Akbari, A. and Ghoshal, S. 2015. Effects of diurnal temperature variation on microbial community and petroleum hydrocarbon biodegradation in contaminated soils from a sub-Arctic site. Environmental Microbiology, 17, 4916–4928.

Andersland, O.B. and Ladanyi, B. 2004. Frozen ground engineering, 2nd ed., Wiley, Hoboken, NJ, USA.

Chang, W. and Ghoshal, S. 2014. Respiratory quotients as a useful indicator of the enhancement of petroleum hydrocarbon biodegradation in fi eld-aged contaminated soils in cold climates. Cold Regions Science and Technology, 106–107, 110–119.

Chang, W. Klemm, S. Beaulieu, C. Hawari, J. Whyte, L. and Ghoshal, S. 2011. Petroleum hydrocarbon biodegradation under seasonal freeze-thaw soil temperature regimes in contaminated soils from a sub-Arctic site. Environmental Science & Technology, 45, 1061–6.

Deming, J.W. 2002. Psychrophiles and polar regions. Current Opinion in Microbiology, 5, 301–9.

Panikov, N.S. Flanagan, P.W. Oechel, W.C. Mastepanov, M. a. and Christensen, T.R. 2006. Microbial activity in soils frozen to below −39°C. Soil Biology and Biochemistry, 38, 785–794.

Rike, A.G. Haugen, K.B. Børresen, M. Engene, B. and Kolstad, P. 2003. In situ biodegradation of petroleum hydrocarbons in frozen arctic soils. Cold Regions Science and Technology, 37, 97–120.

Rike, A.G. Haugen, K.B. and Engene, B. 2005. In situ biodegradation of hydrocarbons in arctic soil at sub-zero temperatures—field monitoring and theoretical simulation of the microbial activation temperature at a Spitsbergen contaminated site. Cold Regions Science and Technology, 41, 189–209.

Rivkina, E.M. Friedmann, E.I. and Mckay, C.P. 2000. Metabolic Activity of Permafrost Bacteria below the Freezing Point. Applied and Environmental Microbiology, 66, 3230–3233.

Snape, I. Acomb, L. Barnes, D.L. Bainbridge, S. Eno, R. Filler, D.M. Plato, N. Poland, J.S. Raymond, T.C. Rayner, J.L. Riddle, M.J. Rike, A.G. Rutter, A. Schafer, A.N. Siciliano, S.D. and Walworth, J.L. 2008. Contamination, regulation, and remediation: an introduction to bioremediation of petroleum hydrocarbons in cold regions, in: Filler, D.M., Snape, I., Barnes, D.L. (Eds.), Bioremediation of Petroleum Hydrocarbons in Cold Regions, Cambridge University Press, Cambridge, Cambridgeshire, England, pp. 1–37.

Walworth, J. Harvey, P. and Snape, I. 2013. Low temperature soil petroleum hydrocarbon degradation at various oxygen levels. Cold Regions Science and Technology, 96, 117–121.

Whyte, L.G. Goalen, B. Hawari, J. Labbé, D. Greer, C.W. and Nahir, M. 2001. Bioremediation treatability assessment of hydrocarbon-contaminated soils from Eureka, Nunavut. Cold Regions Science and Technology, 32, 121–132.

APPENDIX B

Peer-reviewed journal

Published

Kim, J., Lee, A.H. and Chang, W., 2018. Enhanced bioremediation of nutrient-amended, petroleum hydrocarbon-contaminated soils over a cold-climate winter: The rate and extent of hydrocarbon biodegradation and microbial response in a pilot-scale biopile subjected to natural seasonal freeze-thaw temperatures. Science of the Total Environment, 612: 903-913. (Chapter 2)

Kim, J., Chang, W., 2019. Modified soil respiration model (URESP) extended to sub-zero temperatures for biostimulated petroleum hydrocarbon-contaminated sub-Arctic soils. Science of the Total Environment, 667: 400-411. (Chapter 3)

In Preparation

Kim, J., Chang, W., Lee, A.H., 2020. Manipulation of unfrozen water retention for enhancing petroleum hydrocarbon biodegradation in seasonally freezing and frozen soil (submitted to Environmental Science & Technology, Chapter 4).

Published conference paper

Kim, J., Chang, W., 2017, Remediation in Challenging Environments: Numerical Simulation and Measurement of Unfrozen Water Content and its Relationships to Hydrocarbon Biodegradation in Freezing Petroleum-Contaminated Soils, 2017 Canadian Society for Civil Engineering (CSCE) Annual Conference and Annual General Meeting, Vancouver, May 31–June 3.

Kim, J., Chang, W., 2015, Evaluation of Generalized Microbial Respiration Equations for Petroleum Hydrocarbon-contaminated, Cold-climate Soils during Bioremediation, 38th Arctic and Marine Oilspill Program (AMOP) Technical Seminar on Environmental Contamination and Response, Vancouver, June 2–4.

Kim, J., Chang, W., 2014, Site remediation in northern climates: microbial respiration model during bioremediation in petroleum hydrocarbon-contaminated soils at low temperatures, 67th Canadian Geotechnical Conference proceedings, GeoRegina, Regina, September 28–October 1.

Oral presentation

Kim, J., Chang, W., Riess, R., 2017, Bioremediation of Petroleum Hydrocarbon-Contaminated Soils in Cold Climates: a Scaled-Up Field Experiment for the Feasibility of Extending Bioremediation beyond the Conventional Summer Treatment Season, The Fourth International Symposium on Bioremediation and Sustainable Environmental Technologies, Miami, May 22–25.

Kim, J., Chang, W., 2017, An Integrated Soil Respiration Model for Assessing Hydrocarbon Biodegradation Activity in Cold-Region Site Soils, The Fourth International Symposium on Bioremediation and Sustainable Environmental Technologies, Miami, May 22–25.

Poster presentation

Kim, J., Chang, W., Zhu, N., McBeth, J., 2015, Visualizing and Quantifying Bioaccessible Pores in Field-Aged Petroleum Hydrocarbon-Contaminated Clay Soils Using Synchrotron-based X-ray Computed Tomography, AGU's Fall Meeting, San Francisco, December 14–18.

Kim, J., Chang, W., 2015, Physical, Chemical and Microbial Characterization for Bioremediation Feasibility Assessment of Field-ages Petroleum Hydrocarbon-contaminated Soils Shipped from a Large-scale Landfarm Facility, 38th Arctic and Marine Oilspill Program (AMOP) Technical Seminar on Environmental Contamination and Response, Vancouver, June 2–4.

Kim, J., Chang, W., 2014, Cold region bioremediation: Part 2 – The substrate, temperature and unfrozen water dependency of microbial respiration during bioremediation in freezing contaminated soils, 37th Arctic and Marine Oilspill Program (AMOP) Technical Seminar on Environmental Contamination and Response, June 3–5.

APPENDIX C. SUPPLEMENTARY DATA (CHAPTER 2)

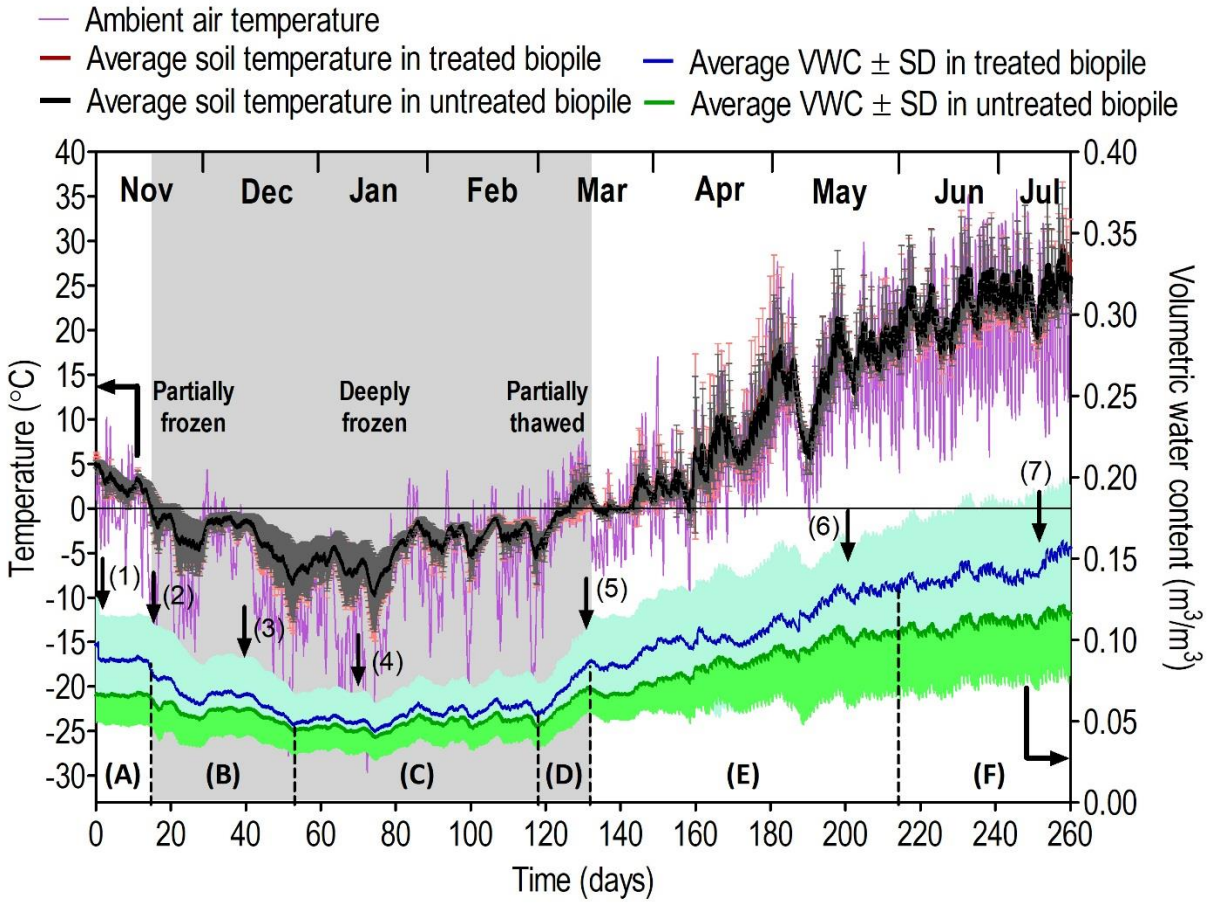

Figure C.1. The monitored ambient air temperature surrounding the biopiles is presented (purple line). The soil temperature and volumetric water content (VWC) averaged for the surface, middle, and bottom layers of the treated and untreated biopiles. The average soil temperatures of the treated and untreated biopiles were nearly identical (soil temperatures for the treated biopile are not visible in Fig. C.1; therefore, they are presented in Fig. 2.3). The portions highlighted in gray and blue or green show the standard deviations (SD) of the mean soil temperature and volumetric unfrozen water content, respectively. The arrows numbered (1) to (7) refer to the soil sampling points related to seasonal soil thermal phase changes. Corresponding time periods for each soil thermal phase are indicated: (A) unfrozen phase, (B) partially frozen, (C) deeply frozen, (D) partially thawed, (E) thawed, and (F) summer.

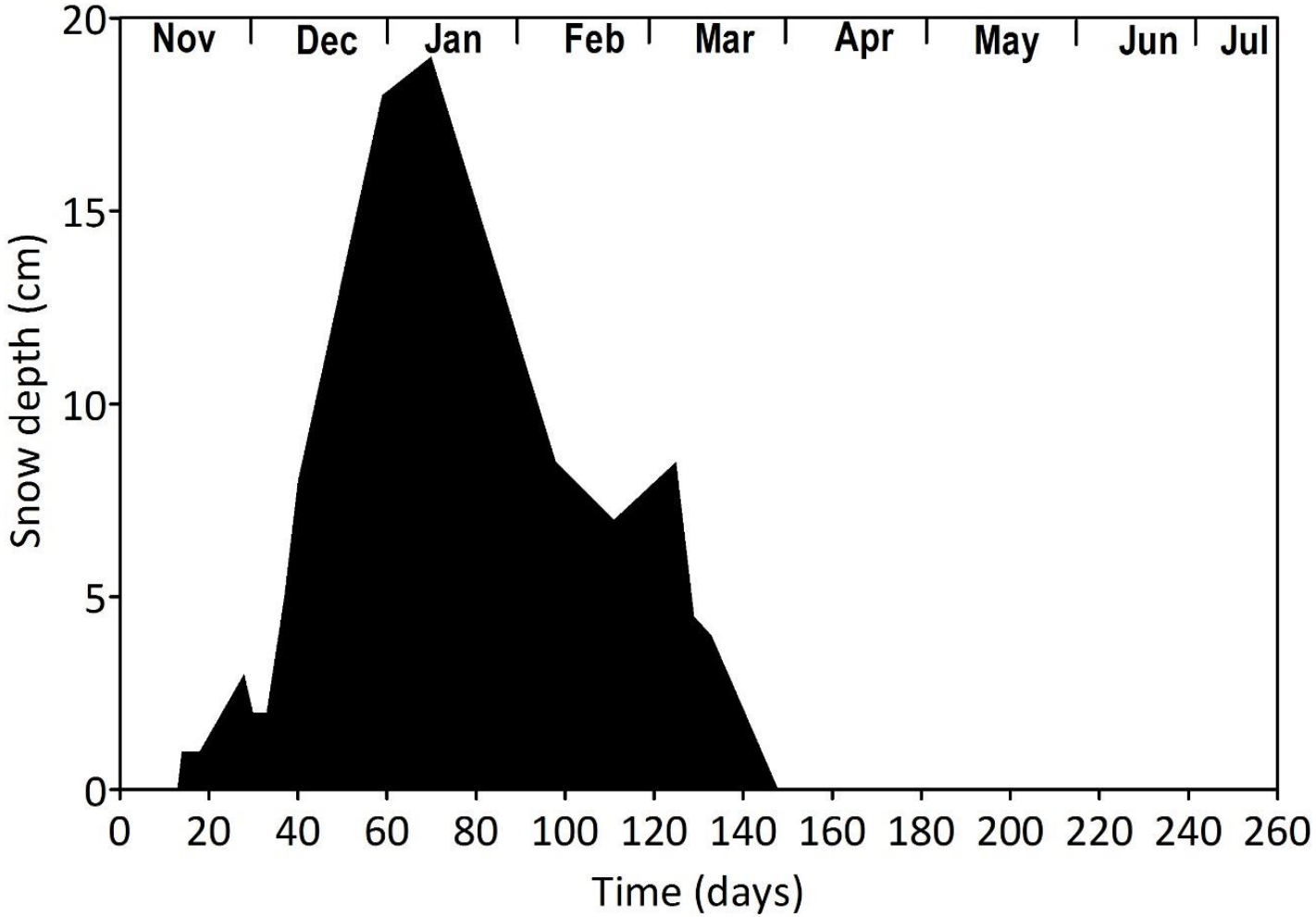

Figure C.2. Snow depth was measured in the area surrounding the biopiles. The biopiles were not covered by snow due to the dry and windy winter conditions of Saskatoon, Saskatchewan (Canada).

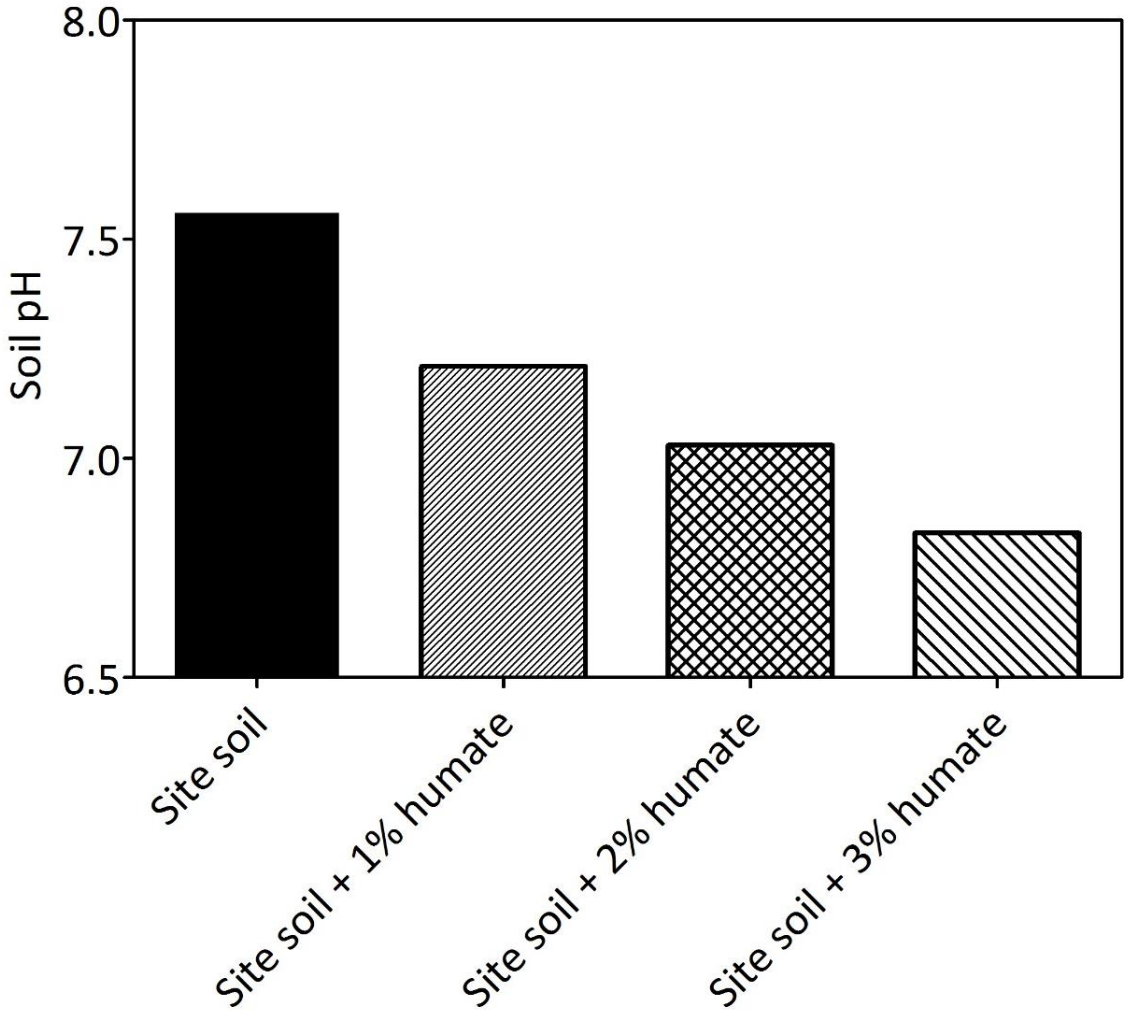

Figure C.3. Change in soil pH due to the humate amendment (%: weight basis, w/w).

Petroleum hydrocarbon analyses

Wet soil samples of ~10 g in cellulose extraction thimbles, along with blank controls (without soils), were loaded into an automatic Soxhlet extractor (Gerhardt Soxtherm, Germany) with 150 mL of a solvent extraction mixture (50:50 v/v mixture of hexane and acetone) and several boiling stones. The extraction efficiency was maintained at over 90%, which was confirmed by spiking the soils with O-terphenyl as a surrogate hydrocarbon. Columns of activated silica gel and sodium sulfate were used to clean the extracts, and were then eluted with a 50:50 mixture of hexane and dichloromethane. The filtered extracts were concentrated using a nitrogen blow-down concentrator (Cole-Parmer, Canada). Final extracts (~4 mL) were analyzed by gas chromatography with a flame ionization detector (GC-FID) (Agilent 7890A) and a capillary DB-1HT column (Agilent J&W GC Column). The extracts were further analyzed using gas chromatography with mass spectrometry (GC-MS; Agilent 7890A and AccuTOF GCV 4G Mass Spectrometer) with a capillary DB-5MS column (Agilent J&W GC Column). Diagnostic ratios of n-alkanes, branched alkanes, and a non-biodegradable biomarker (17α(H),21β(H)-hopane) confirmed that biodegradation in the site soils prevailed over abiotic losses (Prince et al., 1994; Wang et al., 2007). Quality assurance and quality control (QA/QC) operations included routine analytical testing of the chemical equipment (extractor, GC-FID, and N_2 blow-down concentrator), protocols (TPH extractability, calibration, and baseline quality), and sampling quality (storage and replicates). Duplicate or triplicate blank and surrogate controls for extraction, GC-FID operation, and silica-clean-up processes were routinely run, as similarly described in the authors' publications.

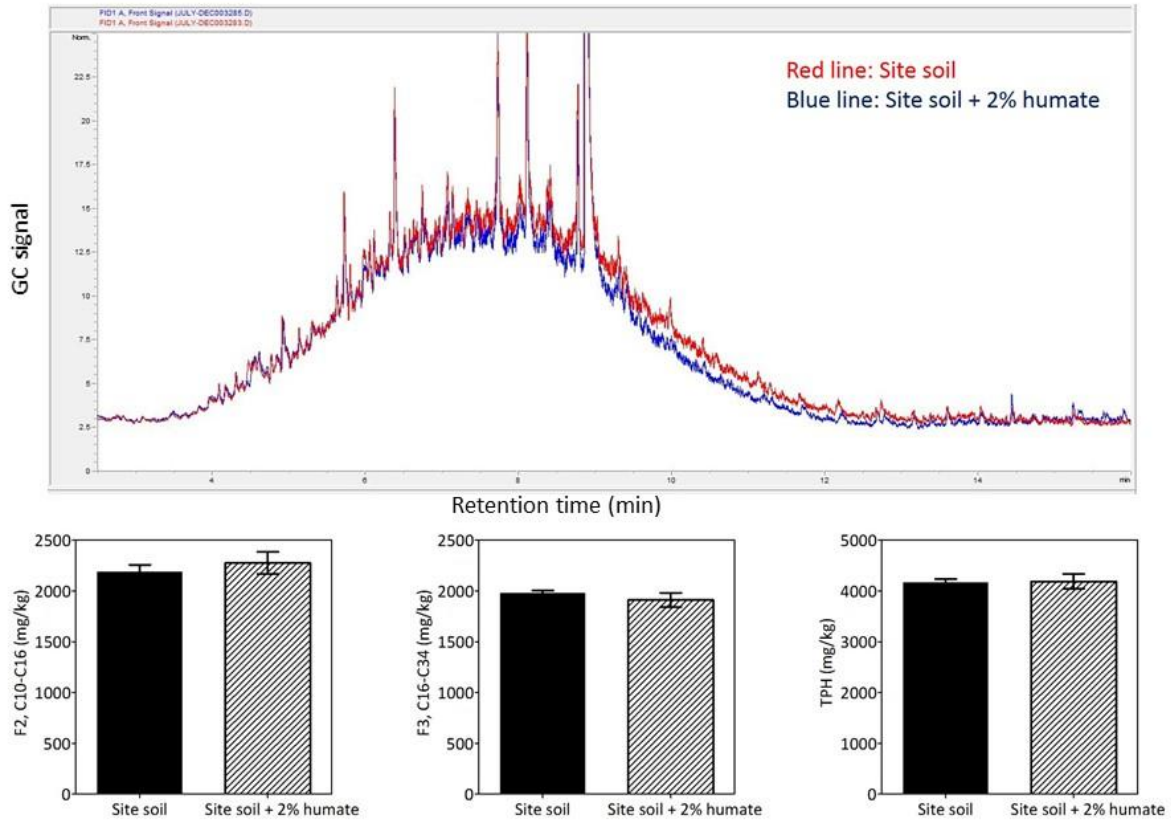

Figure C.4. Petroleum hydrocarbon analyses for the site with and without the 2% (w/w) humate amendment. The humate amendment had no negative effect on hydrocarbon recovery.

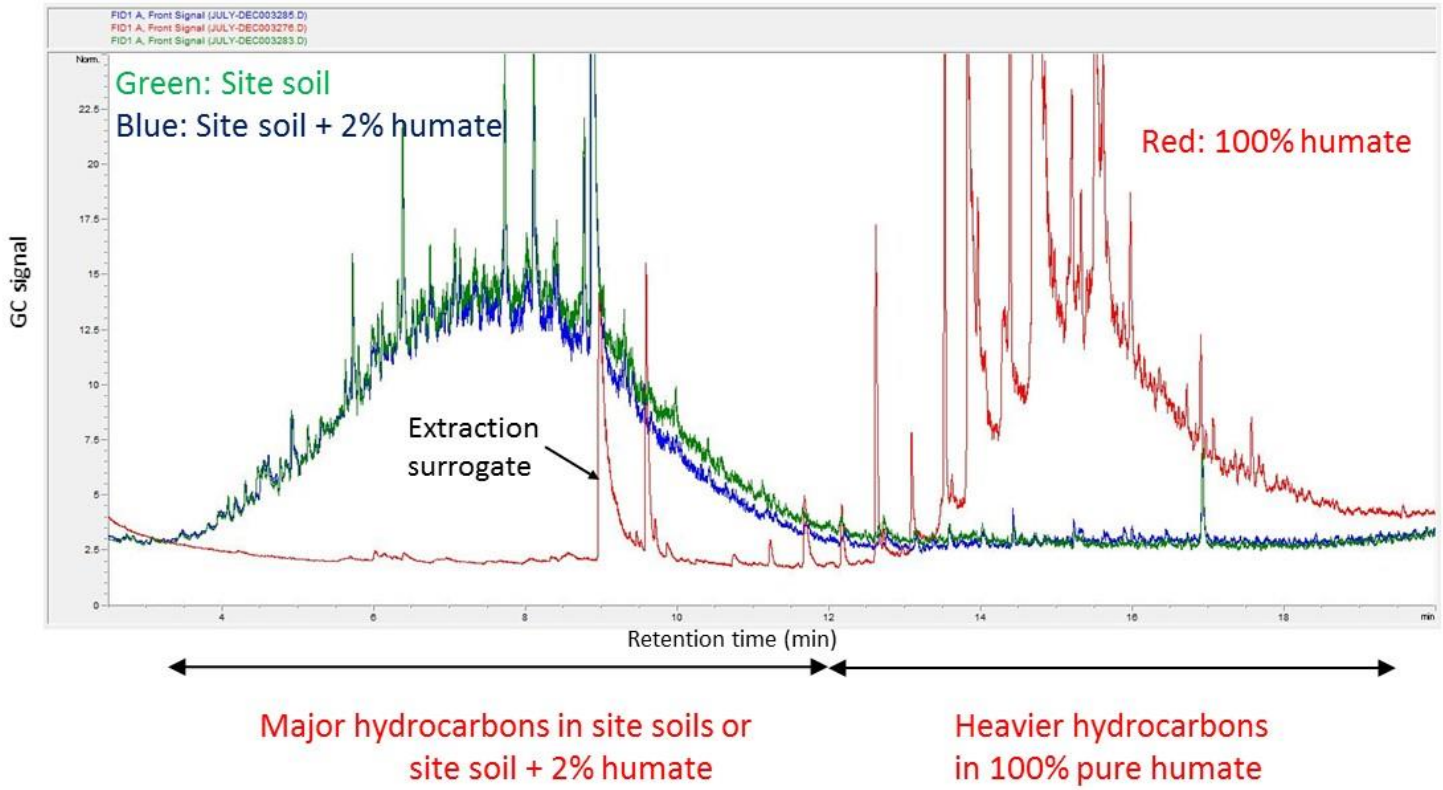

Figure C.5. Different ranges of hydrocarbons in the site soils (with or without the 2% humate admendent) and 100% pure humate.

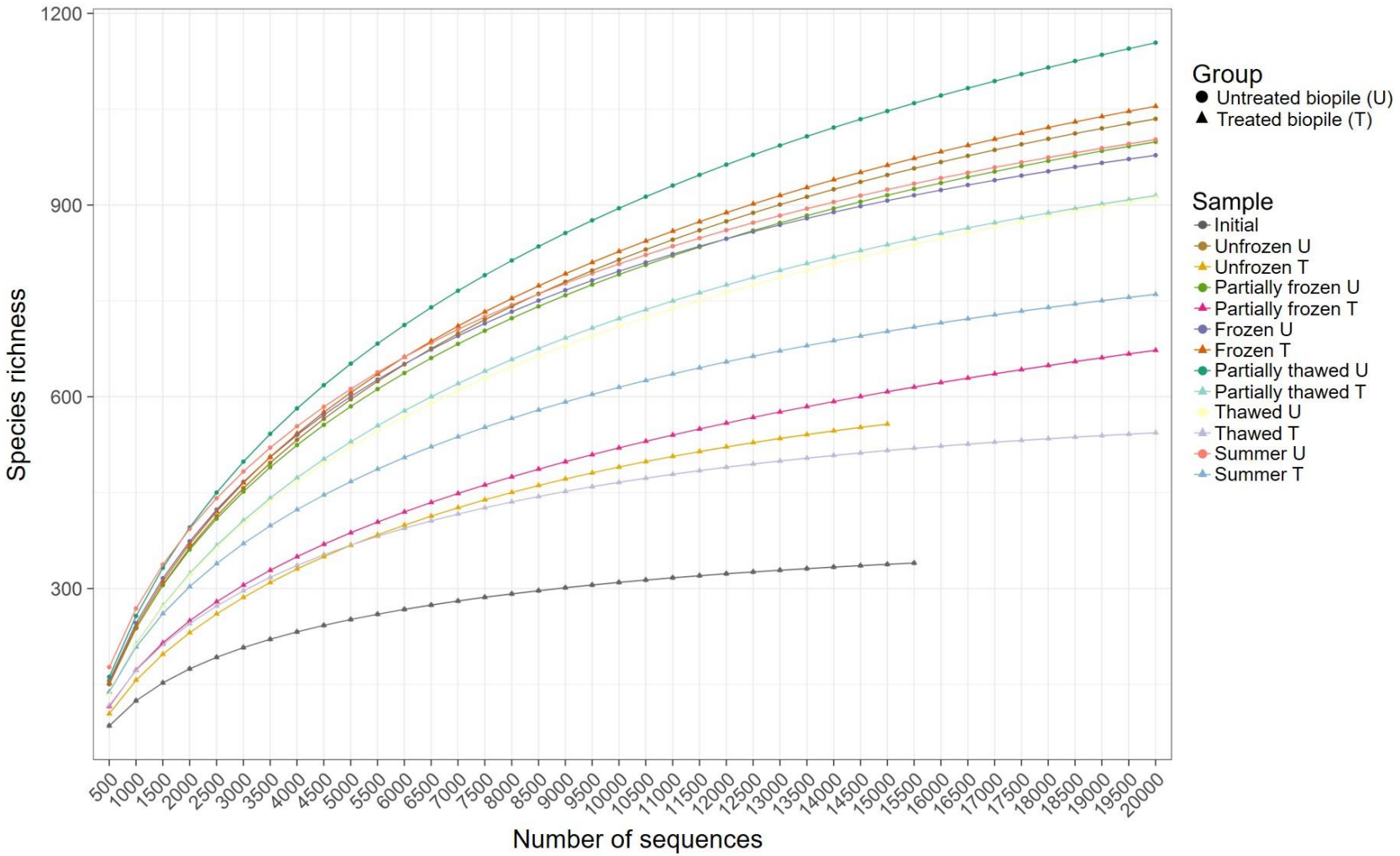

Figure C.6. Rarefaction plot of species richness, with subsampling from 500 to 20,000 reads in increments of 500 reads.

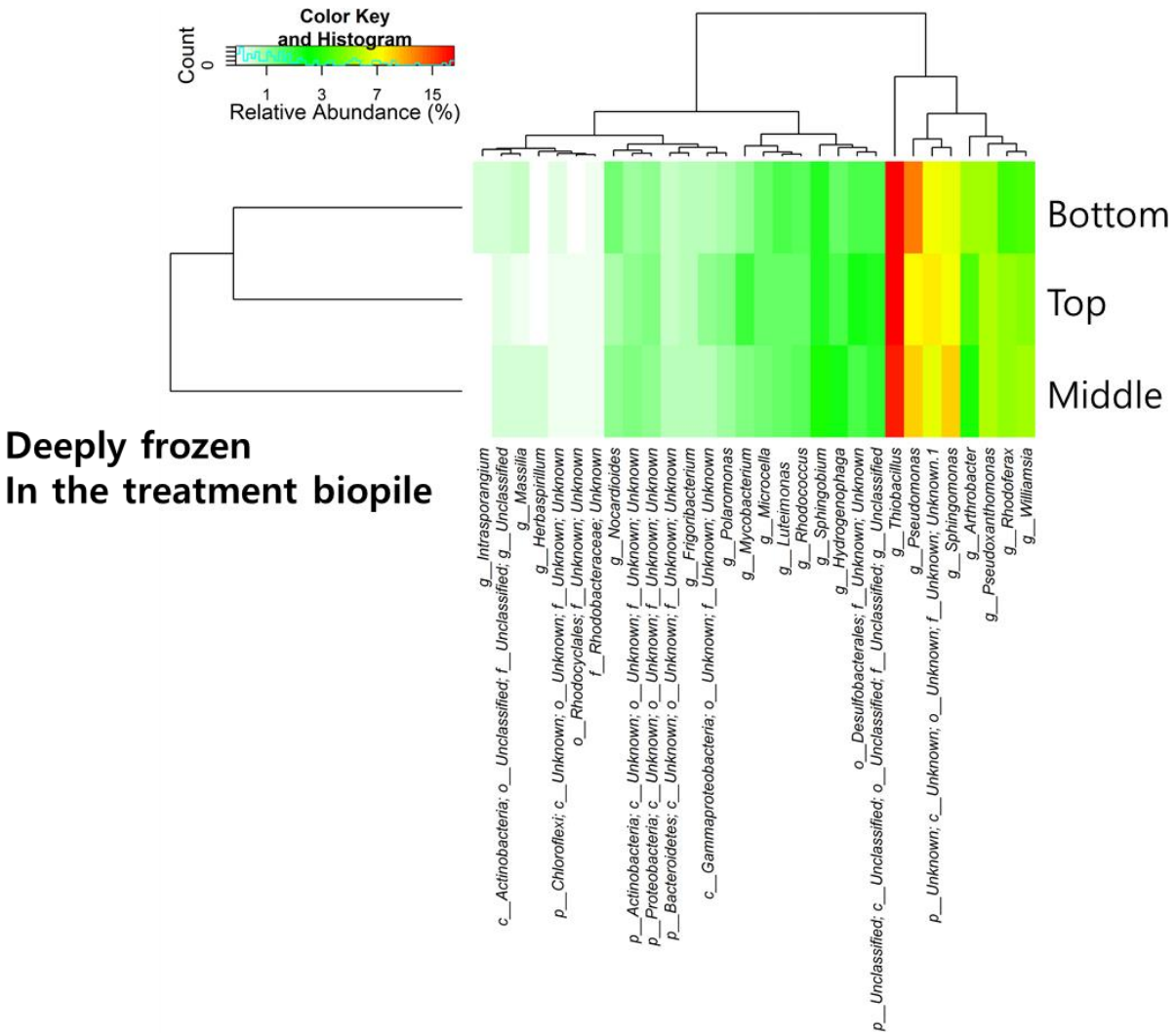

Figure C.7. Heatmap of the relative abundances of the top 30 bacterial genera at each depth in the deeply frozen treated biopile, based on Bray-Curtis dissimilarities and Euclidean distances. Abbreviations indicate taxonomic ranks: p, phylum; c, class; o, order; f, family; g, genus; s, species.

C. References

Prince, R.C., Elmendorf, D.L., Lute, J.R., Hsu, C.S., Haith, C.E., Senius, J.D., Dechert, G.J., Douglas, G.S., Butler, E.L., 1994. 17α(H),21β(H)-hopane as a conserved internal marker for estimating the biodegradation of crude oil. Environ. Sci. Technol. 28, 142–145.

Wang, Z., Yang, C., Fingas, M., Hollebone, B., Yim, U.H. and Oh, J.R., 2007. Petroleum biomarker fingerprinting for oil spill characterization and source identification, In: Wang, Z., Stout, S.A. (Eds.), Oil Spill Environmental Forensics, Academic Press, Burlington, pp. 73–146.

APPENDIX D. SUPPLEMENTARY DATA (CHAPTER 3)

Table D.1. Input parameters used for the several existing soil respiration models.

Respiration model	Input parameter
Arrhenius equation	E_a: 93 kJ/mol A: 1.4×10^7
GRESP (Bunnell et al., 1977)	a_1 (half field capacity): 5.29% a_2 (half maximum retentive capacity): 15.68% a_3 (maximum respiration rate at 10 °C): 1.032 mmol CO_2/kg/min a_4 (Q_{10}): 3.9
BRESP (Schlentner and Cleve, 1985)	a_1 (half field capacity): 5.29% a_2 (half maximum retentive capacity): 15.68% a_3 (scaling factor): 3.1 a_4 (Q_{10}-related fitting constant): 4.1 a_5 (lower limit of respiration rate): 0.057 mmol CO_2/kg/min Upper limit of respiration: 1.54 mmol CO_2/kg/min a_6: 0.67 mmol CO_2/kg/min
FRESP (Carlyle and Than, 1988)	a_1 (half field capacity): 5.29% a_2 (half maximum retentive capacity): 15.68% a_3 (maximum respiration rate at 10 °C): 1.032 mmol CO_2/kg/min a_4 (fitting constant linking Q_{10} to water content): 0.9 a_5 (lower limit of Q_{10}): 3.9 Upper limit for Q_{10}: 10.4 a_6: -3.8

Table D.2. Evaluation of the existing soil respiration models.

	GRESP	BRESP	FRESP
R^2	0.16	0.81	0.19
RMSE	0.272	0.127	0.268
MARE	0.686	0.436	0.659

Table D.3. Results of the sensitivity analysis used to determine empirical fitting constant *F*.

	CO_2 production rate				O_2 consumption rate			
F	0.05	0.15	0.36	0.70	0.50	0.19	0.05	-0.05
R^2	0.62	0.84	0.96	0.79	0.79	0.92	0.82	0.58
RMSE	0.182	0.119	0.058	0.134	0.179	0.114	0.167	0.254
MARE	0.539	0.329	0.132	0.340	0.385	0.724	1.241	1.889

Table D.4. The input soil parameters and soil conditions for the TEMP/W simulation.

Thermal conductivity (kJ/day·m·°C)		Volumetric heat capacity (kJ/m^3·°C)		Soil freeze-thaw characteristic curve
Frozen	Unfrozen	Frozen	Unfrozen	From -5 to 15 °C
38.9	15	1500	1830	$w_u = 0.010 \times \lvert T \rvert^{-0.326}$

w_u: volumetric water content (m^3/m^3), T: temperature (°C).

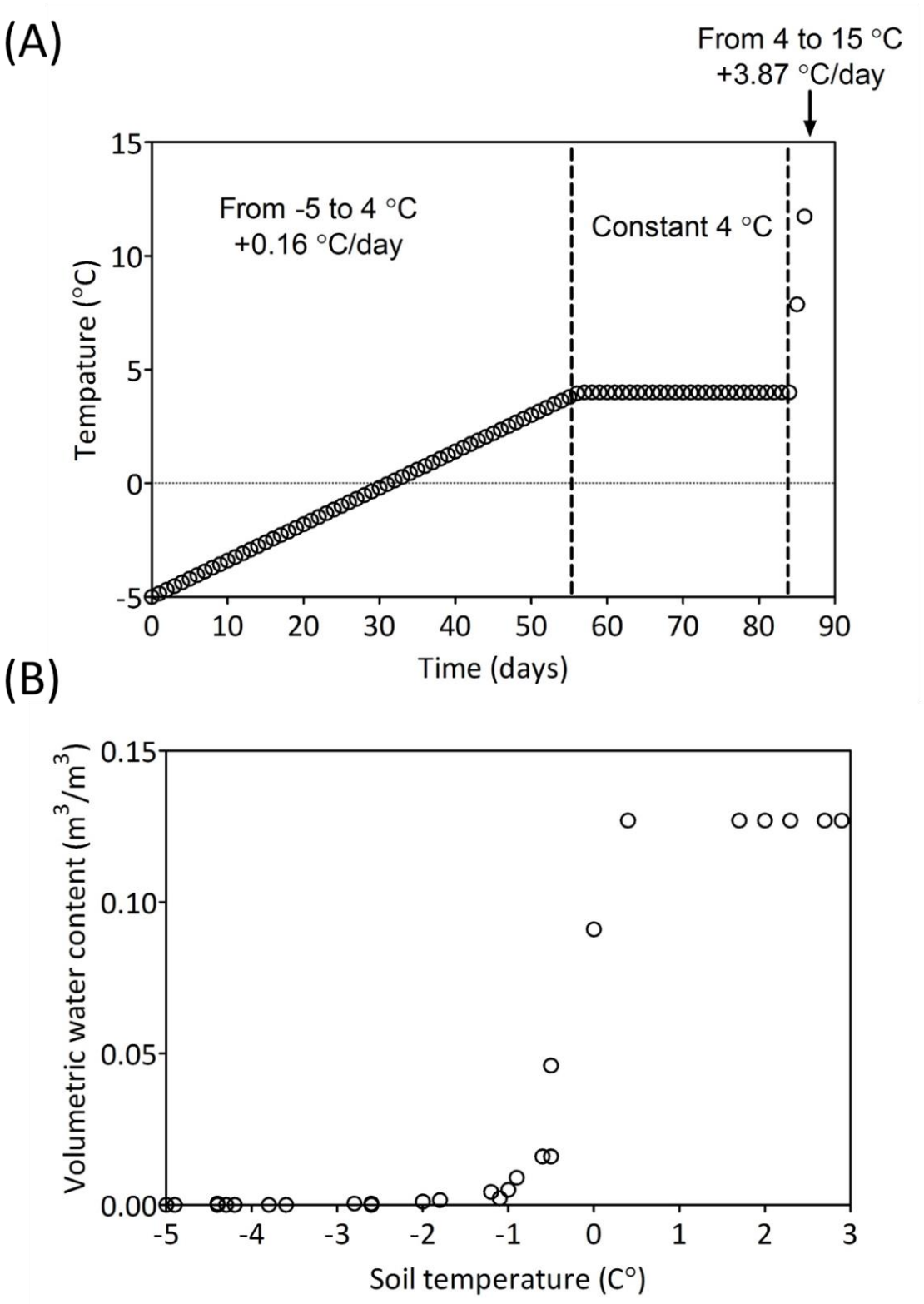

Figure D.1. The input soil conditions (from Chang et al., 2011) for the TEMP/W simulation.

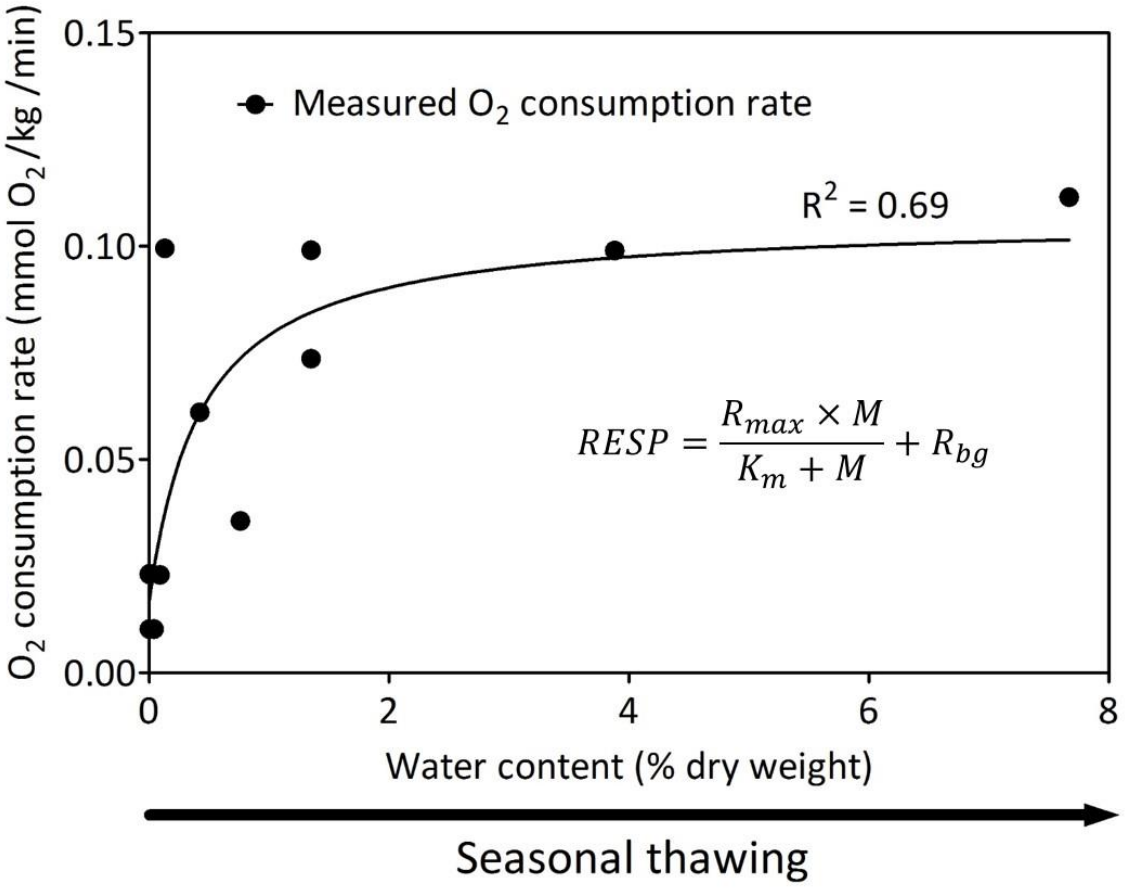

Figure D.2. The Michaelis-Menten equation modified with sub-zero respiration activity term, R_{bg} used to calculate changes in O_2 consumption rates (from Chang et al., 2011) as a function of unfrozen water content in unsaturated, nutrient-amended, petroleum-hydrocarbon-contaminated sub-Arctic soils.

D. References

Bunnell, F., Tait, D., Flanagan, P., Van Clever, K., 1977. Microbial respiration and substrate weight loss—I: A general model of the influences of abiotic variables. Soil Biol. Biochem. 9, 33-40.

Carlyle, J.t., Than, U.B., 1988. Abiotic controls of soil respiration beneath an eighteen-year-old Pinus radiata stand in south-eastern Australia. J. Ecol. 76, 654-662.

Chang, W., Klemm, S., Beaulieu, C., Hawari, J., Whyte, L., Ghoshal, S., 2011. Petroleum hydrocarbon biodegradation under seasonal freeze−thaw soil temperature regimes in contaminated soils from a sub-Arctic Site. Environ. Sci. Technol. 45, 1061-1066.

Schlentner, R.E., Cleve, K.V., 1985. Relationships between CO_2 evolution from soil, substrate temperature, and substrate moisture in four mature forest types in interior Alaska. Can. J. Forest Res. 15, 97-106.

APPENDIX E. SUPPLEMENTARY INFORMATION (CHAPTER 4)

SECTION E.1.0: CONTAMINATED SITE SOIL DATA

Table E.1. Summary of the physical, chemical, and biological properties of petroleum hydrocarbon-contaminated site soil (soil data from Kim et al., 2018).

Soil composition by USCS	Coarse grain (w/w) Fine grain (w/w)	46% 54%
Soil composition by USDA	Sand: 2.0 – 0.05 mm (w/w) Silt: 0.002 – 0.05 mm (w/w) Clay: < 0.002 mm (w/w)	36.6% 30.4% 33.0%
Gravimetric soil moisture	w_w/w_s	15.4%
Soil pH	as $CaCl_2$ extract	7.56
Petroleum hydrocarbons	F1: >C6 – C10 F2: >C10 – C16 F3: >C16 – C34 F4: >C34 – C50 TPH	ND 2858 ± 285.7 mg/kg 3441 ± 141.9 mg/kg ND 6300 ± 305.9 mg/kg
Nutrients	Total organic carbon Soluble nitrite (mg/kg) Soluble nitrate (mg/kg) Soluble ammonia (mg/kg) Total phosphorous (mg/kg)	0.53 ± 0.11% ND 0.18 ± 0.29 mg/kg 0.62 ± 0.07 mg/kg 432.5 ± 12.6 mg/kg
Hydrocarbon-degrading bacteria at 4 °C	Bushnell Hass agar plate spiked with 1% (v/v) diesel	$7.5 \pm 3.9 \times 10^4$ CFU/g soil

USCS: Unified Soil Classification System.

USDA: United States Department of Agriculture.

ND: Not detected.

TPH: Total petroleum hydrocarbons.

CFU: Colony-forming unit.

SECTION E.2.0: MICROCOSM EXPERIMENT SETS

Table E.2. Summary of the microcosm experiment sets prepared in this study.

Microcosm set			Soil amount	Replicates	Total
Soil-sampling microcosm	Treated: 3 types	NPK only, NPK+PM4%, NPK+ZEO5%	200 g	3	9
	Untreated: 2 types	Positive Negative	200 g	3	6
Subtotal	**5**	**5**			**15**
Soil gas monitoring microcosm	Treated: 3 types	NPK only, NPK+PM4%, NPK+ZEO5%	100 g	2	6
	Untreated: 1 type	Positive	100 g	2	2
Subtotal	**4**	**4**			**8**
VWC-Temp-monitoring microcosm	Treated: 3 types	NPK only, NPK+PM4%, NPK+ZEO5%	400 g	2	6
	Untreated: 1 type	Positive	400 g	2	2
Subtotal	**4**	**4**			**8**
Nutrient only or zeolite only	Treated: 7 types	NPK PM4%, ZEO1%, ZEO2%, ZEO5%, ZEO10%, ZEO20%	400 g	2	14
	Untreated: 1 type	Positive	400 g	2	2
Subtotal	**8**	**8**			**16**
Hydrocarbon extractability: Soil amendment effect	Treated: 2 types	PM4%, ZEO5%	200 g	3	6
	Untreated: 1 type	Positive	200 g	3	3
Subtotal	**3**	**3**			**9**

NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®).

PM: Peat moss.

ZEO: Zeolite.

SECTION E.3.0: SOIL TREATMENT DATA

Table E.3. Soil amendment dosages for the microcosm experiments.

Microcosm sets	Treatment
NPK only (Nutrient Only)	N: 49 mg/kg P: 21 mg/kg K: 41 mg/kg C_{TOC}/N: 100:0.8
NPK + 4% peat moss (High Nutrient)	N: 96 mg/kg P: 42 mg/kg K: 80 mg/kg Peat moss 4% (w/w) C_{TOC}/N: 100:0.8
NPK + 5% zeolite (Nutrient + Surface area)	N: 49 mg/kg P: 21 mg/kg K: 41 mg/kg Zeolite 5% (w/w) C_{TOC}/N: 100:0.8
Control	No treatment

Initial water content in all sample sets: 18 ± 0.3 % (w/w)

NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®).

Peat moss: Sphagnum Peat Moss, Premier, Canada.

Zeolite: Canadian Zeolites.

Section E.3.1. Peat Moss Data

Table E.4. Physicochemical properties of peat moss (Sphagnum peat moss, Premier Tech, Quebec).

Physicochemical property	Value
pH	4.2 - 5.2 (1:3, v:v water)
Electrical conductivity	0.09 - 0.30 mmhos/cm
C/N ratio	125 – 155
Cation exchange capacity	150 - 250 meq/100 g
Organic matter	90 - 96%
Total porosity	90 - 97%
Dry bulk density	6 - 8 Lbs. cu./ft. (0.09 - 0.13 g/cm^3)
Water holding capacity	700 - 1100 % by weight
Effective particle size (D10)	3.7 mm

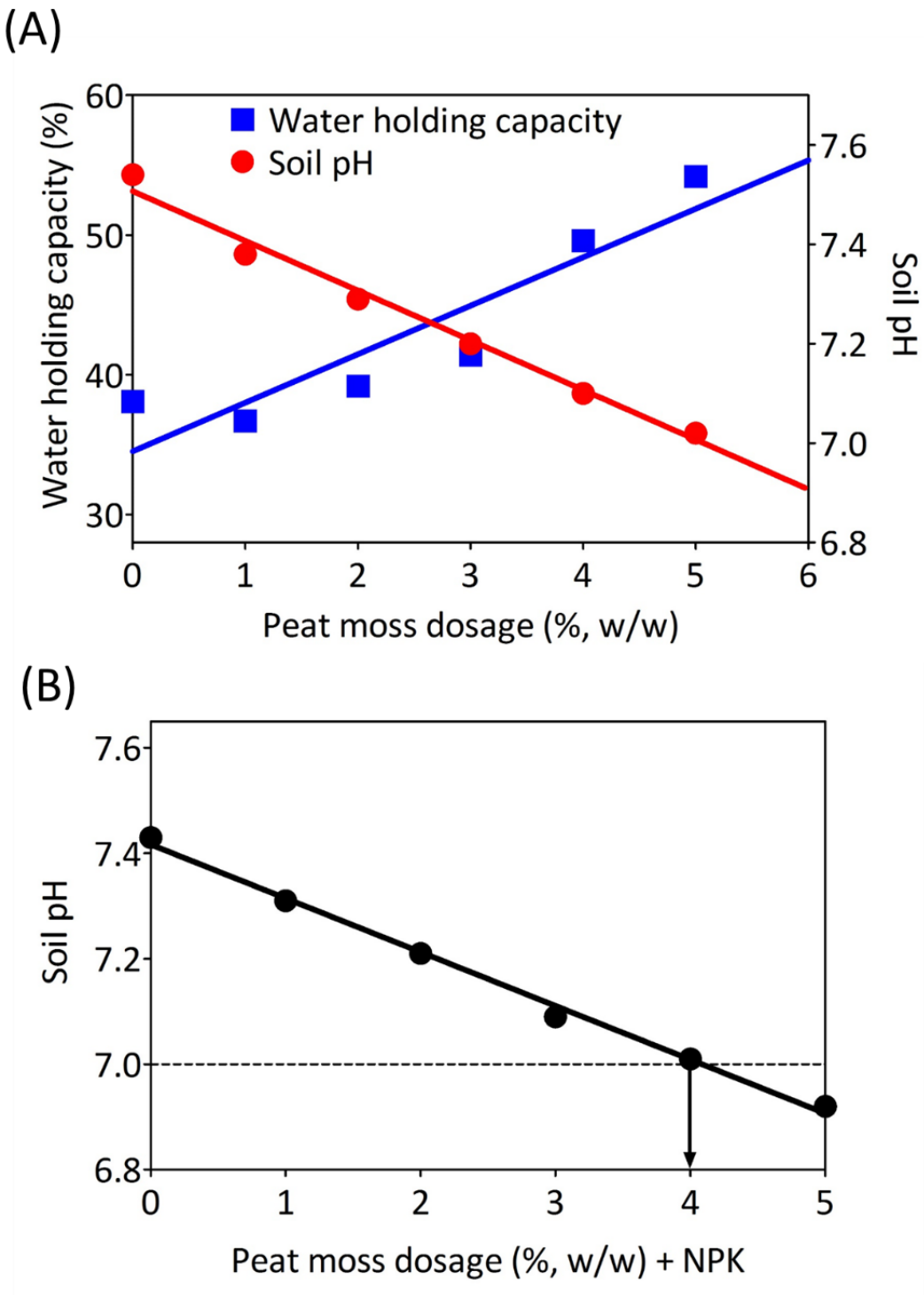

Figure E.1. Effect of site soil treatment using peat moss dosage and nutrient on water holding capacity and soil pH (A), and the combined effect of peat moss and nutrient on soil pH (B). NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®).

Section E.3.2. Zeolite Data

Table E.5. Major constituents, surface area, pore volume, average pore diameter, and effective particle size of zeolite (Paul et al., 2017).

Major constituent (%, w/w)	SiO_2	71.9
	Al_2O_3	10.9
	K_2O	3.3
	Fe_2O_3	2.1
	CaO	1.2
	Na_2O	0.6
	MgO	0.3
	TiO_2	0.15
	MnO	0.01
	S	0.01
Brunauer–Emmett–Teller (BET) analysis	Surface area (m^2/g)	32.2
	Pore volume (cm^3/g)	0.076
	BET-average pore diameter (nm)	9.4
	Effective particle size (D_{10}, μm)	1.9

The major constituents were analyzed using X-ray fluorescence (XRF) by the Geoanalytical Laboratory at the Saskatchewan Research Council (SRC, Saskatoon, Canada).

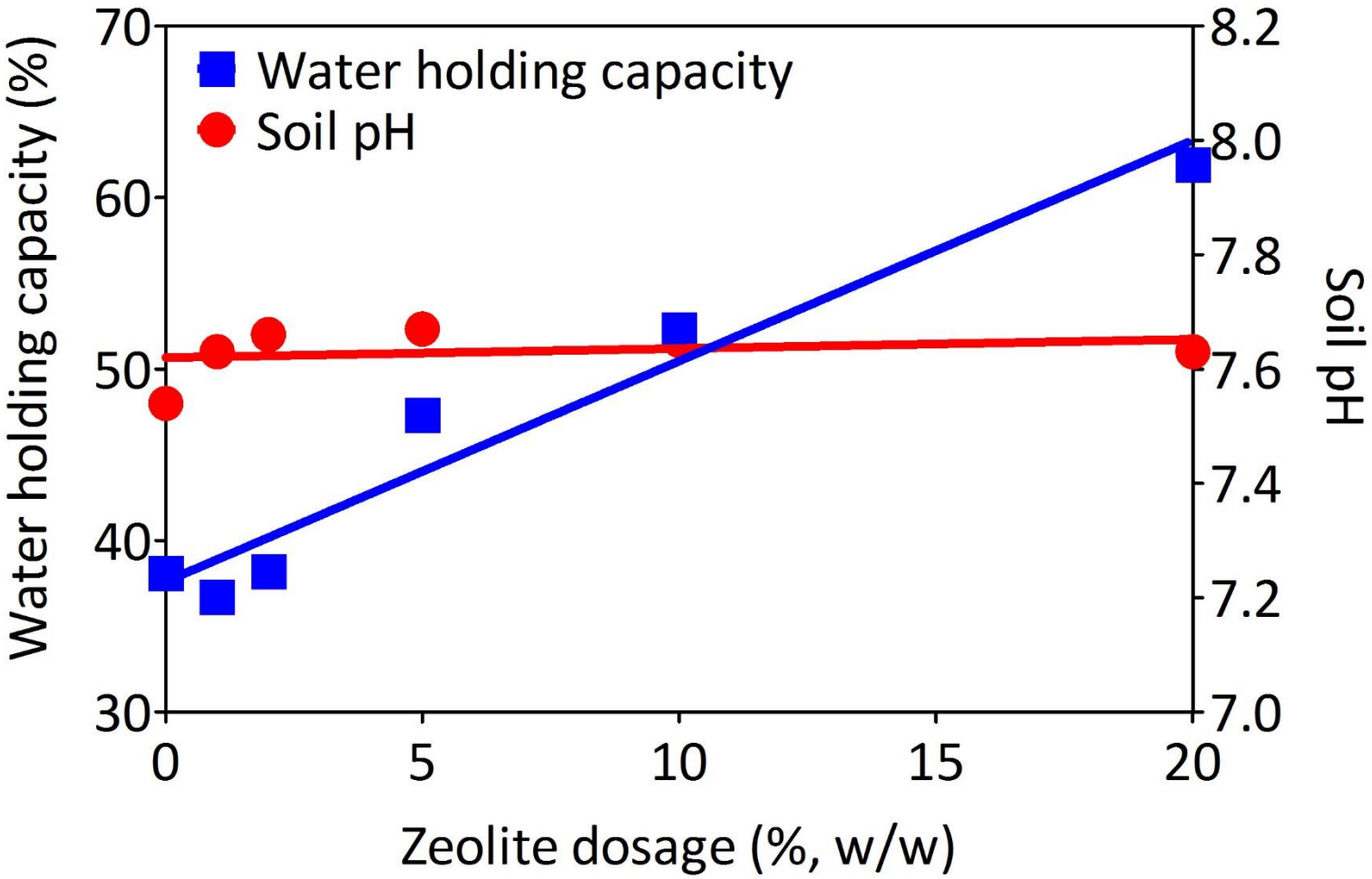

Figure E.2. Relationship between zeolite dosage to water holding capacity and soil pH.

SECTION E.4.0: HYDROCARBON EXTRACTION AND AMENDMENT

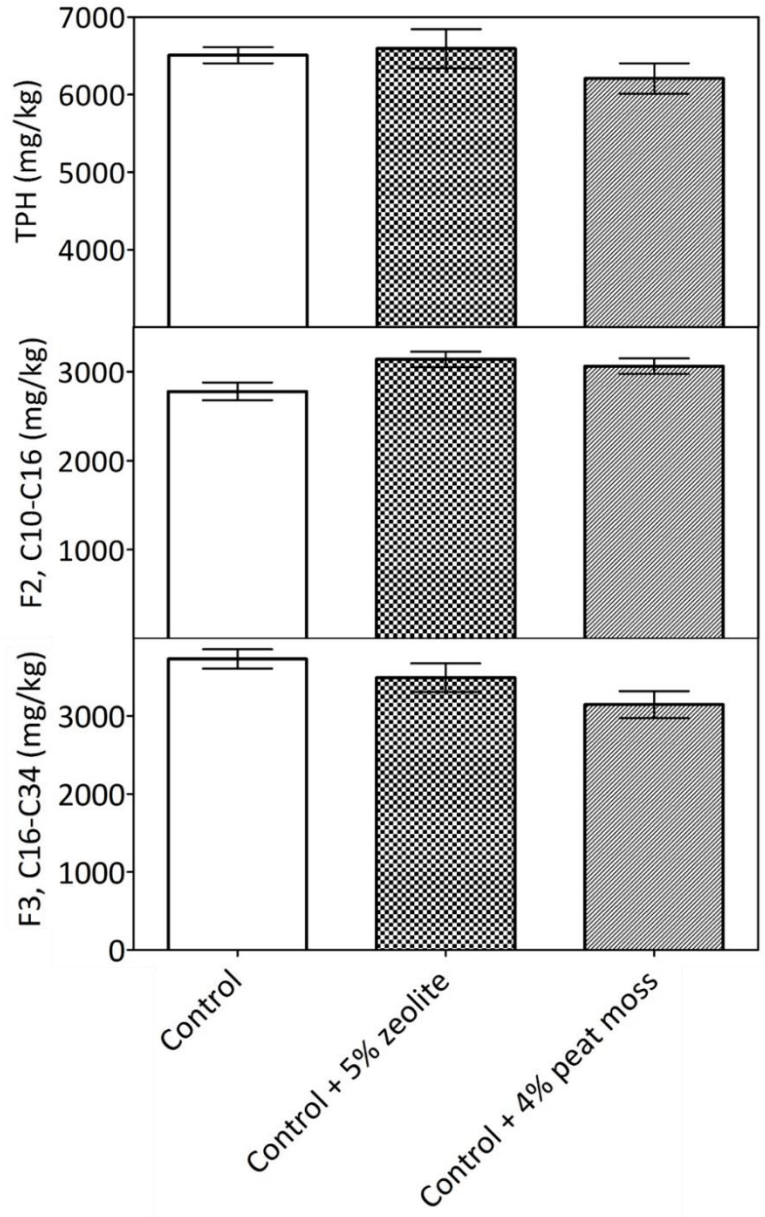

Figure E.3. Sorption and extraction test. Untreated site soil and treated site soils with zeolite and peat moss were incubated at room temperature for 60 d (n = 3). Hydrocarbon recoveries in terms of total petroleum hydrocarbons (TPH) were 101 ± 6.7 and 95 ± 5.3% in the zeolite and peat moss treated soils, respectively. No significant difference ($p < 0.05$) was found among samples by Bonferroni's comparison test.

SECTION E.5.0: SOIL FREEZING CHARACTERISTIC PARAMETERS

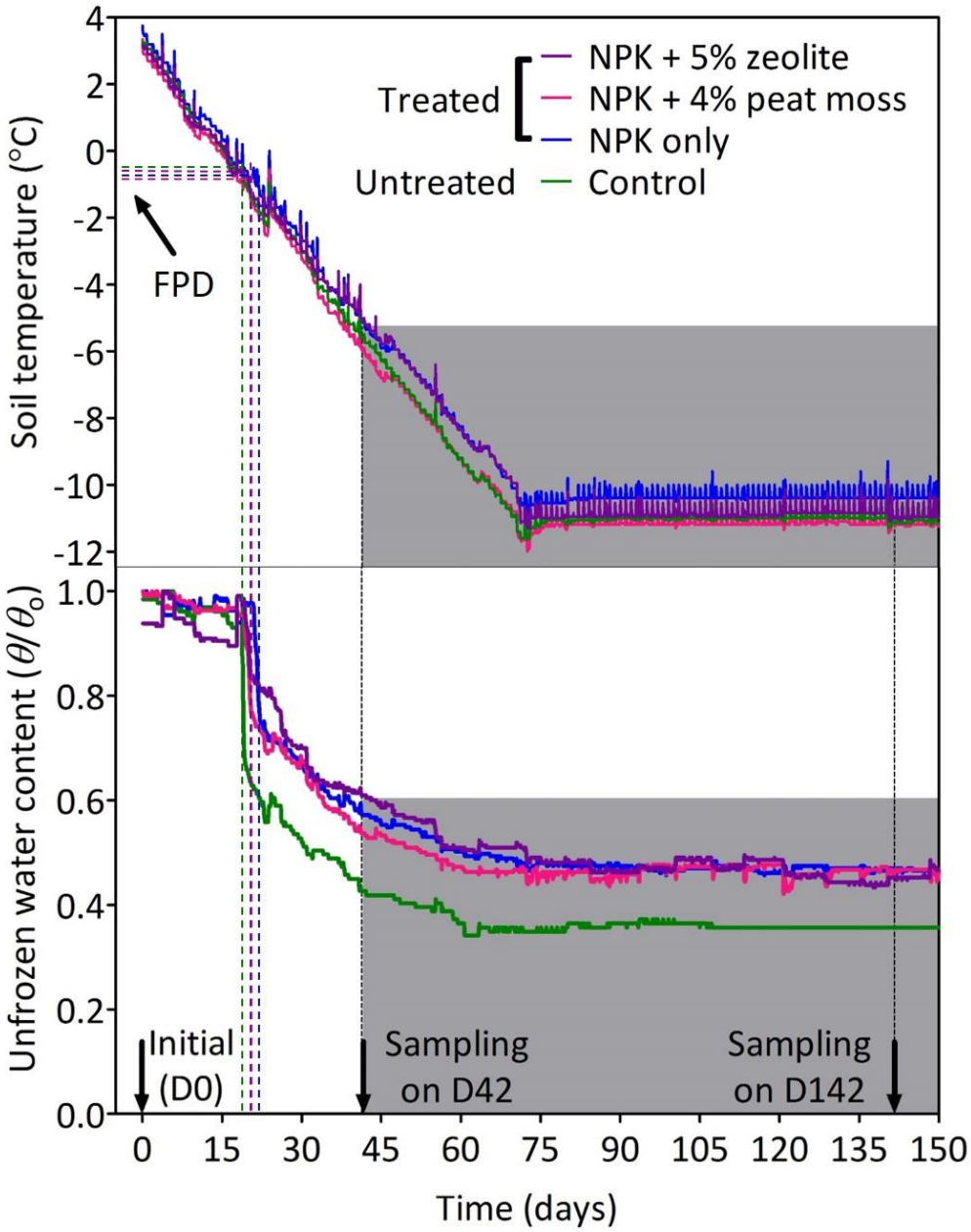

Figure E.4. Profile of soil temperature and unfrozen water content normalized to initial water content in treated and untreated contaminated soils (θ/θ_o) subjected to simulated seasonal (slow) freezing stress (−0.2 °C/day). D0, D42, and D142 refer to the soil sampling day (Days 0, 42, and 142) associated with soil thermal phases indicated by changes in the amount of unfrozen water (unfrozen, phase change, and frozen phases). NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®), FPD: Freezing-point depression.

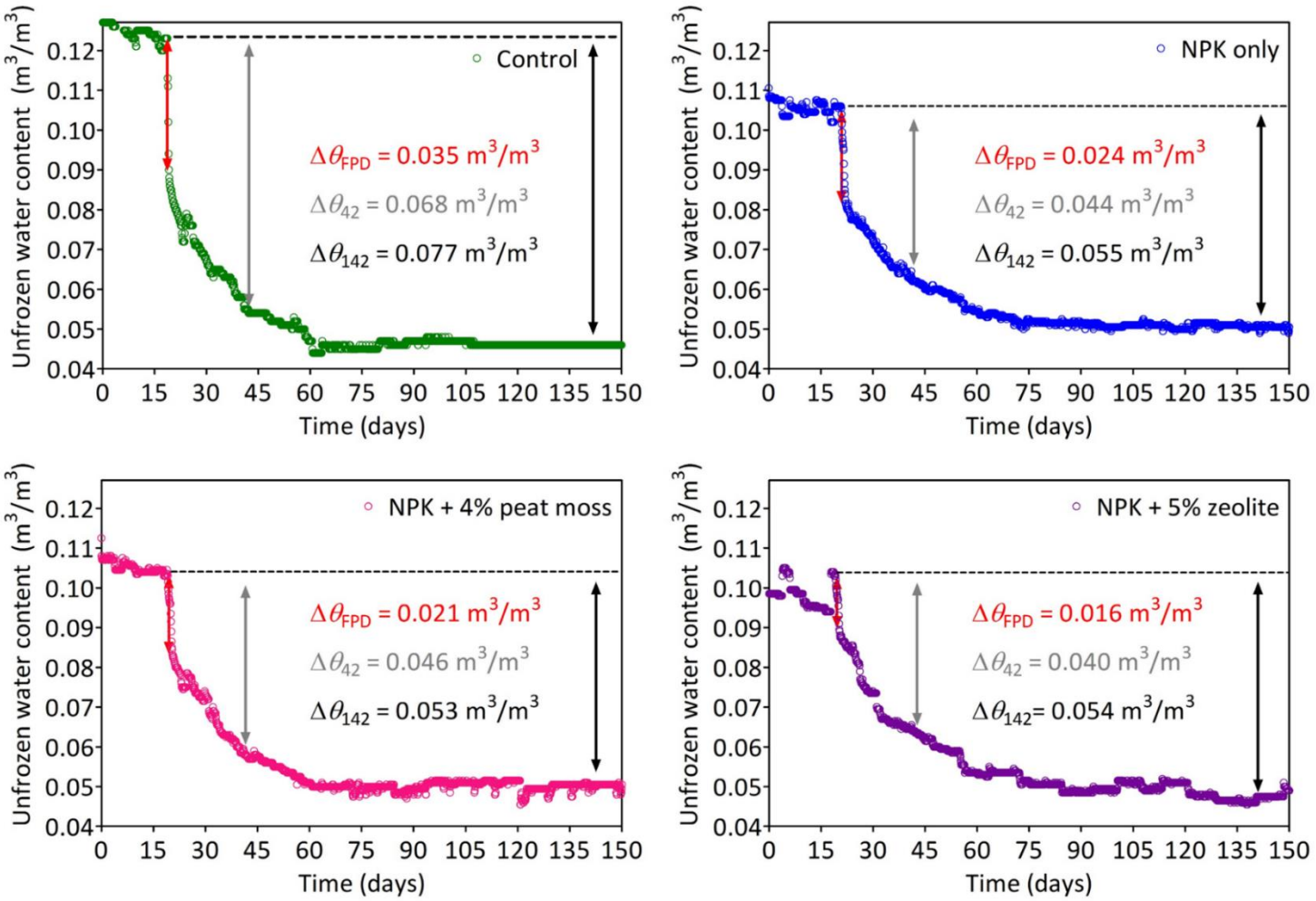

Figure E.5. Decreasing unfrozen water content during freezing-point depression ($\Delta\theta_{FPD}$), freezing phase ($\Delta\theta_{42}$), and frozen phase ($\Delta\theta_{142}$) in treated and untreated contaminated soils subjected to simulated seasonal freezing stress at −0.2 °C/d. NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®).

Table E.6. Decrease of monitored unfrozen water content with soil temperature for phase transition ($\Delta\theta_{FPD}$, D18-D22), freezing ($\Delta\theta_{42}$, D42), and frozen phases ($\Delta\theta_{142}$, D142).

Experiment	Day 18 – 22 Phase transition		Day 42 Freezing phase		Day 142 Frozen phase	
	$\Delta\theta_{FPD}$ (m^3/m^3)	Soil temp. at FPD (°C)	$\Delta\theta_{42}$ (m^3/m^3)	$\Delta\theta_{FPD}$ (m^3/m^3)	Soil temp. at FPD (°C)	$\Delta\theta_{42}$ (m^3/m^3)
Control	0.035	-0.65	0.068	0.035	-0.65	0.068
NPK only	0.024	-0.80	0.044	0.024	-0.80	0.044
NPK + 4% peat moss	0.021	-1.15	0.046	0.021	-1.15	0.046
NPK + 5% zeolite	0.016	-0.80	0.040	0.016	-0.80	0.040

θ: Unfrozen water content (m^3/m^3).

NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®).

FPD: Freezing-point depression.

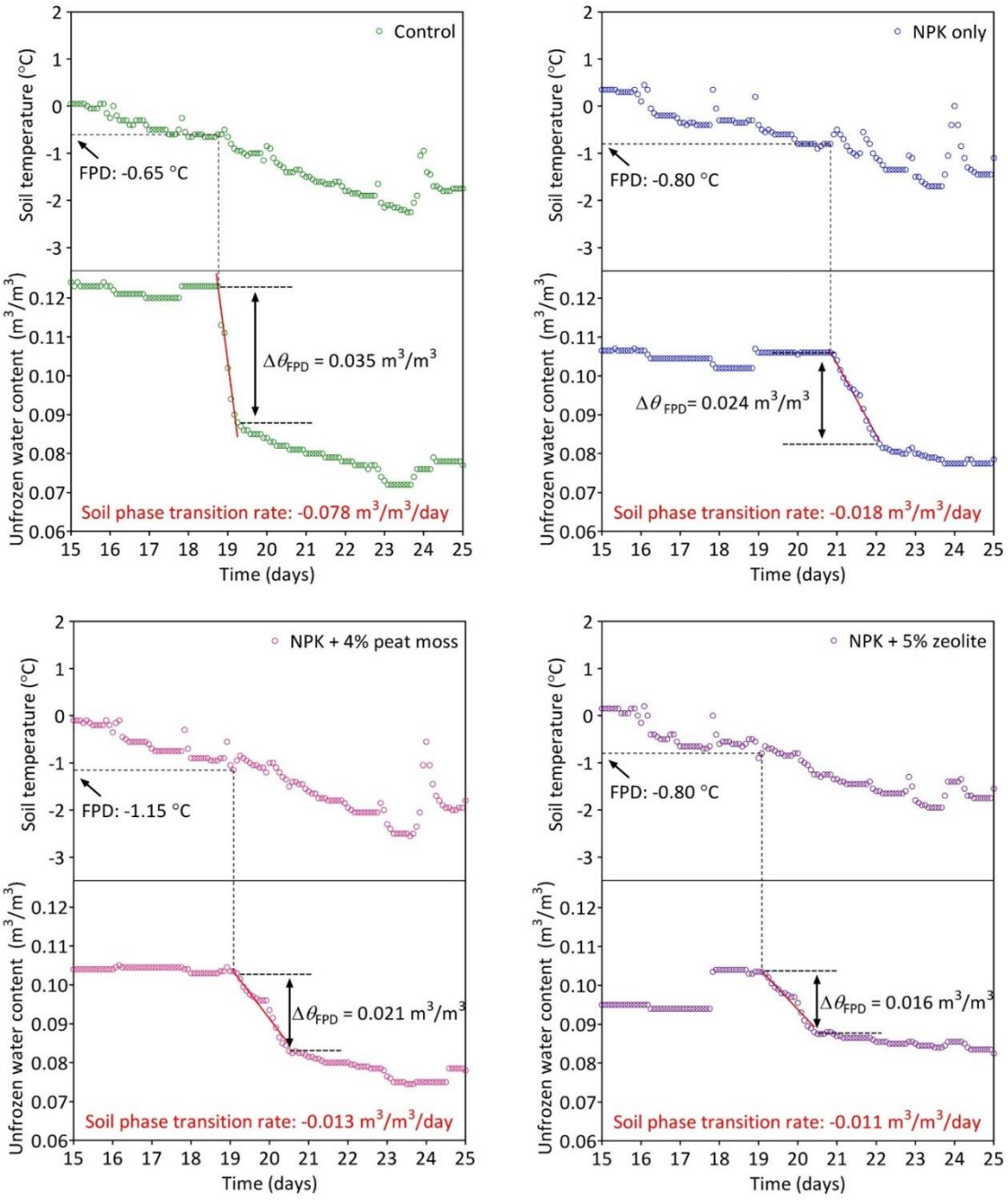

Figure E.6. Estimation of the soil phase transition rates (m^3/m^3/day) in treated and untreated contaminated soils subjected to simulated seasonal freezing stress at −0.2 °C/day. Variation in the soil phase transition rates, freezing-point depression (FPD), and corresponding amount of decreasing unfrozen water content at the FPD ($\Delta\theta_{FPD}$, m^3/m^3) among treated and untreated contaminated soils are indicated. NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®).

Table E.7. Summary of freezing soil parameters: freezing-point depression (FPD), soil phase transition rate (m^3/m^3/day), and decreasing unfrozen water content at FPD ($\Delta\theta_{FPD}$, m^3/m^3).

Experiment	Freezing-point depression (FPD, °C)	Soil phase transition rate (m^3/m^3/d)	$\Delta\theta_{FPD}$ (m^3/m^3)	Phase transition duration (h)
Control	-0.65	0.078	0.035	12
NPK only	-0.80	0.018	0.024	30
NPK + 4% peat moss	-1.15	0.013	0.021	34
NPK + 5% zeolite	-0.80	0.011	0.016	32

Soil phase transition rate (m^3/m^3/d): rate of the decreasing unfrozen water content when soil temperature just passed the freezing-point depression (FPD). $\Delta\theta_{FPD}$ (m^3/m^3): amount of decreasing unfrozen water content at FPD. Phase transition duration: time taken during phase transition at FPD.

The FPD of the contaminated site soil was within the range of previous studies, e.g., −0.07 °C in silty clay (Zhang et al., 2015), −0.2 °C in nutrient-amended sandy soil (Chang et al., 2011), −0.4 °C in clay (Grechishchev et al., 2001), and −1 °C in silty loam (Wu et al., 2015).

NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®).

Table E.8. Surface area (m^2/g) and pore width (nm) determined from Brunauer–Emmett–Teller (BET) analysis as well as soil electrical conductivity (EC, μS/cm) with corresponding α value and residual unfrozen water content (θ/θ_o).

Experiment	Surface area (m^2/g)	Pore width (nm)	Soil EC (μS/cm)	α value	Residual UWC (θ/θ_o)
Control	4.7	20.8	886.8	0.063	0.48
Control + 5% zeolite	6.1	15.5	854.0	0.072	0.58
Control + 10% zeolite	6.9	14.7	840.7	0.083	0.63
Control + 4% peat moss	4.3	17.9	902.7	0.081	0.59

Although the particle size of the peat moss is relatively larger (D_{10}: 3.7 mm) than zeolite (D_{10}: 1.9 μm), its structure is fragile and porous (Goetz and Price, 2015), as confirmed in SEM images (Lee et al., 2015). Thus, the dosage of peat moss up to 5% (w/w) increased soil water retention and water holding capacity from 38.1 to 54.2% (Fig. S4.1). The soil pore width decreased from 20.8 nm in the untreated soil to 17.9 nm in the peat moss-treated soil, indicating peat moss addition likely also induced soil aggregates. Thus, the increase of α value from 0.063 in the untreated soil to 0.081 in the peat moss-treated (4%) soil was likely not due to the effect of surface area but rather to capillary forces, which result in increasing water sorption in the narrow or irregular geometry soil pores by soil aggregates with reduced pore width and the nutrients in the peat moss.

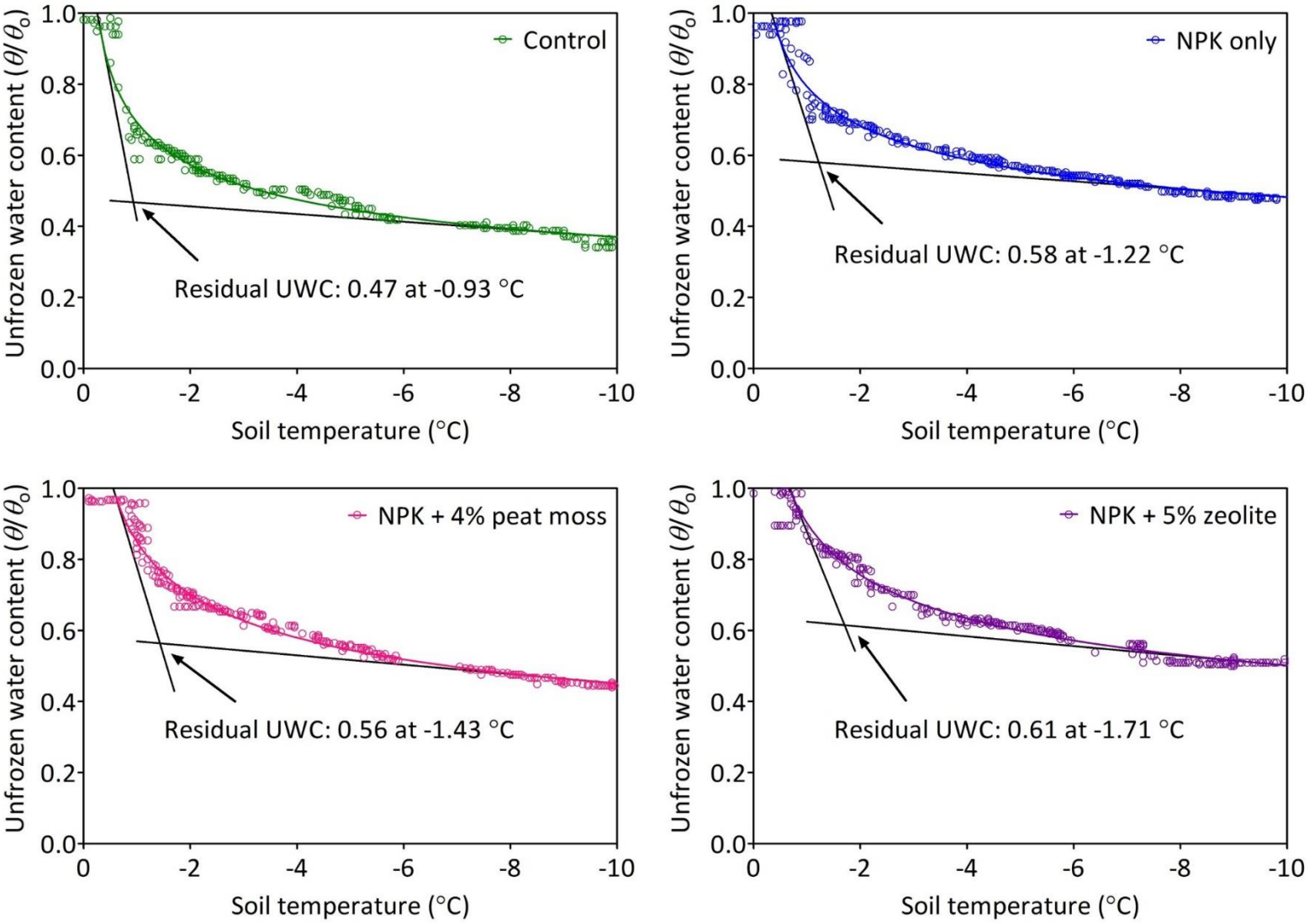

Figure E.7. Residual unfrozen water content (UWC) determined using a previously described method (Ren et al., 2017). NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®).

SECTION E.6.0: HYDROCARBON BIODEGRADATION DATA

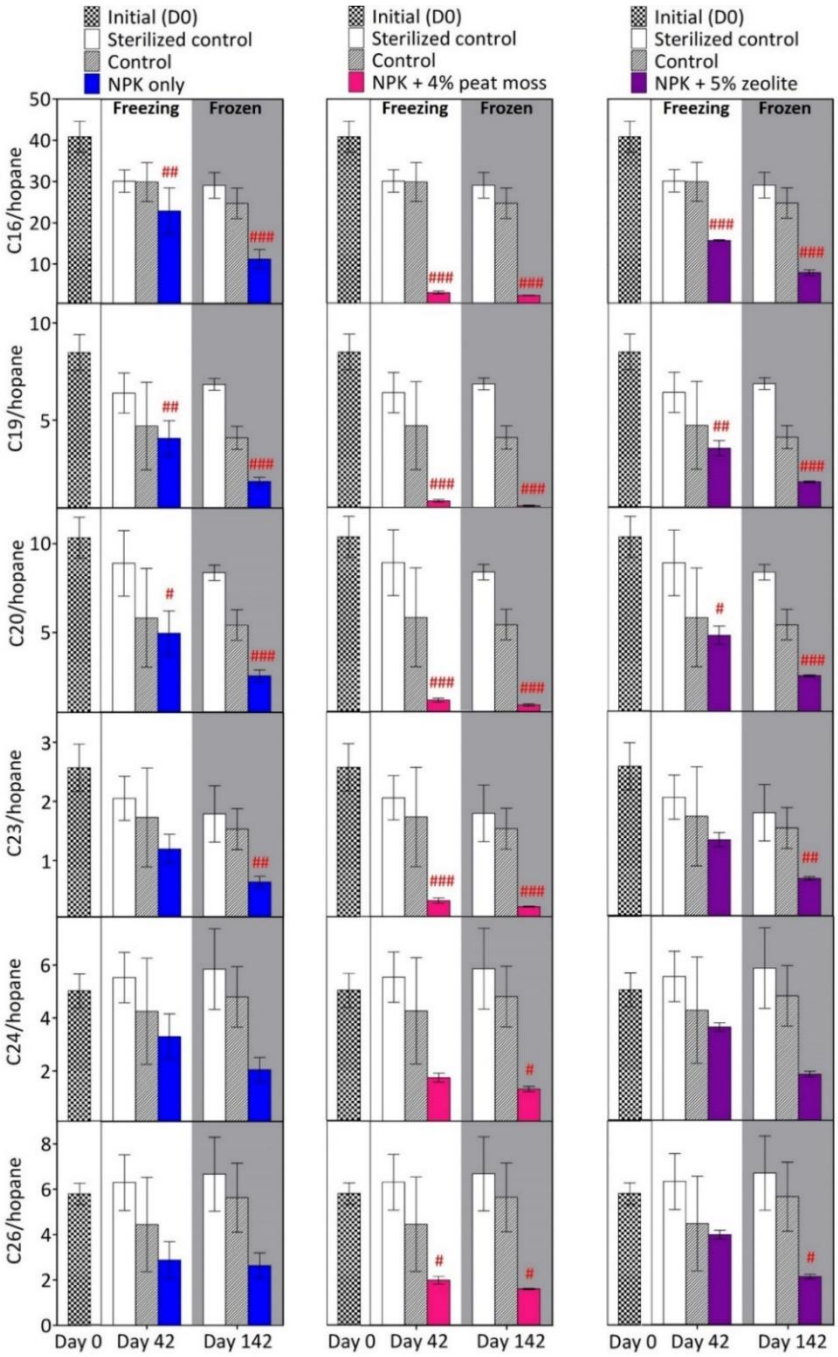

Figure E.8. Evaluation of biodegradation using the ratio of C16, C19, C20, C23, C24, and C26 based on hopane (n = 3). Bars with # above them are significantly different by Bonferroni's comparison test ($p < 0.05$) compared to the initial soil (D0). #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$. NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®).

Table E.9. Petroleum hydrocarbon analysis for the treated and untreated site soils: F2, F3, and total petroleum hydrocarbons (TPH) (n = 3 to 6) with percent removal.

		D42			D142	
		Concentration (mg/kg)	Removal (%)		Concentration (mg/kg)	Removal (%)
Sterilized control	TPH	6015 ± 1326.0	4.5	TPH	6502 ± 158.1	0.0
	F2	3022 ± 641.6	0.0	F2	3135 ± 333.9	0.0
	F3	2993 ± 687.0	13.0	F3	3367 ± 484.7	2.2
Control	TPH	6224 ± 935.2	1.2	TPH	6567 ± 888.0	0.0
	F2	2731 ± 1050.8	4.5	F2	2846 ± 764.2	0.5
	F3	3493 ± 115.8	0.0	F3	3073 ± 379.1	10.7
NPK only	TPH	6312 ± 170.3	0.0	TPH	5746 ± 1153.8	8.8
	F2	3136 ± 171.3	0.0	F2	3063 ± 920.9	0.0
	F3	3176 ± 93.4	7.7	F3	2683 ± 360.7	22.1
NPK + 4% peat moss	TPH	5178 ± 82.2	17.8	TPH	5111 ± 354.4	18.9
	F2	2446 ± 230.7	14.4	F2	2938 ± 215.8	0.0
	F3	2603 ± 64.8	24.4	F3	2173 ± 149.8	36.9
NPK + 5% zeolite	TPH	5849 ± 465.5	7.2	TPH	5119 ± 668.5	18.7
	F2	2847 ± 404.8	0.4	F2	2847 ± 539.5	0.4
	F3	3002 ± 127.4	12.8	F3	2272 ± 193.2	34.0

NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®).

Table E.10. Correlation matrix of freezing parameters of freezing-depression point (FPD), soil phase transition rate, $\Delta\theta$ at FPD, and phase transition duration with F3 removal and α value.

Freezing parameter	F3 removal (D0-D42)	F3 removal (D42-D142)	F3 removal (D0-D142)	α value
Freezing-point depression (FPD)	0.97	0.06	-0.80	-0.53
Soil phase transition rate	-0.76	-0.62	-0.89	-0.98
$\Delta\theta$ at FPD	-0.71	-0.81	-0.92	-0.98
Phase transition duration	0.83	0.54	0.92	0.96

SECTION E.7.0: MICROBIAL ANALYSES

Section E.7.1. Bacterial Community Composition

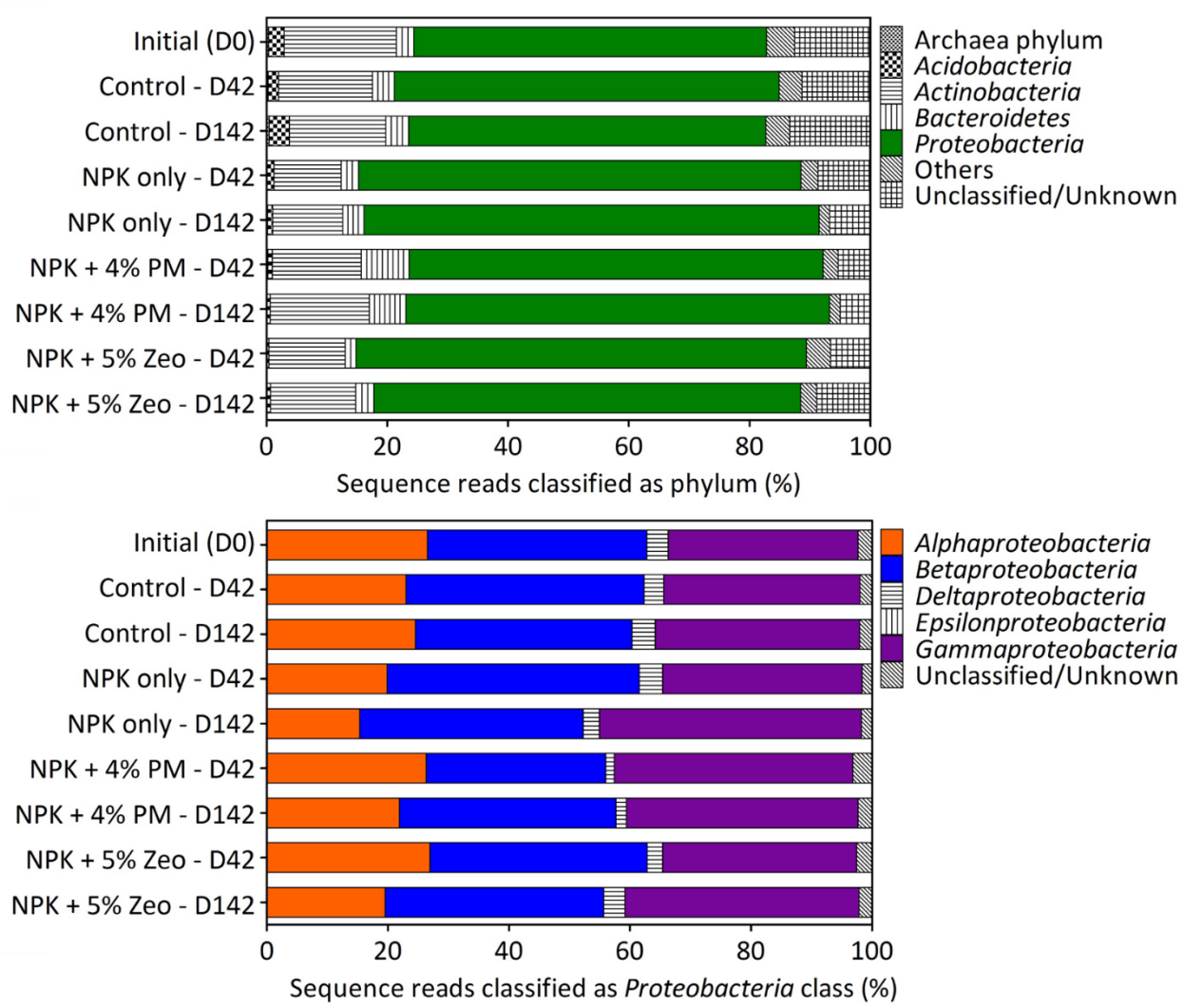

Figure E.9. Community composition at the phylum and *Proteobacteria* class level. NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®), PM: peat moss, Zeo: zeolite.

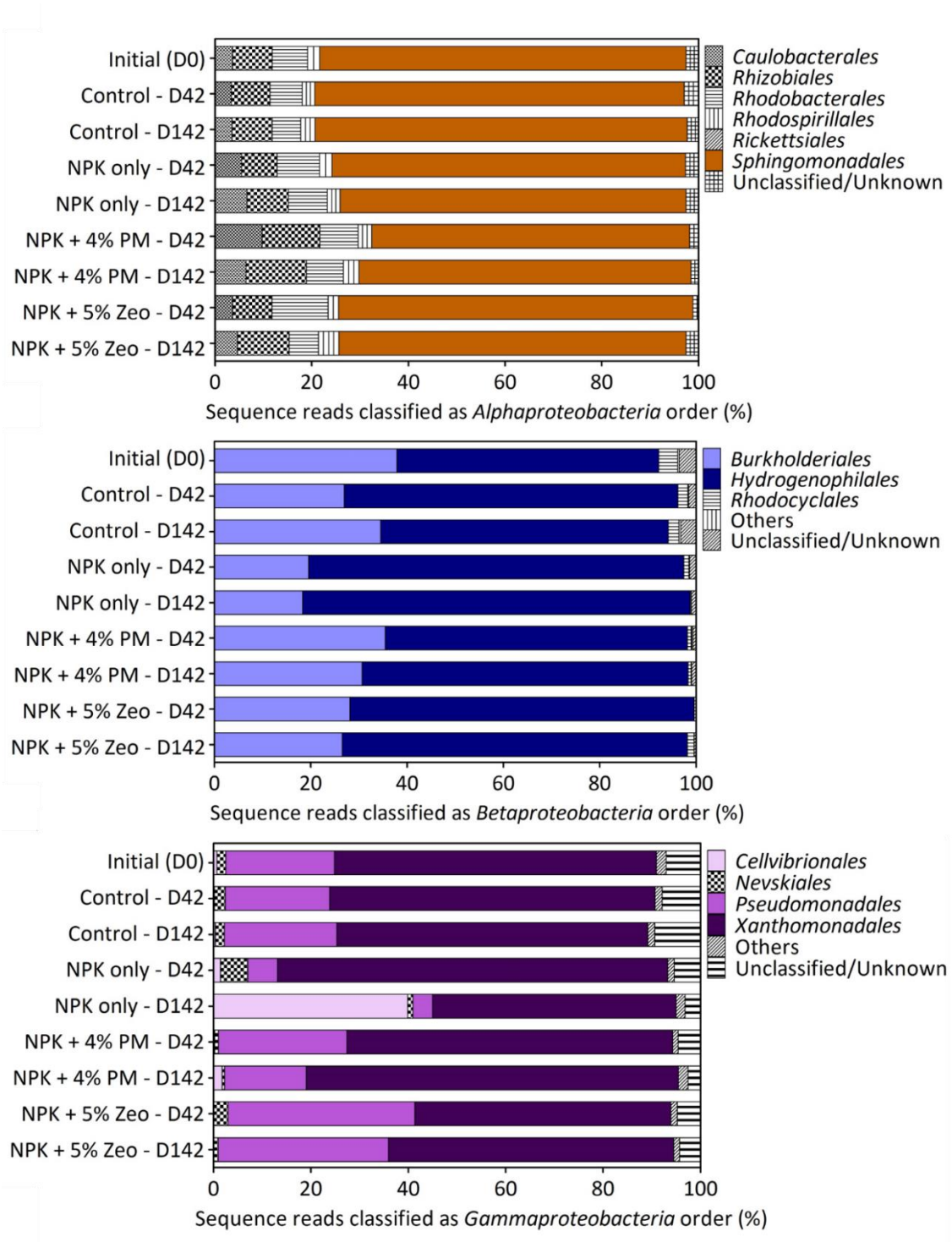

Figure E.10. Community composition at the *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* order level. NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®), PM: peat moss, Zeo: zeolite.

Section E.7.2. Top 20 genera and Principal Coordinate Analysis (PCoA)

The top 20 genera and PCoA plot based on a total of 696,753 effective sequence reads were obtained from all soil samples via high-throughput sequencing using *Illumina* Miseq. The number of operational taxonomic units (OTUs) in all soil samples ranged from 218 to 2,048 out of 2,546. The most plentiful 16S rRNA gene diversity was demonstrated in the initial untreated site soil (D0, 2048 OTUs). The lowest diversity of microbial community was observed in the site soil amended with the nutrient and the zeolite at the phase change (D42, from 4 to −5 °C, 218 OTUs). The highest (153,491) and lowest (61,725) number of sequences were respectively observed in the initial soil (D0) and the nutrient with peat moss-treated soil at the freezing phase (D42, from 4 to −5 °C). The top 20 genera in each sample occupied at least 52.7% of the total number of sequences. The PCoA plot was analyzed in the matrix of the weighted UniFrac distances using the Bray-Curtis similarity to reflect the large variations in bacterial diversity.

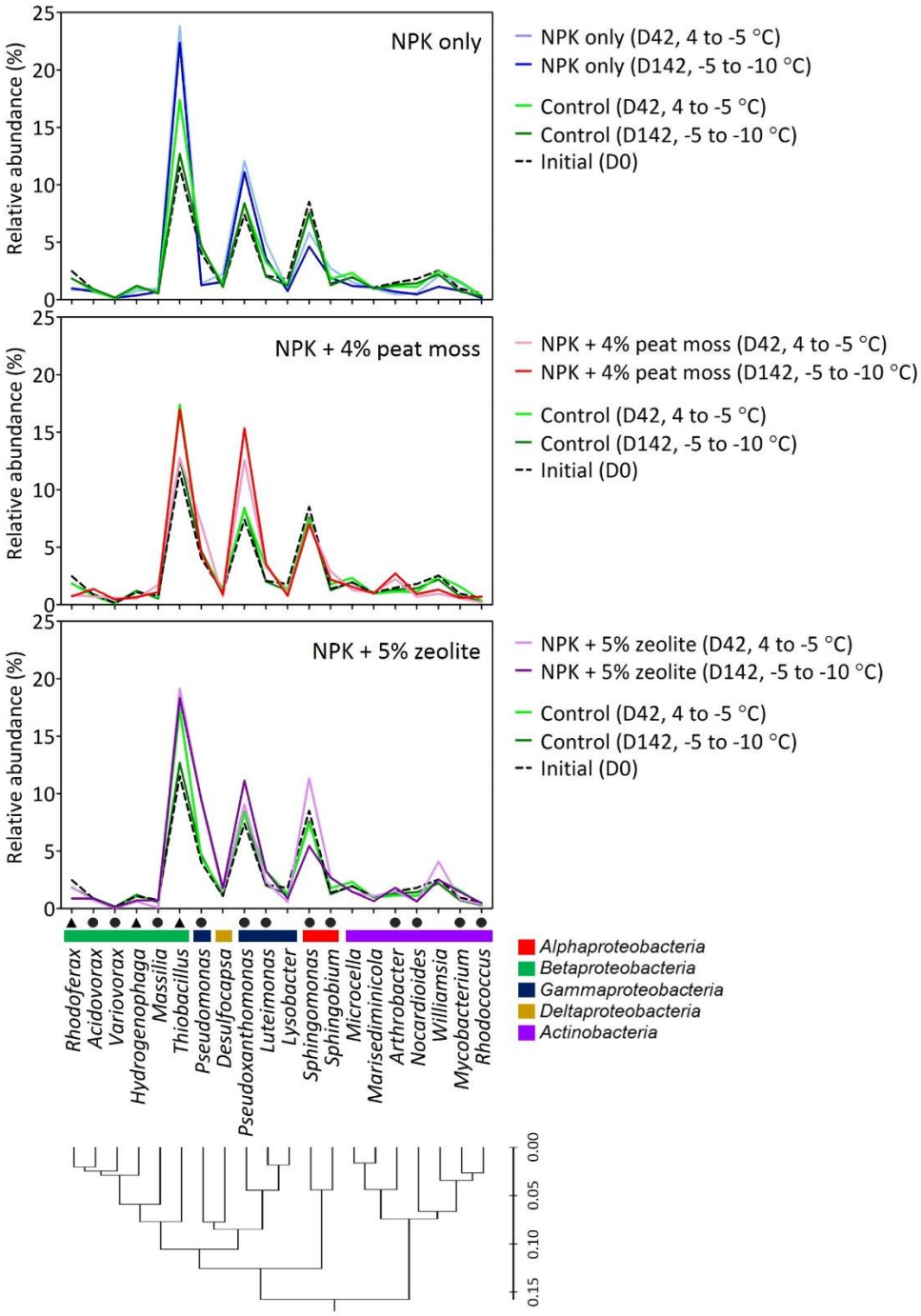

Figure E.11. Relative abundances (%) of the top 20 genera from the gDNA samples corresponding with the soil phases. Genera that include reported hydrocarbon-degrading species are marked using black dots. Genera that include bacterial species previously identified in hydrocarbon-contaminated environments are marked using black triangles. NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®).

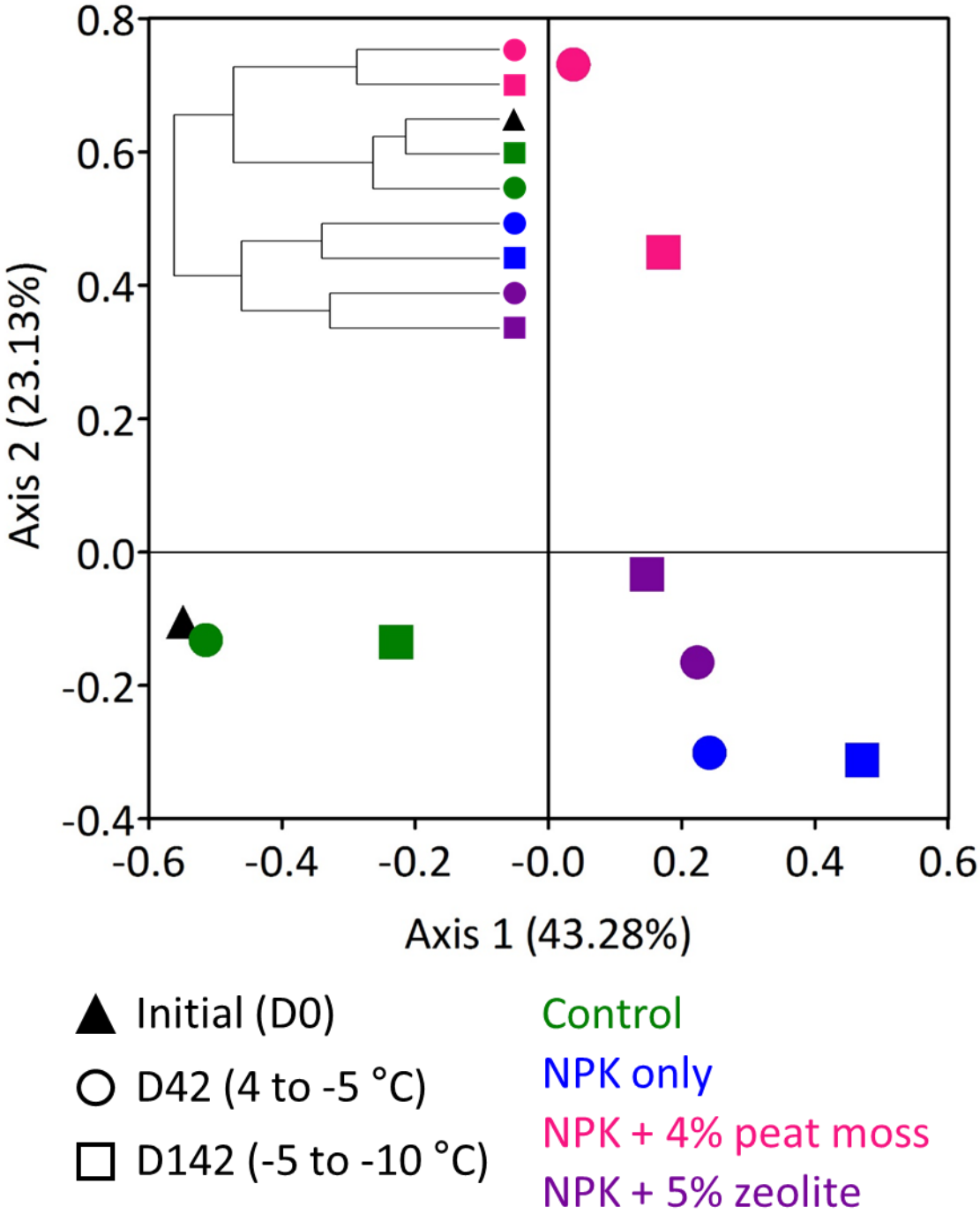

Figure E.12. Principal coordinate analysis (PCoA) plot of Illumina sequence data of soil bacterial communities assessed from the UniFrac distances. NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®).

Section E.7.3. Viable hydrocarbon degraders, CO_2 production, and F3 removal

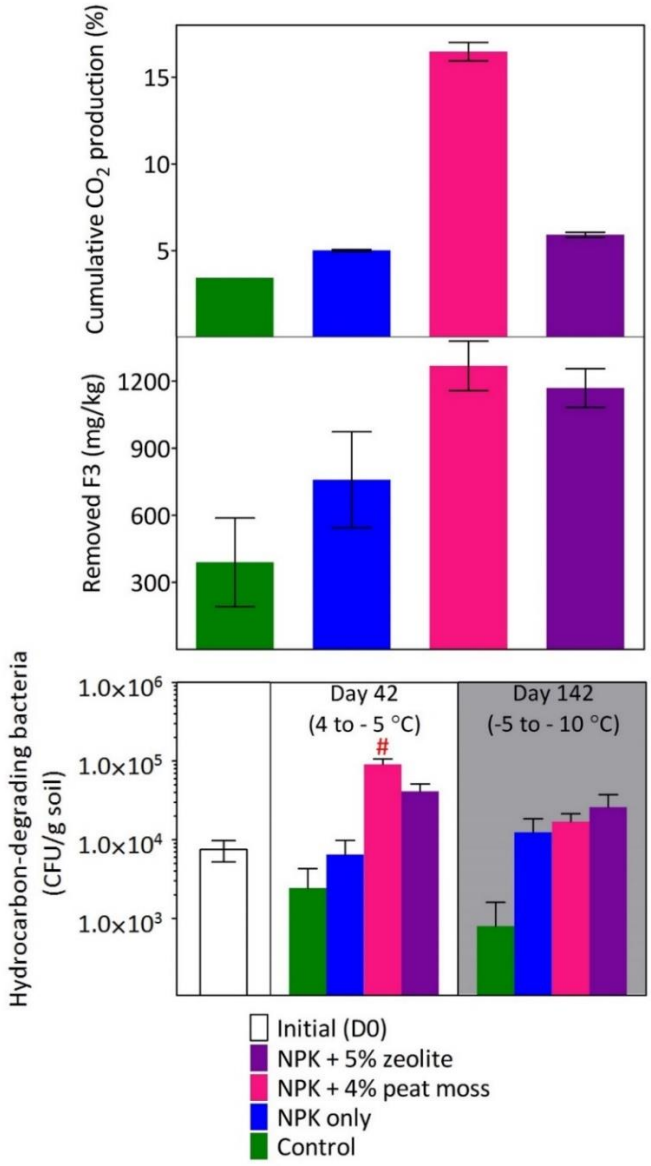

Figure E.13. Cumulative CO_2 production (%) correlated with F3 removal on D142 (Pearson r: 0.74) and the viability of hydrocarbon-degrading bacteria using diesel as a sole carbon source at 4 °C (n = 3, SEM, #: $p < 0.05$, Bonferroni's comparison test, compared to the initial soil (D0)). Despite the second-largest F3 removal in the nutrient with zeolite-treated soil, the cumulative CO_2 production in the nutrient with zeolite-treated soil was similar to the nutrient-treated soil. This might be because produced CO_2 could have been captured in frozen pores and not effectively measured. NPK: N-P-K based 20:20:20 fertilizer (20% total N: 20% P_2O_5: 20% K_2O; Plant Prod®).

E. References

Kim, J.; Lee, A. H.; Chang, W., Enhanced bioremediation of nutrient-amended, petroleum hydrocarbon-contaminated soils over a cold-climate winter: The rate and extent of hydrocarbon biodegradation and microbial response in a pilot-scale biopile subjected to natural seasonal freeze-thaw temperatures. *Sci. Total Environ.* **2018**, 612: 903-913.

Paul, B.; Dynes, J. J.; Chang, W., Modified zeolite adsorbents for the remediation of potash brine-impacted groundwater: Built-in dual functions for desalination and pH neutralization. *Desalination* **2017**, 419: 141-151.

Zhang, L.; Ma, W.; Yang, C., Pore water pressure changes of supercooling and ice nucleation stages during freezing point testing. *Geotech. Lett.* **2015**, 5 (1): 39-42.

Chang, W.; Klemm, S.; Beaulieu, C.; Hawari, J.; Whyte, L.; Ghoshal, S., Petroleum hydrocarbon biodegradation under seasonal freeze−thaw soil temperature regimes in contaminated soils from a sub-Arctic Site. *Environ. Sci. Technol.* **2011**, 45 (3): 1061-1066.

Grechishchev, S.; Instanes, A.; Sheshin, J.; Pavlov, A.; Grechishcheva, O., Laboratory investigation of the freezing point of oil-polluted soils. *Cold Reg. Sci. Technol.* **2001**, 32 (2-3): 183-189.

Wu, M.; Tan, X.; Huang, J.; Wu, J.; Jansson, P.-E., Solute and water effects on soil freezing characteristics based on laboratory experiments. *Cold Reg. Sci. Technol.* **2015**, 115: 22-29.

Goetz, J. D.; Price, J. S., Role of morphological structure and layering of Sphagnum and Tomenthypnum mosses on moss productivity and evaporation rates. *Can. J. Soil Sci.* **2015**, 95 (2): 109-124.

Lee, S.-J.; Park, J. H.; Ahn, Y.-T.; Chung, J. W., Comparison of heavy metal adsorption by peat moss and peat moss-derived biochar produced under different carbonization conditions. *Water Air Soil Pollut.* **2015**, 226 (2): 9.

Ren, J.; Vanapalli, S. K.; Han, Z., Soil freezing process and different expressions for the soil-freezing characteristic curve. *Sci. Cold. Arid. Reg.* 2017**,** 9 (3): 221-228.