Developing and assessing efficient hydrocarbon bioremediation strategies for cold-temperature soils and sediments:

Two case studies in northern Canada

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Abstract

Petroleum hydrocarbon contamination is extensive in northern Canada and very damaging to its fragile environment. Bioremediation is a cost-efficient and sustainable approach for onsite treatment at many contaminated sites in the North. The thesis examines the efficacy of bioremediation of petroleum hydrocarbon-contaminated soils by nutrient biostimulation, under different environmental conditions. The first chapter of this thesis investigated the role of temperature (7 ^{o}C, -5 ^{o}C, and freeze-thaw cycles) and biostimulation regimes on the bioremediation of Arctic diesel in a northern soil from the village Kangirsuk in Nunavik (Northern Quebec). Several metrics were used to study these trends in a microcosm study: hydrocarbon levels, alkane monooxygenase (alkB) and 16S rRNA gene and transcript abundance (qPCR), the community structure (16S rRNA gene and transcript sequencing), and several metrics derived from the 16S sequencing data such as α-diversity (Faith's phylogenic diversity) and representative metabolic pathways (PiCrust2). The key conclusions derived from all these analyses were as follows: Whilst degradation extents in that timescale were small at constantly frozen conditions, some indicators (qPCR, PiCrust2) showed bacteria capable of degrading diesel were appearing. Both the freeze-thaw and the 7 ^{o}C systems had high degradation, but whilst nutrients had a large role in degradation in the summer (7 ^{o}C), the main determinant for bioremediation in the freeze-thaw was concluded to be access to hydrocarbons. *Pseudomonodales* order were overwhelmingly correlated with hydrocarbon degradation. The study also indicates nutrient addition could cause osmotic toxicity and bioremediation treatability studies should follow a plan that maximizes treatability extents whilst minimizing additions to the site. The second chapter of this thesis studied the role of temperature (5 ^{o}C, 0-1 ^{o}C), oxygen, and nutrient addition on bioremediation of F3 hydrocarbons in three northern sediments in a

water management pond for the water treatment plant of a diamond mine in the Northwest Territories. This was studied in a microcosm study where hydrocarbon levels were tracked and related to site microbiology (16S rRNA gene qPCR/sequencing) and physio-chemical properties. The key findings were that degradation slowed at around 700-1000 ppm, that degradation could occur at winter temperatures but much slower than in the summer, and that the nickel and chromium levels across the three sites were correlated with inhibiting hydrocarbon bioremediation and microbial growth. Thus, the thesis analyzed and illustrated some key trends in hydrocarbon bioremediation in two separate and contrasting case studies in northern Canada.

Résumé

La contamination des hydrocarbures pétroliers est large et très endommageant à l'environnement fragile du nord du Canada. La bioremédiation est une approche soutenable et peu couteuse pour le traitement in situ de sites contaminés dans le nord. Cette thèse examine l'efficacité de la bioremédiation de sols contaminés en hydrocarbures pétroliers par biostimulation avec des nutriments, sous différentes conditions environnementales. Le premier chapitre de cette thèse étudie le rôle des températures (7 ^{o}C, -5 ^{o}C, et cycles de gel-dégel) et les types de biostimulations sur la bioremédiation de diesel Arctique dans une terre nordique du village Kangirsuk en Nunavik (Nord du Québec). Plusieurs métriques furent utilisées pour étudier ces tendances dans des études en microcosme : les niveaux d'hydrocarbures, les abondances de gènes et de transcrits des gènes monooxygénase d'alcanes (alkB) et ARNr 16S (qPCR), la structure des communautés microbiennes (sequencage de gènes et transcrits d'ARNr 16S), et plusieurs métriques dérivées du séquençage 16S comme la diversité α (diversité phylogénique Faith) et les chemins métaboliques représentatifs (PiCrust2). Les conclusions primaires dérivées de cette étude sont les suivantes : Les dégradations dans cette échelle de temps étaient petites en condition de gel constant, mais des indicateurs (qPCR, PiCrust2) démontraient néanmoins que des bactéries capables de dégradation de diesel sont apparues. Les systèmes à 7 ^{o}C et en cycle de gel-dégel avaient tous deux de la haute dégradation, mais alors que les nutriments avaient un large rôle à jouer pour la dégradation en été (7 ^{o}C), le déterminant principal pour la dégradation dans la situation de gel-dégel fut conclus d'être l'accès aux hydrocarbures. L'ordre *Pseudomonodales* étaient corrélés à la dégradation d'hydrocarbures. Cette étude indique aussi que l'addition des nutriments pourrait causer de la toxicité osmotique et que les traitements en bioremédiation devraient suivre un plan qui maximise la dégradation mais minimise les additions au site. Le deuxième chapitre de cette thèse étudie le rôle de la température (5 ^{o}C, 0-1 ^{o}C), l'oxygène, et l'addition de nutriments sur la

bioremédiation des hydrocarbures F3 dans trois sédiments nordiques dans un étang de gestion des eaux pour la station de traitement des eaux usées d'une mine de diamant dans les territoires du Nord-Ouest. Ce fut étudié dans une étude en microcosmes ou les niveaux d'hydrocarbures furent mesurés et liés à la microbiologie du site (gène 16S ARNr qPCR/séquençage) et aux propriétés physico-chimiques du sol. Les découvertes principales furent que la dégradation ralentissais autour de 700-1000 ppm, que la dégradation pouvait se passer en hiver mais bien plus lentement qu'en été, et que les niveaux de nickel et chromium dans les trois sites étaient corrélés avec l'inhibition de la bioremédiation des hydrocarbures ainsi que l'inhibition de la croissance microbienne. Ainsi, cette thèse a analysé et illustré des tendances majeures en bioremédiation d'hydrocarbures dans deux études de cas séparés au nord du Canada.

Thesis Introduction

Petroleum hydrocarbons are extensively used in northern remote regions for many key human activities, such as for power generation (e.g., diesel generators) for domestic use, industry, and commercial buildings and transportation. Accidental spills as well as disposal practices have led to hydrocarbon contamination in the environment (1). Toxicity of various hydrocarbon compounds has been demonstrated for a large number of receptors including humans, plants, invertebrates, and microbes (2). Even in remote regions with no permanent settlements, hydrocarbons can cause risks for humans and biota by bioaccumulation in the food chain (3).

There are several methods for hydrocarbon remediation, both in-situ and ex-situ. The destruction methods can be classified in three main categories: physical such as incineration, chemical such as UV oxidation, and biological bioremediation methods (4). The remoteness of arctic regions make on-site treatment desirable, making bioremediation a sustainable and cost-efficient option (1). Contrary to some other organic pollutants, hydrocarbons are naturally found in the environment and therefore several bacterial genera have developed to degrade these compounds by biological processes (5). Hydrocarbon bioremediation by cold-adapted bacteria in these remote northern sites has been demonstrated in many studies, with these cold-adapted bacteria being able to degrade hydrocarbons during the relatively cold temperatures of the arctic summers, with reports of degradation occurring even at sub-zero temperatures (5-8). The degradation can occur in both aerobic conditions and anaerobic conditions and has been shown to occur for both aromatic and aliphatic components of petroleum products (9-13).

Whilst this may be the case, there are several factors limiting the rates and extents of hydrocarbon bioremediation in the arctic. These include cold temperatures, nutrient availability, and soil moisture (1).

Some tools have been developed to bridge these limitations. For instance, biostimulation through moisture and/or nutrient amendment has been successful in enhancing hydrocarbon degradation rates and extents in many cases (14-17). Bioaugmentation which is adding microbes to the site has also been conducted and been successful in some cases (18). Whilst these tools exist, there are risks associated with implementing these strategies in the environment without careful restraint. For biostimulation, adding too much fertilizer to a soil has been shown in several cases to cause inhibition of biodegradation (14, 19, 20), and more generally can adversely affect soil properties and lead to eutrophication in water bodies through runoff (21). Bioaugmentation may also lead to unpredictable impacts and risks disturbing soil ecology (22). Thus, a careful balance needs to be reached between maximizing hydrocarbon biodegradation rates and extents, and minimizing the ecological risk associated with onsite treatments. To minimize both costs and environmental impacts of the site remediation requires a deep understanding of the factors determining the success of a bioremediation treatment. Many of the factors at play in limiting/enhancing hydrocarbon bioremediation in the north warrant further understanding. This thesis attempts to explore the role of some of these factors, through two very different biotreatability studies in northern Canada.

My first chapter is on bioremediation of Arctic diesel in a sub-arctic soil in Kangirsuk in Nunavik. The objective of that chapter is to explore the role of temperature, freeze-thaw, nutrient amendment, and moisture on the degradation of hydrocarbons. It attempts to further the understanding of the microbiology and biochemistry at play through analyses of genes and

transcripts of the 16S rRNA and the alkane monooxygenase (alkB) gene, which respectively serve as markers for total bacteria and for alkane degraders.

My second chapter is on bioremediation of F3 (carbon length C16-C34) hydrocarbons in clayey sediments in a water management pond for a diamond mine in the Northwest Territories. The objective of this chapter is to explore the role of nutrients, temperature, and oxygen in the degradation. This was done with three sites in that pond, which differ in depth, physicochemical parameters such as heavy metals, original hydrocarbon concentration, and overall biodegradation behavior. The fact that these sites are directly connected but differ in several key properties enable the investigation of how the tested independent variables are influenced by the differing site parameters.

Both chapters are connected to real contaminated sites in northern Canada. Finding sustainable and cost-efficient strategies is important for the implementation of remediation plans for these sites and to protect their surrounding environment. These chapters also serve to increase the understanding of bioremediation of hydrocarbons in the north, thus providing information to design efficient and sustainable strategies for future site cleanup. Both chapters were written as manuscripts to be submitted to peer-reviewed journals.

Chapter 1: The impact of seasonal temperatures and biostimulation on diesel bioremediation in northern soils: insights gained from multi-approach nucleic acid analyses of the alkB and 16S genes

1. Introduction

Energy access in remote communities in northern Canada remains a challenge. There is currently a high reliance on diesel generators for power supply and transportation in the communities, leading to intensive diesel use and associated spills and leakages leading to many instances of diesel contamination on land (23). Diesel fuel has been associated with several toxic effects in humans, such as renal failure, skin-related diseases, and various mutagenic impacts (2). Diesel contamination also causes many risks for the surrounding ecosystem at large. For instance in the soil ecosystem, it has been found that diesel could lead to nitrogen cycle disruption through bacterial species richness reduction (24), and have deleterious impacts on macro and microbiota (25). The vicinity of northern villages to the coastline also increases risks of seepages into water bodies, posing additional risks to aquatic organisms and up the dependent food chain (26).

Bioremediation of petroleum hydrocarbons in contaminated soils remains an attractive option for hydrocarbon remediation in the north given its low environmental footprint and cost (27). However, nutrient and organic matter of arctic soils can often be low, so biostimulation through nutrient amendment is often necessary to achieve effective bioremediation rates (28). Biostimulation through nutrient amendment has been successful in many cases (14-17). On the contrary, some studies have found no increase or even a small decrease in degradation through biostimulation (14, 19, 20). A meta-analysis of 58 biostimulation studies using soils from

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northern sites showed there was no clear correlation between doses added and degradation enhancements (29). Laboratory biotreatability studies using soils from a sub-Antarctic site conducted by Walworth et al. at 6°C showed that biostimulation by addition of 125 mg/kg of nitrogen reduced total petroleum hydrocarbon (TPH) concentrations from 5250 to 1520 mg/kg after 123 days compared to 2753 mg/kg without nutrient addition. Although a low dose helped, adding more nutrients led to degradation that was still higher than when no nutrients were added, but less degradation than for a small dose. For instance, adding 250 mg/kg led to final values of 2160 mg/kg (19).

Some studies have attributed the lack of enhancement in petroleum biodegradation at higher nutrient doses to osmotic toxicity caused by increasing soil salinity (14, 19). Increases in salinity have been associated with lower microbial growth and inhibition of microbial degradation of hydrocarbons in soil (30). Increases in salinity and/or water potential were for instance found to decrease soil respiration and microbial biomass (31), to reduce amino acid uptake and protein synthesis (32), and the lowered osmotic potential caused by salinity was found to lead bacteria to synthetize osmolytes to protect themselves, adding a metabolic burden taking away energy that could be used for growth (33).

The majority of cold-region soils bioremediation activity is targeted for the 2-4 summer months (34) when temperatures are consistently above freezing, but the length of the summer is short, and reaching acceptable pollutant level the earliest possible to minimize downstream and long-lasting contamination is preferred. Studies have shown that cold-adapted bacteria are still metabolically active at sub-zero temperatures (35), and petroleum treatability studies have been conducted at sub-zero temperatures close to freezing point such as would be found in the spring and the fall, with some finding degradation (19-24). In cold regions, fall and spring are

associated with diurnal or several days long temperature oscillations around the freezing-point, leading to recurring freeze-thaw cycles. The frequency of these freeze-thaw cycles can be exacerbated by the increased frequency of warming event observed in the arctic in recent years (36-38). Several studies have been done regarding hydrocarbon degradation in cold weather soils under freeze-thaw, both seasonal freeze-thaw and cyclical freeze thaw (39-41). Some have found an increase in bioremediation extents when thawing regularly as opposed to constantly semi-frozen conditions (39).

Freezing raises aqueous salinity in the available water where biotic activity takes place (42), which may lead to toxicity. Because soil freezing occurs for much of the year in cold regions, adding the same nutrient levels as in the summer could lead to even higher salinities during semi-frozen conditions where bioactivity is viable (42). Freeze-thaw events have implications on microbial community structure (21), soil aggregate structure (29-33) and soil nutrient profiles (27, 38). The impact of temperature on all these soil properties suggests the impact of biostimulation on microbial activity in soils needs to be investigated under different temperature regimes in cold regions soils. Such knowledge could help optimize treatability for the (semi-)frozen months that shoulder the summer.

The objective of this study was to determine the impacts of nutrient addition at different temperature regimes on petroleum degradation, as well as on the total and active microbial communities. An associated objective of the study was to gather a wide array of biochemical information to build theories for the observed differences in petroleum degradation extents.

As such this study does a side-by-side comparison of degradation extents with either no biostimulation, only water addition, or water and a low dose of N, P added. The three systems also provide 3 different levels of salinity. To assess the effect of different temperature regimes,

the experiments were conducted at a constant unfrozen (average summer) temperature at 7°C, a constant semi-frozen (average spring/fall) temperature at -5°C, and a temperature regime which mimicked the instability in the spring and the fall and as spurred by characteristic seasonal or climate warming events, which consisted of 3.5 days at -5°C followed by 14 hours thawing at 7°C. The abundance (qPCR) and expression (RT-qPCR) in all these conditions were tracked for both the 16S rRNA and the alkane monooxygenase (alkB) genes, which were considered population markers for all bacteria and alkane degraders respectively. The microbial community structure was analyzed by sequencing the 16S rRNA gene and transcripts. An attempt to gain an understanding of the large-scale metabolic switches occurring was done by first predicting the key metabolic pathways through phylogeny associated unobserved states prediction (PiCrust), then categorizing marker pathways for each hydrocarbon degrading situation. All this knowledge serves to increase understanding of the underlying factors leading to a cold site's treatability, to lead to an a priori understanding of situations where bioremediation may be successful to inform an adequate treatment methodology.

2. Methods and materials

2.1 Soil characterization

Soil was obtained from an uncontaminated site in Northern Quebec, Canada, in the remote village Kangirsuk (60°01'N 70°01'W). The soil was collected using sterile methods, shipped by Hydro-Québec in containers with sterile liners, and stored at -10 ºC until use. Before use in experiments, soil was sieved with an autoclaved sieve with a mesh size of 4.5 mm to filter out rocks. Soil characteristics are reported in our prior study (43), and are summarized in SI Table 1. Briefly, the total N, total P, total organic carbon, water holding content and original moisture content were 640 mg/kg, 540 mg/kg, 12000 mg/kg, 24.6%, and 6.09% respectively.

2.2 Microcosm Experiments for Total Petroleum Hydrocarbon (TPH) Biodegradation

Treatability experiments were carried out for a period of 52 days, using three different temperature regimes: (i) constant 7°C, representing the summer temperature at the site (June-September); (ii) constant -5°C representing the spring/fall temperatures (April-May, October-November) and (iii) at -5°C, but thawed at 7°C over 14 hours every 4 days, to represent freeze-thaw conditions. Additionally, the systems DW and DWNP at -5°C were also sampled at 80 and 100 days, as these systems were degrading slowly, and more time was needed to assess the impact of the nutrients. The temperatures were chosen by surveying the temperature data from the last 4 years from Environment Canada, which can be seen plotted as SI Figure 1. All glassware and soil handling tools used for setting up the microcosms were cleaned by acid wash followed by deionized water rinse, and autoclaved. For all systems, 10 g of soil (dry weight) were added to 120 mL serum vials and closed with PTFE-lined silicone septa crimped with aluminum caps. All system amendments are found in Table 1. The systems were opened briefly on a weekly basis in a biosafety cabinet to allow for oxygen replenishment.

Table 1: Microcosm set up in treatability experiment.

System name	Arctic Diesel addition (per dry weight)	Water addition	Nutrient addition
D: Diesel added	2500 mg/kg	No water addition	No nutrient addition
DW: Diesel and water added		70% of water holding capacity	
DWNP: Diesel water and N&P added			100:2:1 C:N:P molar ratio
U: Unamended	No diesel addition	No water addition	No nutrient addition
W: Water added		70% of water holding capacity	
WNP: Water and N&P added			100:2:1 C:N:P molar ratio

For molar ratios, C in diesel was approximated using hexadecane as a reference compound. N was added as NH_4NO_3, P as 1:1 molar ratio of KH_2PO_4 and K_2HPO_4.

At day 0, 24, 36 and 52 and additionally 80 and 100 for -5°C DW and DWNP, 1.4 g were removed from the microcosms and TPH was extracted and quantified from the remaining soil sacrificially from at least 3 replicates using methods reported in our prior studies (43). Full details of the extraction and the Gas-chromatography flame ionization detector (GC-FID) parameters can be found in the SI.

2.3 RNA/DNA co-extractions and cDNA synthesis

RNA/DNA co-extractions were done from triplicate microcosms at day 12 and 36 of the microcosm experiments using the 1.4 g of soil removed prior to TPH extraction. For the freeze-thaw systems, the extraction occurred after having thawed at 7°C overnight. The extraction was performed in a biosafety cabinet using the NucleoBond® RNA Soil extraction kit along with the DNA Set for NucleoBond® RNA Soil (Macherey-Nagel.) Then, a one-pot DNASE1 and reverse-transcriptase reaction was done on a 14 µL aliquot of the RNA using iScript gDNA Clear cDNA synthesis Kit (BioRad). All nucleic acids were kept at -80°C until further analyses.

2.4 qPCR/RT-qPCR quantification of AlkB and 16S genes and transcripts

Standards for both genes were synthetized by PCR-purification of full genes from site environmental DNA, with the primer combination Ba27F and Ba1492R (44) for 16S and the combination alkB-1r and alkB-1f for alkB (45, 46). The PCR products were gel purified using QiAquick gel extraction kit (Qiagen), and the copy numbers in the standards were quantified by using the PicoGreen method. For the qPCR analysis, a sub-section of the gene was quantified for the 16S gene using the primer set Ba519F and Ba907R (47), whilst for alkB the same primer

set as for the standard was used (45, 46). All reactions were done using a CFX connect real-time PCR detection system (BioRad). The specificity of each reaction was verified after each run through a melting curve analysis between 65 °C and 95 °C. No template and no reverse-transcriptase controls were run. The efficiency of the 16S reaction was 92%. For alkB it was 85%, a value previously published for these primers (46). For alkB the quantification was either done with 1 µL of undiluted DNA/cDNA or the standard at concentrations 10^{0}-10^{-7}. For 16S the quantification was either done with 1 µL of 100-fold diluted DNA/cDNA or the standard at concentrations 10^{0}-10^{-5}. All sample quantifications were done using biological triplicates and analytical duplicates. The primer sequences thermocycling conditions and mastermix compositions can be found in the SI. Measuring the levels of the genes in DNA gives an estimate of the potential of these genes. Measuring them in cDNA gives a measure of the actual activity of the genes. In terms of 16S rRNA, DNA-based measurements give an approximate measure of cellular density, whilst cDNA-based measurements give a measure of soil metabolic activity. For alkB, DNA-based measurements give a measure of the potential of the microbial system to degrade alkanes, whilst cDNA-based measurements give a measure of the alkane-degrading activity. AlkB is not the only gene responsible for hydrocarbon degradation in diesel, as there are other components in diesel beyond alkanes. But alkanes are very abundant in diesel and given that we are investigating some systems where little degradation is expected to occur (e.g. -5°C), alkB was picked as a marker as it would be abundant and as such would be expressed above limits of detection (LOD) for many scenarios, thus allowing comparisons to be made.

2.5 Sequencing

For sequencing, the DNA and cDNA were sent to the Genomic analysis platform of Laval University in Quebec City Canada. For all samples, the V3- V4 region of the 16S gene was amplified using primers Bact341F (3'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTCC

TACGGGNGGCWGCAG-5') and Bact805R (3'-GTGACTGGAGTTCAGACGTGTGCTCTTC CGATCTGACTACHVGGGTATCTAATCC-5'). The amplified sequences were sequenced on an Illumina MiSeq. The reads have been submitted to the SRA database under PRJNA946730.

2.6 Bioinformatic analyses

Reads were analysed using qiime2 (48). Reads were trimmed at 240bp both forwards and backwards, denoising was done using dada2, and classified using a naïve Bayes classifier trained against the full 16 gene Greengenes database. Diversity metrics were run using a sequencing depth of 1100, which contained 46.34% of the sequences and 98.63% of the samples. A PiCrust2 analysis was done on the 16S cDNA data (49-54). To identify functional markers, the following approach was taken: pathways relative abundances for systems D, DW, and DWNP were divided by the U value for equivalent temperature regimes and timesteps. Pathways were identified as degradative environment markers if elevated by a minimum of 2 in diesel-containing systems, and with a P-value under 0.05 between their values and values for the U systems. The pathway markers were then identified on MetaCyc (55). Pathways plotted P-values and fold-changes for this analysis are in SI Table 2-4.

3. Results and discussion

All statistical analyses in this section refer to the Student's T-test.

3.1 Dynamics of the 16S and alkB genes

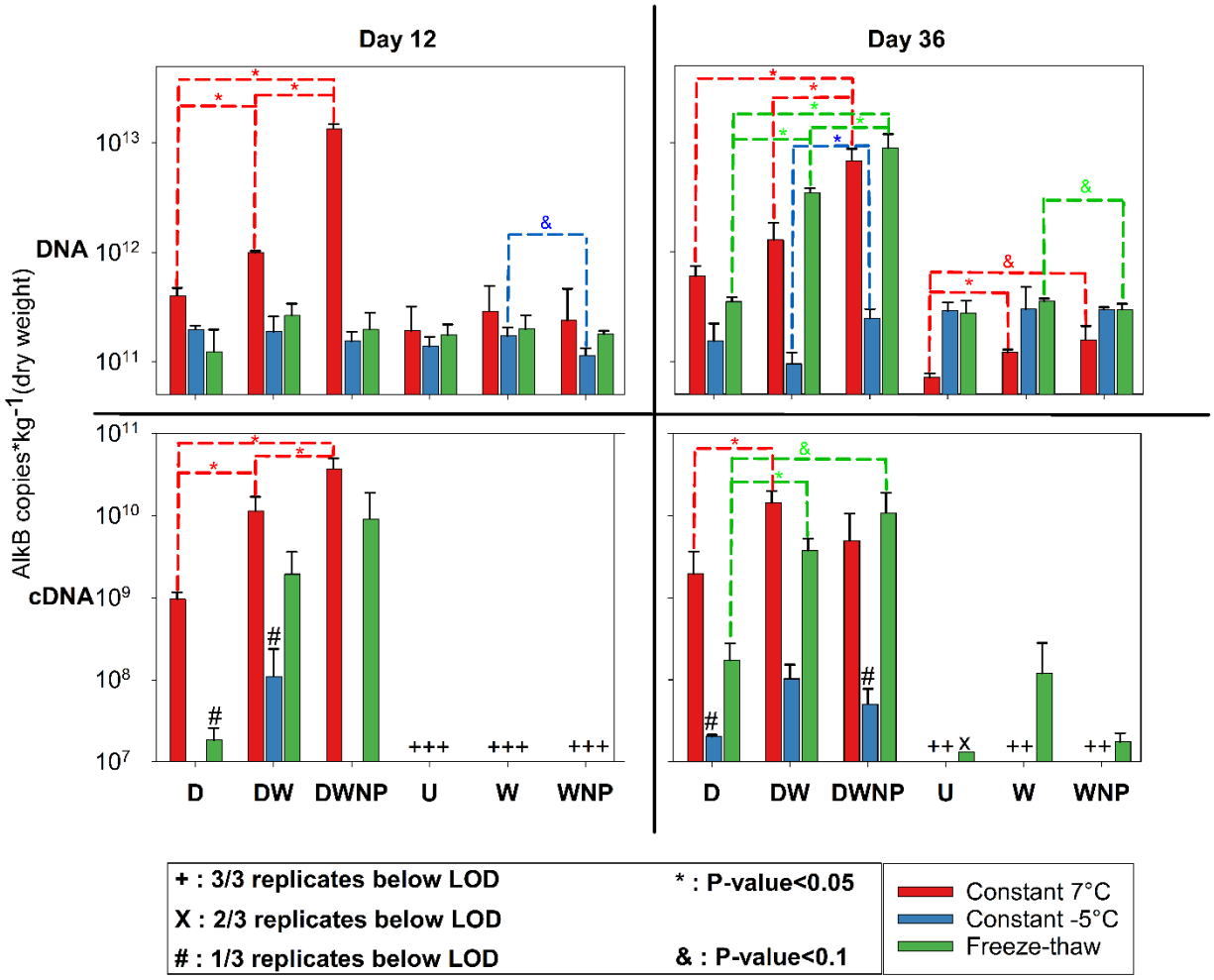


Figure 1: **AlkB abundance and transcripts across biostimulations and temperature patterns.** Average values for copy numbers are plotted on a logarithmic scale. LOD refers to limit of detection.

Nutrients and water impacted alkB cDNA and DNA levels at 7°C, while water was the key factor for freeze-thaw systems. The cDNA levels at -5°C were close to the limit of detection (LOD) but increased between day 12 and 36.

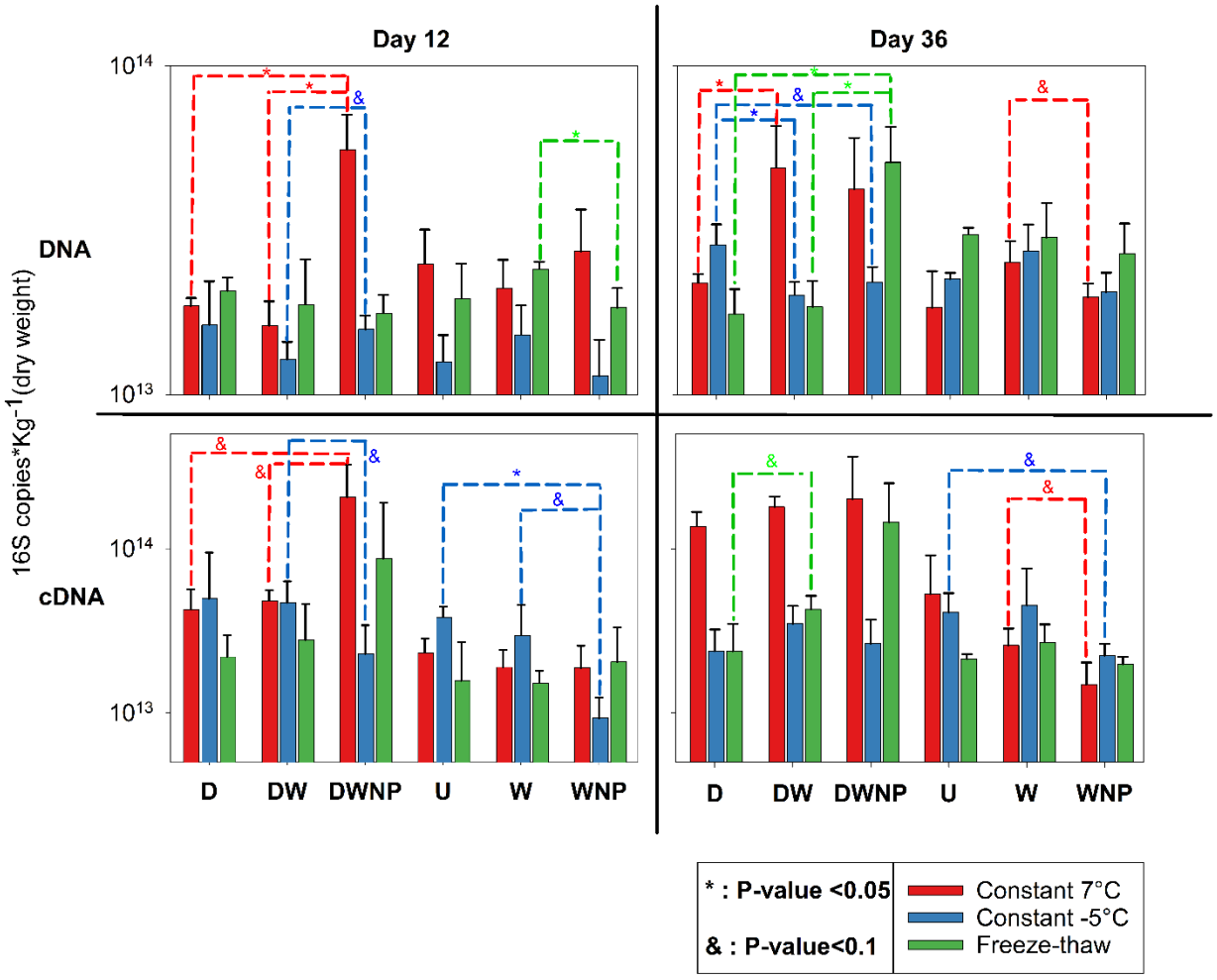


Figure 2: 16S abundance and transcripts across biostimulations and temperature patterns. Average values for copy numbers are plotted on a logarithmic scale.

For 16S, nutrients had a large impact in increasing levels of genes and transcripts in both the freeze-thaw and the constant 7°C systems. The nutrients surprisingly seemed to decrease the levels of the transcripts (cDNA) at -5°C. The temperature being constantly -5°C did not dramatically impact 16S levels and the overall range of values was small. Having an overall range of value being smaller for 16S than alkB is expected, given that 16S is universal and as such would be less disturbed by stressors.

3.2 Hydrocarbon Degradation Patterns

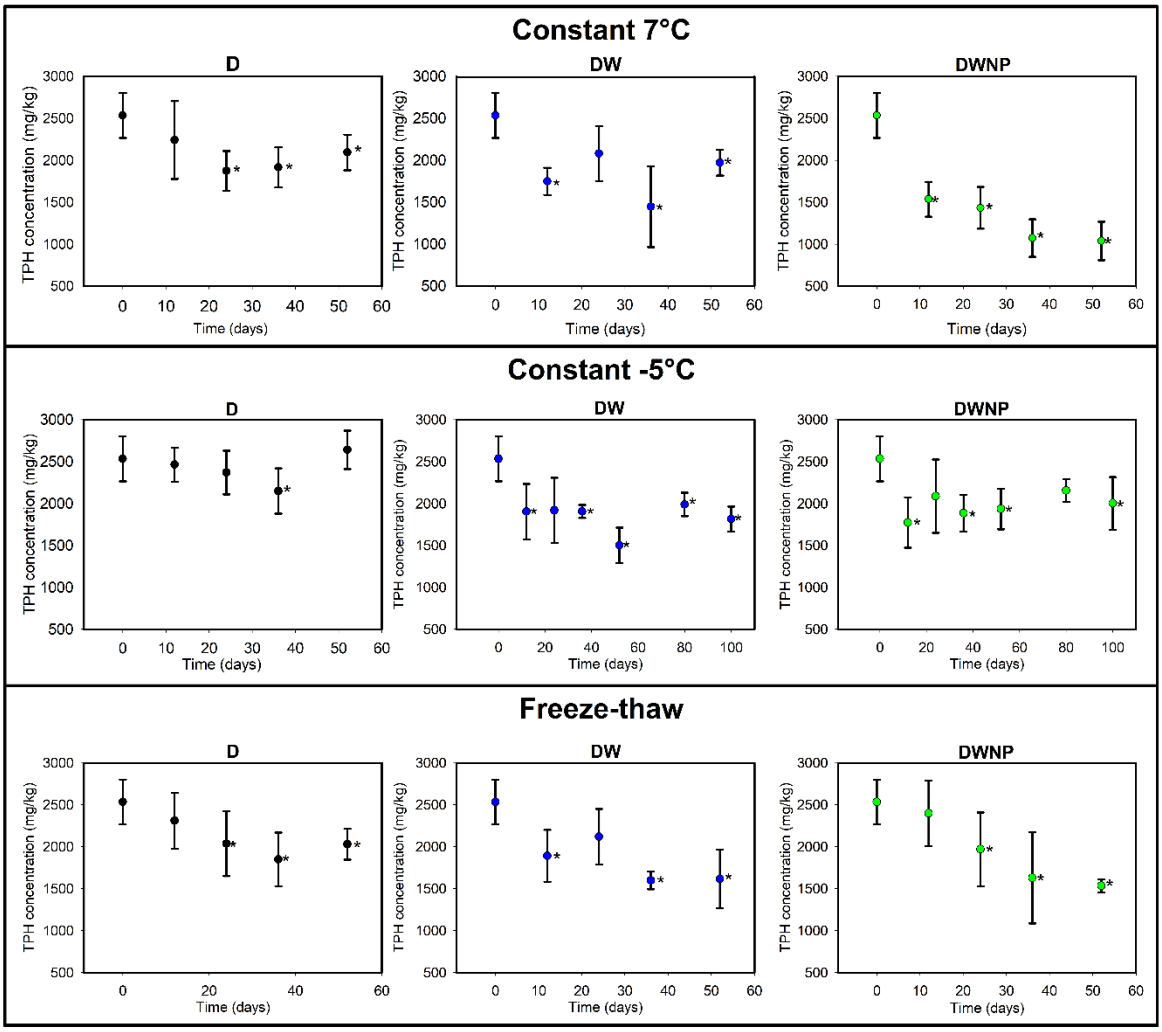


Figure 3: Hydrocarbon biodegradation extents. The averages were plotted against time with error bars ± standard deviation. The * symbol denotes a statistically significant decrease from day 0. Values are considered statistically significant for a $P<0.05$.

For all 3 temperature regimes and amendments, statistically significant decreases in TPH concentrations were observed by the end of the experiments (day 52), except at a constant -5°C degree for D in which degradation was limited. Except for that system, statistically significant TPH degradation was observed by day 24.

The results agree with some previously published trends, namely that nutrient biostimulation (43) and regular thaw (39) can help bioremediation.

3.3 Nutrients are helpful at the start of diesel metabolism at constant 7°C

Nutrients had a high impact early in the study for DWNP at a constant 7°C, but a more limited impact as time went on. Hydrocarbon degradation extents (Figure 3) showed that adding N&P resulted in a large statistically significant increase in degradation between final values for DWNP and the other systems (DW, D). Across the data set the lowest values for D DW and DWNP were 1873 ± 237 mg/kg (day 24), 1445 ± 481 mg/kg (day 36), and 1037 ± 229 mg/kg (day 52) respectively. But the large impact of nutrient addition is mostly seen in the beginning, with 67% of the total degradation for DWNP occurring in the first 12 days followed by a plateau. The impact of nutrient being primarily at the start is also seen in the alkB data (Figure 1). For both alkB DNA and cDNA at day 12, all differences in diesel-amended systems are significant, with values DWNP>DW>D. At day 36, for DNA, the levels for DWNP are still elevated compared to DW, but the difference between DW and D is no longer significant. In contrast, for cDNA at day 36, DWNP is no longer elevated compared to either other diesel-containing amendment. This suggests that in our study, cDNA based alkB trends offer an instantaneous view of the degradation rates whilst DNA based trends offer a long-term view of the degradation extents. Indeed, the instantaneous rates at day 36 for DWNP and thus the alkB activity (cDNA) is similar than for the other diesel-amended system, given most of the DWNP degradation occurred in the first 12 days. The disparity between the level of alkB in the two nucleic acids in DWNP relative to DW and D when degradation has started plateauing in DWNP may be due to the larger environmental persistence of DNA, or perhaps the following: A lot of degradation of diesel has already occurred, producing many metabolites. This in turn would lead to increased overall activity, increasing both alternative carbon sources and dead bacteria accumulating in SOM necromass. It is possible that hydrocarbon degraders that were originally stimulated by the

alkanes in the diesel are still getting sustained by other carbon sources but are not actively degrading alkanes. They would be captured in alkB DNA but not alkB cDNA.

Trends in 16S offer similar but slightly contrasting trends (Figure 2). At day 12 in DNA, DWNP values are statistically higher than for D and DW. For cDNA they are also higher and would be statistically significant using 0.1 as a threshold, with P for D-DW and D-DWNP being 0.077 and 0.083 respectively. At day 36, DWNP is elevated in neither DNA nor cDNA. While for both genes the difference between DWNP and other diesel-containing systems decreases over time in both cDNA and DNA, the reasons for that change differ. For alkB, DWNP levels decrease significantly between 12 and 36 days and the other diesel containing-systems do not change. For 16S, D and DW statistically increase over time and DWNP does not change. Overall soil metabolism as captured by 16S peaked at the carrying capacity of the system after 12 days for DWNP, and after 36 days the system is still operating at that same capacity. This suggests there are still enough nutrients around for the cell to be operating at the same capacity after 36 days, it is specifically alkB harbouring-bacteria that decrease over time in the DWNP systems. This could be due to several reasons such as a lower bioavailability of the remaining contaminants. After the most degradable hydrocarbons have been taken up, the energy costs for the degraders to degrade remaining hydrocarbons may place them at a competitive disadvantage for other nutrient sources, for example N&P.

3.4 Adding nutrients may inhibit degradation under constant semi- frozen conditions (-5°C)

The result of this study suggests that adding nutrients may have inhibited degradation as well as microbial activity at -5°C. This is first seen for hydrocarbon degradation trends (Figure 3). The only statistically significant difference for hydrocarbon values at day 52 was between the

systems D (2640 ± 229 mg/kg), and the systems DW (1502 ± 210 mg/kg). For day 80 and 100 which followed, we may see that day 80 was a statistically significant from day 0 for DW (P=0.0144) and not for DWNP (P=0.0594). The values for day 100 were both statistically significant decreases from day 0, but the value for DW (1816 ± 147 mg/kg) was lower than DWNP (2002 ± 313 mg/kg). Indeed, it appears there was more degradation in the DW systems than in the DWNP systems.

The gene analysis reflects this even further, for both alkB (Figure 1) and 16S (Figure 2). For alkB, at Day 12, while there are no statistically differences between amendment in alkB DNA, for alkB cDNA the only levels above LOD are for DW. At day 36, alkB cDNA can be seen above LOD for all 3 diesel-amended systems, suggesting that alkB activity is occurring under constant semi-frozen conditions but may be slower to appear. Still, the only amendment for alkB cDNA that has all three replicates above LOD is DW. Surprisingly at day 36 in alkB DNA, the levels are statistically higher for DWNP than DW (P=0.011). That is not necessarily surprising, given that cDNA Is more sensitive than DNA. Under low degradation and activity of the alkB gene (alkB cDNA), bacteria able to degrade alkB (alkB DNA) may still subsist degrading other sources, which may lead to unexpected trends comparing the two nucleic acids.

For 16S, similar trends can be seen in both diesel containing systems and controls. The 16S gene is universal and is as such less sensitive to changes, as we can see by the smaller range in values. As such, this is best captured in cDNA which is more sensitive. Similarly, the trend is most accurate to analyze at day 12, where added nutrients are expected to be higher than day 36. In cDNA day 12 the 16S levels are higher in DW than DWNP (P=0.1), and in W than WNP (P=0.095).

The results suggest a decrease in hydrocarbon degrading activity and overall metabolism when nutrients are added at constant -5°C. The salinity of salt in the unfrozen water in the constantly semi-frozen systems (-5°C) is expected to be larger than at 7°C, and as such it is likely that there is more osmotic toxicity of adding nutrients at -5°C. Osmotic toxicity of salt on bacteria has been reported before (31-33).

3.5 The importance of regular thaw for hydrocarbon biodegradation

Regular thaw has a large impact in this study. Although the systems in the freeze-thaw are kept at the same temperature as the constant -5°C systems for 87.5% of the time, the results between -5°C and freeze-thaw are different in terms of both hydrocarbon degradation extents (Figure 3) and genes and transcript abundances and activity (Figure 1-2). The results also suggest that the impact of thawing is larger on alkane degraders than bacteria generally. The impact of nutrients and the extents also differ between the freeze-thaw and the 7°C systems, suggesting a truly unique degradation scenario under freeze-thaw.

A first trend that showcases the different impacts of nutrients at 7°C and in the freeze-thaw, is the difference in biodegradation kinetics for DWNP, with second-order kinetics for 7°C (R^2=0.91, k=1×10^{-5} $g^{-1}day^{-1}$, $t_{1/2}$= 39.45 days) and pseudo-zero order kinetics for the freeze-thaw (R^2=0.94, k=21.37mg·day^{-1}, $t_{1/2}$=59.29 days). A pseudo-zero order kinetics occurs when the rate is independent of reactants, which we may simplify for our systems as being the carbon source the nutrients and the bacteria. Illustrating this, whilst adding water has a clear impact on the hydrocarbon degradation extents for freeze-thaw, nutrient addition has no statistically significant impact, with final hydrocarbon degradation extents for D DW and DWNP being 2029 ± 78.4 mg/kg 1616 ± 352 mg/kg and 1534 ± 78.4 mg/kg respectively. This suggests that adding water under freeze-thaw is important and necessary, but once that is done for both DW and DWNP the

degradation is likely primarily controlled by the thawing. This may be for instance be due to a physical limitation caused by the freezing (35) or reduced metabolism under cold (56).

Reduced metabolism under cold is unlikely to be the only reason, as there is clear and visible metabolic activity at a constant -5°C, as captured by 16S cDNA (Figure 2). This suggests that 0°C is not a primary limit to metabolism in these systems, which has been found in previous studies (35). Indeed, having a constantly semi-frozen structure seems to be having a larger impact on alkane degraders than bacteria generally, as can be seen by looking at the impact of regular thaw on alkB and on 16S levels. For instance, for DWNP, the ratios $\frac{\text{Freeze}-\text{thaw}}{\text{Constant}-5°\text{C}}$ for levels of alkB and 16S respectively are 36.1 and 2.32 for DNA, and 213 and 5.46 for cDNA. This suggests there is a greater influence of freezing on alkane than on the general bacterial population.

Adding nutrients did not change the hydrocarbon degradation extents under freeze-thaw but had some influence on the presence and activity of alkB in diesel-containing systems. Indeed, for DNA at day 36 the differences are all statistically significant with values DWNP>DW>D. In cDNA, the levels were again DWNP>DW>D, but the only significant difference was from DW to W. Small non statistically significant differences in the thawed period could have led to accumulation of more bacteria in DWNP which may explain that there is more alkB DNA for DWNP than DW at 36 days. Given that nutrients heavily impact thawed behavior and the overall degradation extents do not differ when adding nutrients under freeze-thaw, this suggests that thawed behavior does not dominate the freeze-thaw degradation. As such there must be significant degradation occurring when at -5°C.

Every time the systems were thawed and enhanced access to hydrocarbons occurred, quick alkB activity followed. This can for instance be seen in Figure 1 when there are are few alkB-harbourers (DNA) at day 12 in diesel-containing systems and there is still large activity (cDNA). The levels of alkB in DNA for freeze-thaw go up between day 12 and 36, which may be due to spikes in alkB metabolic activity (cDNA) increasing levels of degraders (DNA) over time. Periods of activity increasing abundance of bacteria over time could be due to many reasons. For instance, formation of a stable bacterial community, or solubilization of diesel during thawed period leading to higher access in the frozen periods. Bacteria may also be killed every freezing event leading to accumulation of DNA over time which is more persistent than RNA so would have a higher tendency to accumulate.

A possibility the data suggests, is having consecutive freeze-thaw cycles helps circumvent some physical limitations placed on the constantly semi-frozen systems (‘constant -5°C’). Most biotic hydrocarbon-degrading activity at -5°C is assumed to take place in the unfrozen water films surrounding soil aggregates, where bacterial contact with the diesel enables its solubilization and consumption. At a constant -5°C, the same contacts of diesel pools with unfrozen water films are maintained through the experiment, which may be seen as a limitation. In the freeze-thaw, every freeze-thaw event changes these contacts and exposes new diesel pools and may also redistribute pockets of low oxygen diffusivity. Both these would yield higher degradation in the semi-frozen periods when ongoing regular thaw. Indeed, both DW and DWNP have large degradation under freeze-thaw, with final levels after 52 close to 1500 mg/kg, while the systems at -5°C have final levels after 100 days of 1816 ±147 mg/kg and 2002 ± 313 mg/kg for DW and DWNP respectively.

Consecutive freeze-thaw cycles may over time also increase surface area of contact of aggregates (and thus oil) with unfrozen water films by reducing aggregate size. In the literature, it has been demonstrated that having many freeze-thaw events may reduce aggregate stability and size (57-61), especially at higher water content (58, 62). The first result supporting this occurring for this study is the results for alkB cDNA in controls at day 36 under freeze-thaw. The appearance of alkB transcripts in uncontaminated soil is due to soil organic matter. Soil organic matter contains alkane-like moieties (46), and freeze-thaw is known to lead to the release of easily degradable organic matter leading to its microbial decomposition (63). Systems containing water (WNP, W) in these controls had higher quantities of alkB cDNA than the system without water (U) at day 36, with levels for U W and WNP being $1.31x10^7$, $1.20x10^8$, and $1.75x10^7$ copies respectively. As seen in the literature adding water further reduced aggregate stability upon successive freeze-thaw, enabling more decomposition of organic matter containing alkane-like moieties**.** This trend in aggregate stability may also influence the bioavailability of the diesel. At day 36, the levels for the alkB DNA and cDNA in the freeze-thaw were significantly higher than at constant 7°C in diesel containing systems when water was added (DWNP, DW), and much lower when no water was added (D). Instead for 16S in cDNA and DNA the two diesel-containing systems without nutrients (DW, D) were lower in the freeze-thaw than in the constant 7°C and the systems with nutrients (DWNP) were equivalent under both temperature regimes. Indeed, for 16S it was not water that made the largest impact under freeze-thaw, it was nutrients, as the levels of D and DW were similar for all nucleic acids and timesteps, while the levels of DWNP were higher.

This specific impact of water on alkB under freeze-thaw suggests diesel-containing systems may be impacted similarly than the controls, and surface area of contact between unfrozen water films

and diesel may increase over time. For the general population (16S) there is not such a limitation placed on carbon access given some carbon sources will always be soluble in unfrozen water, and as such nutrients may make more of a difference.

For hydrocarbon degraders, unfrozen water film surface area of contact with soil aggregates increasing over time may have had a large impact, as oil may be sorbed to aggregate surfaces.

3.6 Community analyses

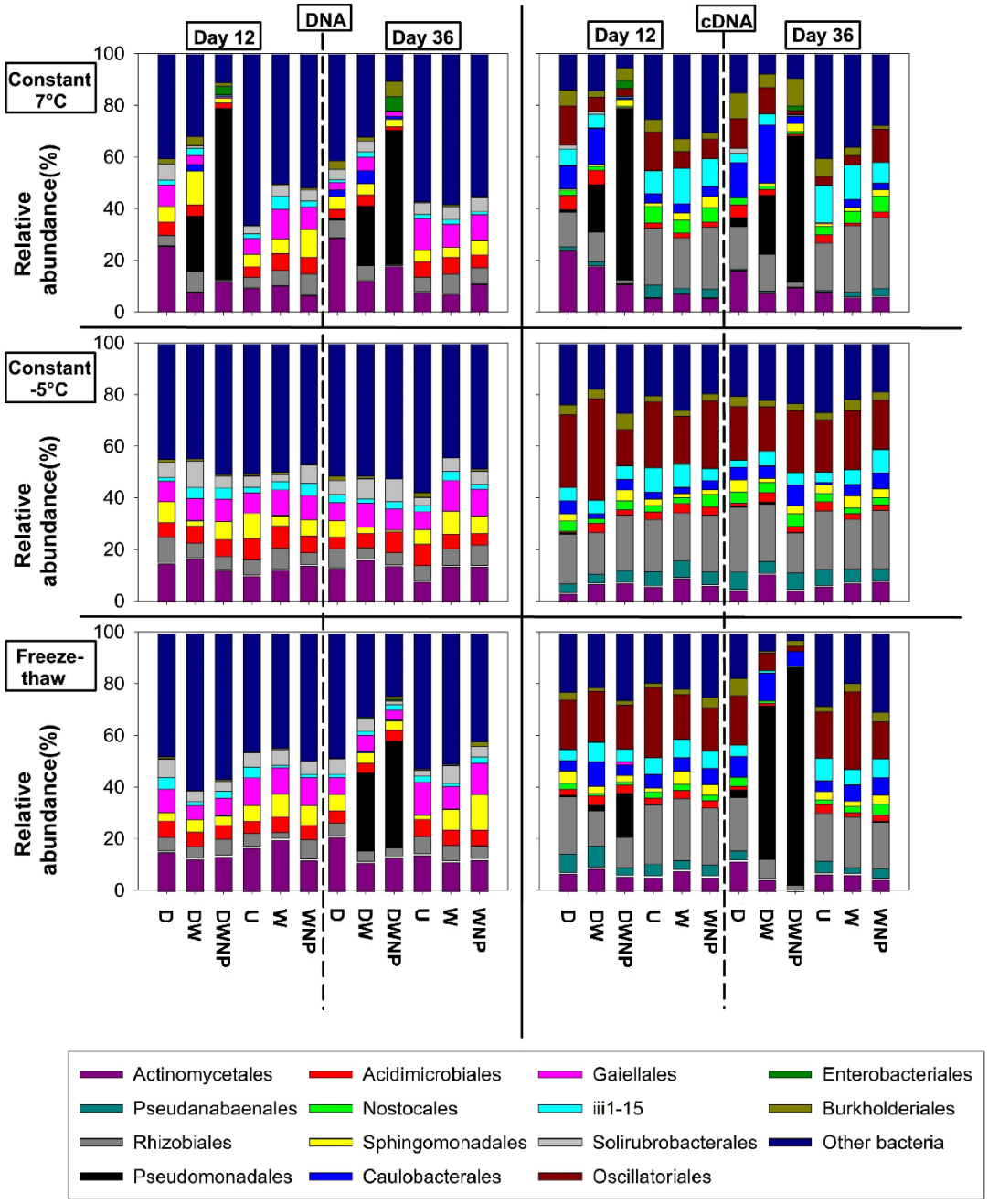


Figure 4: Most representative bacterial orders. The most representative orders are represented. Bacteria were considered representative if they represented above 5% of any samples.

The patterns seen in the day 36 for the constant 7°C and the freeze-thaw suggest that Pseudomonadales is associated to hydrocarbon degradation, which has been suggested before (64, 65).

For freeze-thaw at both day 12 and day 36, the relative abundance of Pseudomonadales is larger in cDNA than DNA. This is constrasted to the constant 7°C, where at each timestep there is similar Pseudomonadales in cDNA and DNA. The activity (cDNA) mirrors the abundance (DNA) at 7°C, where both temperatures and metabolism are relatively stable.

In the freeze-thaw conditions, activity (cDNA) is larger than the abundance (DNA). The systems may be having spikes in Pseudomonadales activity followed by slight reversions to closer to original community activity. This follows the previous argument that the thawing controls the degradation of hydrocarbons.

For both freeze-thaw and 7°C in DNA and cDNA, all diesel-containing systems clearly have higher levels of Pseudomonadales than the ecological controls incrementing as D<DW<DWNP. As such, this shows that diesel water and nutrients all have an incremental role in increasing Pseudomonadales and suggests that degradation may occur at all three conditions.

At a constant -5°, Pseudomonadales appeared for all diesel containing systems in cDNA at day 36. The levels were 0.532±0.482 % for D, 0.936 ±1.08 %, for DW, and 0.335±0.332 % for DWNP. These differences are not statistically significant, but the levels for DW were higher than the other diesel containing systems. This showcases that the bacteria found associated with hydrocarbon degradation in this study appear over time even at constant -5°C, and may again suggest that under constant semi-frozen conditions the system DW is the best option.

In the freeze-thaw, controls at day 36 had alkB cDNA (Figure 1), but Pseudomonadales are negligible in their community. Either alkB production was by other bacteria in controls, or the production was too small to drastically change the community structure.

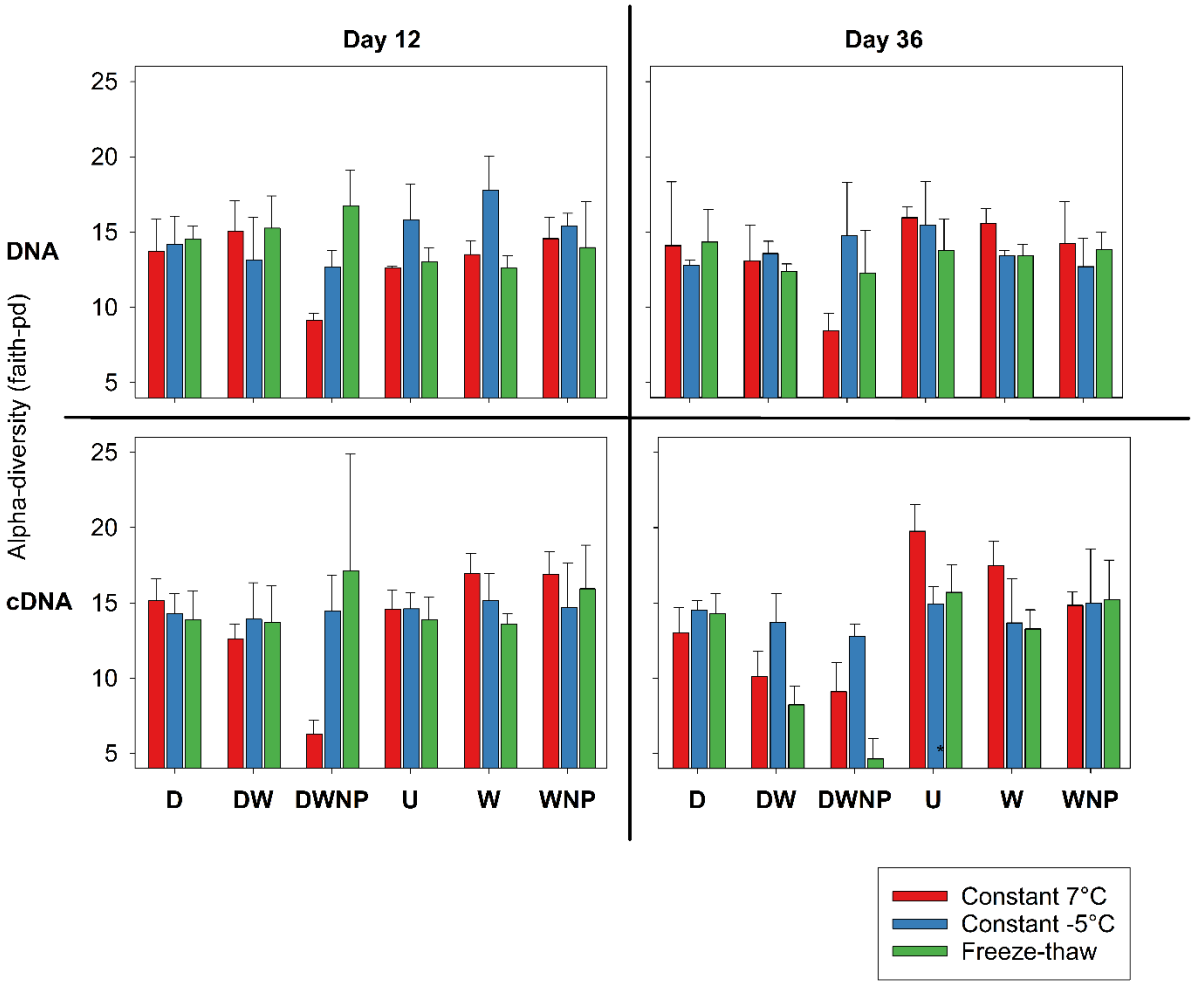


Figure 5: Faith's PD alpha-diversity. Faith-pd values generated by QIIME analysis were averaged and plotted by amendment.

The alpha-diversity results showcase a few trends. Firstly, the increased sensitivity yielded by analyzing community changes in cDNA against DNA. In the most metabolically active case that is constant 7°C in DNA at both day 12 and 36 the only set with lower diversity is DWNP. In cDNA at both timesteps every addition to the soil incrementally lowers the alpha diversity, for

only diesel-containing systems at day 12, but for every single addition across both control and test groups at day 36, with diversity decreases in order U>W>WNP>D>DW> DWNP. Changing microbial diversity is neither good nor bad (66), but this result shows that every addition incrementally stimulated specific bacteria.

For freeze-thaw at day 36, upon thawing the diversity of active functions (cDNA) lowers drastically, while the DNA diversity does not. This implies a quick access to the new hydrophobic nutrient source, which rapidly alters the active community before some stabilization upon freezing.

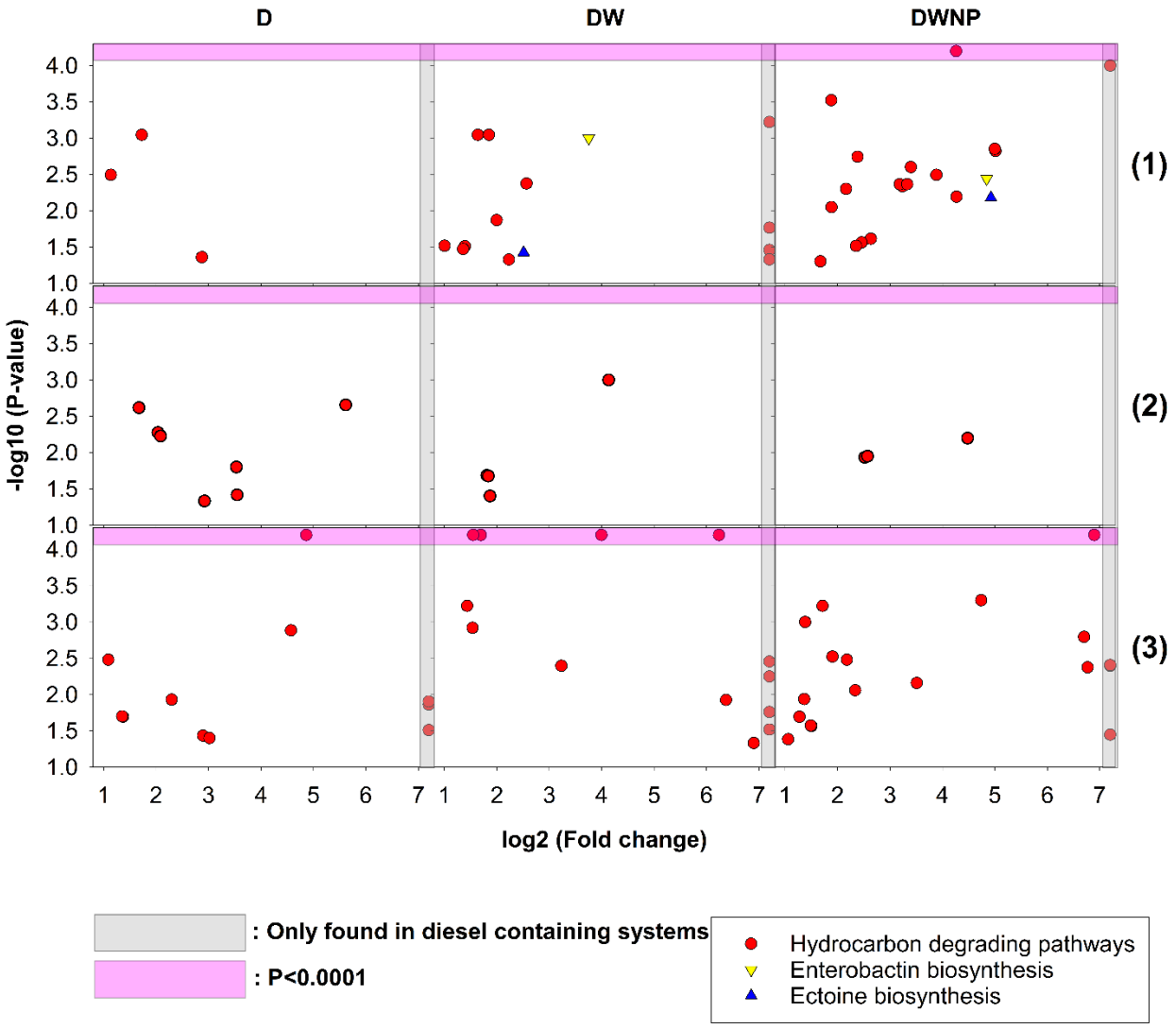


Figure 6: Environmental pathways in active stage diesel degradation predicted using cDNA. (1) refers to constant 7°C day 12, (2) to constant -5°C day 36, and (3) to freeze-thaw day 36. The timesteps that were picked to plot were the ones in most active degradation under each temperature regimes, as determined by the hydrocarbon degradation trends (Figure 3).

Marker pathway changes in Figure 6 are predictive but still showcase overall microbiome function. PiCrust has been suggested to be less reliable for soil than for human samples (67), but it is used here not to make new conclusions but to support some conclusions made through the rest of the data.

At constant -5°C levels of several hydrocarbon degrading pathways across all three diesel-amended systems make them markers at day 36, which was not the case at day 12 (data not shown). This with previous data suggests level of degraders increase over time, and that the community can degrade hydrocarbons at a constant -5°C.

Patterns seen at constant 7°C day 12 and freeze-thaw day 36 look similar, except for presence of the markers ectoine and enterobactin at constant 7°. Ectoine is an osmolyte has been shown to be related to increases in external salinity (68), and enterobactin is a siderophore which facilitates iron access to microbial systems (69).

The patterns for ectoine can be explained as follows: At day 0 nutrients are added to the DWNP and adding water in the systems DW is likely solubilizing nutrients from soil. In the freeze-thaw, nutrients are used by all bacteria gradually, and the limitation placed on diesel uptake make degraders capture a lesser proportion of the added nutrients than at constant 7°C. The active degrader phase is also later in freeze-thaw, so the lower nutrient level would make the water less ionically charged during the active period and lower the need for the osmolyte. The levels of the osmolyte at 7°C are higher for DWNP than DW, suggesting nutrient addition selects for salt-tolerant degraders.

The patterns for enterobactin can be explained as follows: Freeze-thaw has an external control on metabolism, but at 7°C the growth will go until reaching the system's carrying capacity, and bacteria able to compete for other nutrients such as iron are selected.

4. Conclusions

In this study, a fixed dose of nutrients stimulated hydrocarbon degradation in a constant 7°C condition, had a slightly inhibitory effect on hydrocarbon degradation at -5°C, and had marginal effects on hydrocarbon degradation in a freeze-thaw temperature regime (cycles: -5°C 3.5 days, 14 hours 7°C). Our results suggest that extending the bioremediation into the fall/spring would be possible with regular thaw and water helping increase bioremediation. The results also showed that adding nutrients under constantly frozen conditions may cause bacterial inhibition, likely by increasing salinity in the unfrozen films. Using both cDNA and DNA to track abundance and activity of the 16S and alkB gene as well as sequencing 16S DNA and cDNA to track the present and active bacterial communities enabled us to map both instantaneous and long-term trends.

5. Supplementary information

5.1 TPH extraction and GC-FID conditions

40 mL of dichloromethane and 50 µL of 5-α androstane (a chemical surrogate) were added to the soil microcosm vials. The vials were then shaken at 18°C and 180 rpm in a vertical shaker overnight (14 hours). Systems were decanted, then 10 mL was added to the empty vials they were stirred, and the systems were decanted again. The extracts were then blown down using a N_2 evaporator. Then a column purification was done using an elution solvent of 1:1 hexane:dichloromethane, and a solid phase consisting of (bottom to top): glass wool, 2g silica, and 4.5g anhydrous sodium sulfate. The sample was concentrated down to 2 mL, and 1 mL was loaded into auto-sampler tubes and quantified using an Agilent 5890 Gas-chromatography flame ionization detector (GC-FID) fitted with a DB-1 column (30 m x 530 µm x 5 µm). The injection temperature was 280°C in a splitless injection mode with a helium career gas, with a column flow of 10 mL/min, a Run time of 27 minutes, and a detector temperature of 300°C. The oven program was as follows: Hold at 40°C for 1min, Temperature ramp from 40°C to 280°C at the rate of 15°C/min, Hold at 280°C for 10min. The TPH concentrations were determined by running a standard curve prepared with known concentrations of kerosene.

5.2 qPCR primers thermocycling protocols and mastermix composition

alkB-1f	3’-AAYACIGCICAYGARCTIGGICAYAA-5’
alkB-1r	3’-GCRTGRTGRTCIGARTGICGYTG-5’
Ba27F	3’-AGAGTTTGATCMTGGCTCAG-5’
Ba1492R	3’-GGYTACCTTGTTACGACTT-5’
Ba519F	3’-CAGCMGCCGCGGTAATWC-5’
Ba907R	3’-CCGTCAATTCMTTTRAGTT-5’

For the 16S gene standard: each 20 µL reaction contained 10 µL of iTaq Universal SYBR Green Supermix (BioRad), 5% Dimethyl sulfoxide (Fisher), 500 nM of each primer (Ba27F and Ba1492R), and 1 µL of environmental DNA from the site. The thermocycling parameters were denaturation at 95°C for 3 minutes followed by 40 cycles of amplification (20 s at 95 °C, 30 s at 50 °C, and 105s at 72 °C).

For the 16S qPCR sub-target: Each 20 µL reaction contained 10 µL of iTaq Universal SYBR Green Supermix (BioRad), 5% Dimethyl sulfoxide (Fisher), 500 nM of each primer (Ba519F and Ba907R), and 1µL of a 100-fold dilution of unknown cDNA or DNA, or the synthetized standard (10^{0}-10^{-5}). The thermocycling parameters were denaturation at 95°C for 3 minutes followed by 40 cycles of amplification (10 s at 95 °C, 30 s at 50 °C, and 60 s at 72 °C).

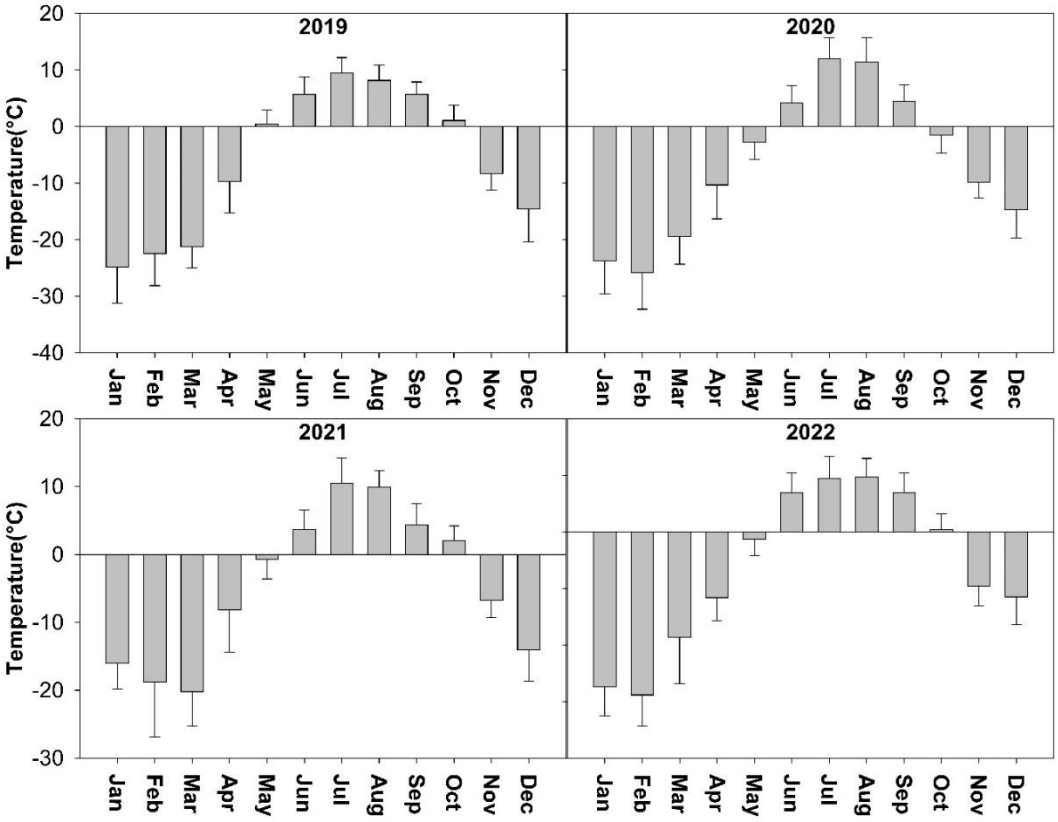
For alkB: Each 20 µL reaction contained 10 µL of iTaq Universal SYBR Green Supermix (BioRad), 500nM of each primer, and 1µL unknown cDNA or DNA, or the synthetized standard (10^{0}-10^{-7}) The thermocycling conditions were as follows: 3 mins at 95°C, followed by 40 cycles of amplification (20s 95°C, 30s 53°C, and 60s at 72°C).

5.3 Physiochemical data

SI Table 1: Physiochemical data * indicates the tests done by Bureau Veritas

Test	**Value**	**Analytical method**
TOC (mg/kg)*	12000	MA.310–CS 1.0 R3 m (70)
Total Kjeldahl Nitrogen (mg/kg)*	640	MA.300–NTPT 2.0 R2 m (71)
Nitrogen ammonia NH_3-N (mg/kg)*	<5	MA.300–N 2.0 R2 m (72)
Nitrates N-NO_3- (mg/kg)*	3.7	MA.300–Ions 1.3 R3 m (73)
Nitrites N-NO_2- (mg/kg)*	<0.20	MA.300–Ions 1.3 R3 m (73)
Total P (mg/kg)*	530	MA.300–P.Ino2.0 R2 m (74)
Inorganic P (mg/kg)*	180	MA.300–P.Ino2.0 R2 m (74)
Moisture (%)	6.09 +/- 0.62	Gravimetric
WHC (%)	24.6	Gravimetric

5.4 Temperature data



SI Figure 1: Temperature data Kangirsuk. These are the averages for daily temperature values from Environment Canada.

5.5 PiCrust2: Pathways picked P-values and fold-changes

SI Table 2: PiCrust2 data for day 12 constant 7°C. # is for when it is only present when diesel is added.

Amendment	Pathway class	Pathway name	Fold change	p-value
Diesel added	Hydrocarbon degrading pathways	toluene degradation III (aerobic) (via *p*-cresol)	2.20	0.0032
		catechol degradation II (meta-cleavage pathway)	3.32	0.0009
		Octane oxidation	7.34	0.0436
Diesel and water added	Hydrocarbon degrading pathways	toluene degradation IV (aerobic) (via catechol)	#	0.0171
		superpathway of aerobic toluene degradation	#	0.0347
		meta cleavage pathway of aromatic compounds	#	0.0006
		naphthalene degradation to acetyl-CoA	#	0.0468
		4-methylcatechol degradation	5.92	0.0042
		3-hydroxyphenylacetate degradation	4.69	0.047
		toluene degradation III (aerobic) (via *p*-cresol)	3.11	0.0009
		catechol degradation III (ortho-cleavage pathway)	2.63	0.0308
		catechol degradation to 2-hydroxypentadienoate II	2.00	0.0304
		catechol degradation II (*meta*-cleavage pathway)	3.61	0.0009
		aromatic compounds degradation via β-ketoadipate	2.63	.00308
		superpathway of salicylate degradation	2.56	0.0335
		Octane oxidation	4.00	0.0134
	Enterobactin biosynthesis		13.48	0.001
	Ectoine biosynthesis		5.69	0.0375
Diesel water and nutrients added	Hydrocarbon degrading pathways	toluene degradation IV (aerobic) (via catechol)	#	0.0001
		3-hydroxyphenylacetate degradation	6.22	0.0242
		Gallate degradation I	9.42	0.0046
		Gallate degradation II	14.8	0.0032
		Methylgallate degradation	9.10	0.0043
		protocatechuate degradation I (*meta*-cleavage pathway)	10.06	0.0043
		catechol degradation I (meta-cleavage pathway)	5.50	0.0273
		catechol degradation III (ortho-cleavage pathway)	5.22	0.0018
		aromatic compounds degradation via β-ketoadipate	5.22	0.0018
		4-methylcatechol degradation	19.2	<0.0001
		superpathway of vanillin and vanillate degradation	32.3	0.0015
		Syringate degradation	10.6	0.0025
		vanillin and vanillate degradation I	32.3	0.0015
		vanillin and vanillate degradation II	32.0	0.0014
		nicotinate degradation I	5.11	0.0305
		catechol degradation to β-ketoadipate	3.70	0.0089
		toluene degradation I (aerobic) (via o-cresol)	3.20	0.050
		toluene degradation III (aerobic) (via *p*-cresol)	3.68	0.0003
		toluene degradation II (aerobic) (via 4-methylcatechol)	3.20	0.050
		superpathway of salicylate degradation	4.48	0.005
		Octane oxidation	19.3	0.0063
	Enterobactin biosynthesis		28.7	0.0036
	Ectoine biosynthesis		30.4	0.0066

SI Table 3: PiCrust2 data for day 36 constant -5°C.

Amendment	Pathway class	Pathway name	Fold change	p-value
Diesel added	Hydrocarbon degrading pathways	3-hydroxyphenylacetate degradation	11.6	0.0158
		Catechol degradation I meta cleavage	7.6	0.0463
		Meta cleavage pathway of aromatic compounds	49.1	0.0022
		4-methylcatechol degradation (*ortho* cleavage)	11.7	0.0382
		Catechol degradation II (meta-cleavage pathway)	3.20	0.0024
		2-nitrobenzoate degradation I	4.11	0.0053
		2-aminophenol degradation	4.25	0.0059
Diesel and water added	Hydrocarbon degrading pathways	2-nitrobenzoate degradation I	3.51	0.0206
		2-aminophenol degradation	3.58	0.0209
		Nicotinate degradation I	3.66	0.0396
		3-hydroxyphenylacetate degradation	17.6	0.001
Diesel water and nutrients added	Hydrocarbon degrading pathways	3-hydroxyphenylacetate degradation	22.3	0.0063
		2-nitrobenzoate degradation I	5.74	0.0116
		2-aminophenol degradation	5.95	0.0112

SI Table 4: PiCrust2 data for day 36 freeze-thaw. # is for when it is only present when diesel is added.

Amendment	Pathway class	Pathway name	Fold change	p-value
Diesel added	Hydrocarbon degrading pathways	superpathway of aerobic toluene degradation	#	0.0307
		naphthalene degradation (aerobic)	#	0.0137
		naphthalene degradation to acetyl-CoA	#	0.0124
		Gallate degradation I	2.59	0.0201
		Methylgallate degradation	2.56	0.0199
		catechol degradation II (*meta*-cleavage pathway)	2.13	0.0033
		Gallate degradation II	7.45	0.0366
		toluene degradation IV (aerobic) (via catechol)	29.2	<0.0001
		meta *cleavage* pathway of aromatic compounds	23.8	0.0013
		4-methylcatechol degradation (*ortho* cleavage)	8.12	0.0395
		Octane oxidation	4.92	0.0117
Diesel and water added	Hydrocarbon degrading pathways	superpathway of aerobic toluene degradation	#	0.0302
		syringate degradation	#	0.0056
		mandelate degradation to acetyl-CoA	#	0.0173
		Pathway: mandelate degradation I	#	0.0035
		catechol degradation to β-ketoadipate	2.90	0.0012
		toluene degradation III (aerobic) (via *p*-cresol)	2.70	0.0006
		aromatic compounds degradation via β-ketoadipate	3.23	<0.0001
		superpathway of salicylate degradation	2.93	<0.0001
		Gallate-degradation II	9.39	0.004
		toluene degradation IV (aerobic) (via catechol)	75.5	<0.0001
		meta cleavage pathway of aromatic compounds	82.9	0.0118
		4-methylcatechol degradation (*ortho* cleavage)	120	0.0463
		Octane oxidation	16.0	<0.0001
Diesel water and nutrients added	Hydrocarbon degrading pathways	Superpathway of aerobic toluene degradation	#	0.004
		Syringate degradation	#	0.0039
		naphthalene degradation to acetyl-CoA	#	0.0356
		catechol degradation to β-ketoadipate	3.75	0.003
		Gallate degradation I	2.84	0.027
		Methylgallate degradation	2.81	0.0266
		Toluene degradation III (aerobic) (via p-cresol)	2.42	0.0201
		Catechol degradation III (*Ortho*-cleavage pathway)	4.53	0.0033
		catechol degradation II (*meta*-cleavage pathway)	2.085	0.0412
		aromatic compounds degradation via β-ketoadipate	4.53	0.0033
		superpathway of phenylethylamine degradation	3.28	0.0006
		superpathway of salicylate degradation	2.58	0.0115
		phenylacetate degradation I	2.61	0.001
		Gallate degradation II	11.4	0.0069
		Protocatechuate degradation	5.06	0.0087
		toluene degradation IV (aerobic) (via catechol)	119	<0.0001
		meta cleavage pathway of aromatic compounds	104	0.0016
		4-methylcatechol degradation (*ortho* cleavage)	109	0.0042
		Octane oxidation	26.7	0.0005

Bridge between chapters

The first chapter of this thesis used fresh contamination of soil with a hydrocarbon source to investigate biostimulation under several different temperature regimes. Whilst freshly spiking hydrocarbons enables high replicability, it underestimates any bioavailability limitations and/or contaminant weathering that may occur at a real site. Using already contaminated samples risks having less easily interpretable results due to a larger starting heterogeneity but may give a more realistic understanding of what is happening in-situ.

A large incentive for studying so many conditions in only one soil in chapter 1, was that there is inherently a difficulty in controlling confounding variables in environmental samples. i.e., comparing studies that occur at different soils may be difficult, given that there are many variables we cannot control that will impact the resulting trend. But if we were to heavily limit the quantity of variables that differ between sites and treat them under the same conditions, it would be possible to attribute differences in the resulting trends to these variables we know differ.

As such, the second chapter of this thesis is focused on already contaminated sites, namely three closely related sediments from a freshwater pond in the Northwest Territories. These three sediments differ in some variables of interest. Similarly to the first chapter, the second chapter studies the role of temperature and biostimulation on bioremediation of hydrocarbons. But the goals of the two chapters differ. The first chapter aimed to deeply investigate the microbiology and biochemistry underlying this topic in a relatively-well studied environmental matrix that is soil. Instead, the second chapter aimed to understand on a larger scale the impact of certain

variables in sites with aged contamination using the relatively-poorly studied environmental matrix that is cold-climate freshwater sediment.

Chapter 2: Testing multiple strategies for bioremediation of F3 hydrocarbons from northern Canadian sediments with elevated chromium nickel and aluminium

1. Introduction

Sediments often act as a sink for recalcitrant hydrocarbon compounds, which can lead to toxicity for both the sediment biota, and for aquatic biota and receptors. This may occur through contact with both hydrocarbons at the sediment-water interface (75) and with hydrocarbons transiently associated to suspended particulate matter (76). Aquatic organisms, including benthic organisms, are sensitive to chemical pollution, and it has been shown that it takes considerable time for these communities to recover after oil exposure (76-78).

Timely remediation of hydrocarbon contamination in sediments is essential to minimizing eco-toxicological impacts that hydrocarbons pose (2), as well as avoid exposure to a wider range of biota through bioaccumulation in aquatic and benthic organisms. Bioremediation is an attractive option for clean up of hydrocarbon contamination, given it is both sustainable and cost-effective (27). Even more so for remote sites, where displacing the contaminated samples for offsite treatment may prove to be even more cost prohibitive. Whilst this is the case, applying this strategy in cold climates does have its challenges, such as lower metabolism of bacteria and lower nutrient content of the sediments (1). There have been numerous studies on bioremediation of cold climate oil-contaminated soils (43, 79), as well as marine environments (80-82). Much fewer studies have been done on contaminated cold climate freshwater sediments (83). Oxygen access to sediments may vary depending on sediment depth as well as lake surface

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freezing trends. As such, degradation may sometimes be expected to occur through aerobic pathways, or through anoxic pathways.

Firstly, sediment degradation has been found to occur aerobically. For instance, Kalneniece et al. in a freshwater sediment found that biostimulation using beet molasses, cabbage leaf extract, yeast extract and a biosurfactant in a lake sediment sludge along with daily mixing and aerating at 23 ^{o}C led to 79.8% degradation in a site of a depth of 4-5 m and 63.4% in a site of a depth of 8 m in 32 days. Without biostimulation, the degradation was 22.7% at the site 4-5 m deep and 9.8% at the site 8 m deep (83). Some other freshwater sediment studies found no increase in degradation upon biostimulation (84). For instance Venosa et Al. conducted a bioremediation study using sediments from a freshwater wetland adjacent to the St-Lawrence river, where they freshly contaminated the sediments with Mesa light crude oil, and saw that across a wide set of conditions (including unamended as well as several biostimulations) there was 35% degradation across all plots after 147 days (84). This highlights the fact that the success of biostimulation will depend on the specific site and many factors. In-situ hydrocarbon degradation in sediments is often catalyzed by anaerobic pathways given low oxygen (85), and anaerobic degradation has been shown to occur in several studies (85-89). For instance, Dell'Anno et al. incubated anoxic marine sediments 60 days at 20°C and found that without any biostimulation TPH concentrations decreased from 1010 to 464 mg/kg (87).

Whether hydrocarbon contamination in a sediment will be remediated significantly under aerobic or anaerobic conditions will depend on many factors such as the availability of the right terminal electron acceptors and the microbial community composition of the site. It was for instance shown that with iron sulfate and nitrate present, benzene could be degraded under anaerobic conditions (90). Nitrogen is often added for aerobic bioremediation with phosphorus, making

nitrate both an electron acceptor for anoxic conditions as well as a source of N for biomass growth for either aerobic or anaerobic conditions (91). Oxygen access in sediments has also been shown to cause large changes in both microbial communities and metabolic activities in coastal sediments (92), suggesting that different communities may be at play when oxygen is involved.

Another factor that may be influence sediment bioremediation is temperature, which has been studied previously. For instance, Garret et al. found that in some arctic marine sediments the degradation of crude oil after 90 days was 48% at 6°C and 61.5% at 20°C, which is not that large a difference (81). This study suggests that whilst temperature plays a role, the bacteria in northern sites are cold-adapted and can function under cold conditions.

Another factor that may influence the efficiency of bioremediation is the presence of co-contaminants. Hydrocarbon contaminated sediments may often have heavy metals present as co-contaminants (93, 94) . Heavy metals have generally been associated with a large loss of functional diversity in other similar matrices such as soils (95), which has wide ranging implications for soil health but also for microbial processes such as biodegradation. Studies have also shown that microbial diversity and microbial community structure significantly changed after heavy metal contamination (96). A study by Ma et al. found that out of a list of tested metals (Zn, Mn, Cu, Al, Ni, Fe), the hydrocarbon degrader *B.Subtillis* had the lowest tolerance for aluminium and nickel, and during a degradation experiment in water using *B.Subtilis* and *Acremonium sp.*, addition of these at a concentration of 5 mmol/L led to significantly less degradation of some polycyclic aromatic hydrocarbons against a control (97). As such, heavy-metal co-contamination may hinder hydrocarbon biodegradation.

In this study, F3-hydrocarbon degradation was assessed in three freshwater sediments from a pond at a mining site. Studies investigated the following: The role of temperature on degradation

(1°C and 5°C); degradation in aerobic conditions; degradation in anaerobic conditions; and the impact of heavy metals on degradation. A few knowledge gaps are fulfilled by this study. Firstly, bioremediation of hydrocarbons has rarely been studied in cold-temperature freshwater sediments. The three sites are found in close vicinity in the same freshwater pond but do differ in several characteristics such as physio-chemical properties, microbiology, and depth. Comparing bioremediation experiments between different sites can be difficult given that the heterogeneity and complexity of environmental samples may lead to a variety of uncontrollable confounding variables. Having three neighboring communicating sites enables correlation of any difference in trends with the few differing variables between the sites. The degradation was monitored through microcosm experiments, hydrocarbon levels were tracked, and to give insights as to any difference in trends, sediment physio-chemical and microbiological (16S qPCR, 16S sequencing) properties were measured.

2. Methods and materials

2.1. Site location and history

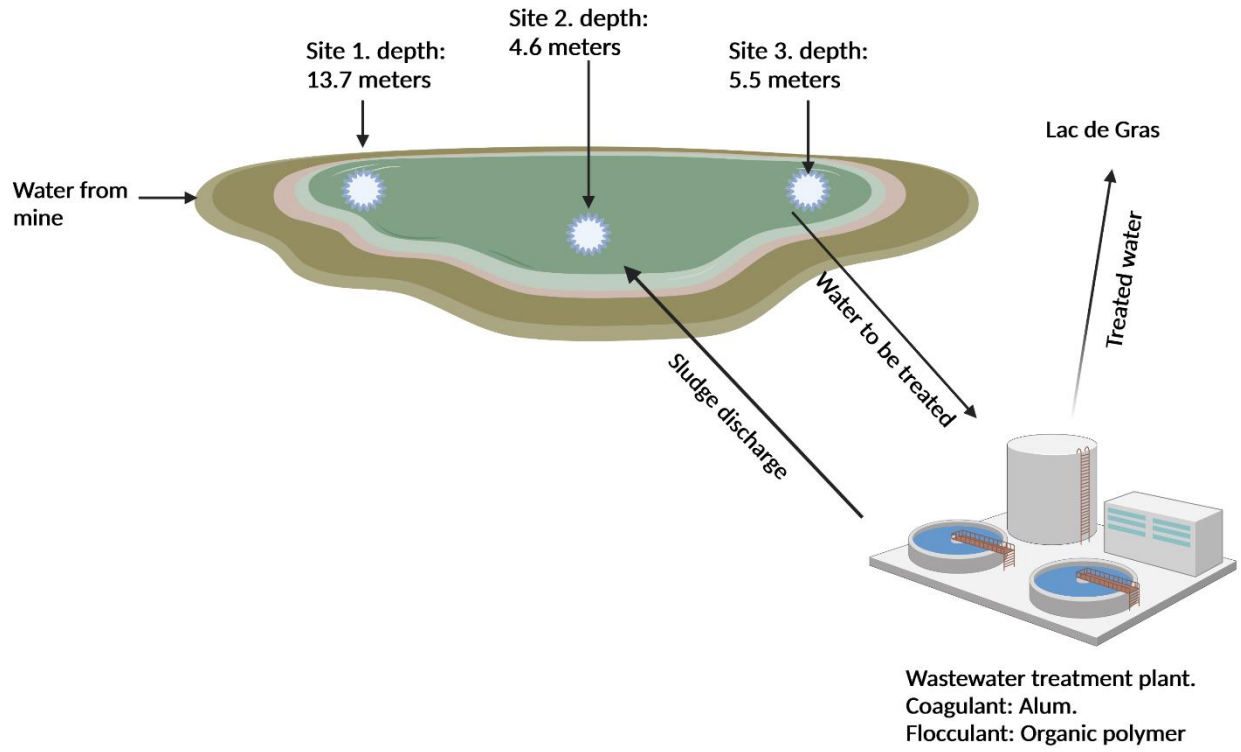


Figure 7: Schematic showing location and depths of the 3 sites. The figure was created using BioRender.com.

The 3 sediment sites are in the same pond in the northwest territories in Canada, at the Diavik diamond mine (64°30'20.6"N 110°17'15.6"W). The sites are labelled 1 to 3 in Figure 7. Sediment samples and site information was provided to McGill by Golder WSP. The pond in question is a water management pond for the mine's water treatment plant. The water around the site and the water from the underground mine get directed into this pond, the water is then treated in their wastewater treatment plant using alum as a coagulant and an organic polymer as a flocculant. The sludge from this treatment is then added back into the pond at Site 2, and the treated water goes to the De Gras Lake. There is a source of F3 hydrocarbons in the pond sediments that is

hypothesized to enter through the lake through the underground mine water due to spills of either hydraulic or lubricating oil from heavy machinery operating in the mine. There are also elevated levels of nickel and chromium especially at the sludge discharge point, from unknown sources. The Diavik diamond mine is set to shut down in 2025 (98), and efficient bioremediation of the sediments is therefore sought.

2.2. Soil sampling and shipment

The sediments were sampled from the 3 sites and shipped to Golder in Montreal and then to McGill University in coolers with ice packs, where they were stored at -30°C until the start of the experiment. The sediments were shipped in plastic bags that were placed in plastic buckets.

2.3. Sediment chemical characterization

Sediments were sent to the commercial lab Bureau Veritas (BV) to have several parameters measured: Chlorides, Nitrates, Total Kjeldahl Nitrogen, Orthophosphate, Total Phosphorus, Sulfates, and Total Organic Carbon (TOC), and several key metals. Methods used by BV are listen in the SI as SI Table 5.

2.4. Sediment size and dispersion dynamics

After witnessing the ease at which the sediment suspended, investigations into the size of the sediment and the impact of varying salinity at the site on sediment size were done. To measure the size, both light microscopy measurements and Dynamic light scattering (DLS) measurements were performed. The light microscopy measurements concluded that the most representative size were sub-micron particles, therefore likely clay. DLS and, ζ-potential measurements were performed on a Nano Brook Omni instrument at a sediment concentration of 800 mg/L in triplicate, at the McGill Chemistry Department characterization facility. Particles were resuspended 30 minutes prior to the ζ-potential measurements.

2.5. Microcosm set-up

For all 3 sites two seasons were modeled using sacrificial microcosms, and at these two seasons both natural attenuation and biostimulation was tested. The overlaying water present in the buckets was removed using a sterilized metal ladle, and the sediments at water holding capacity were then mixed with the ladle to maximize homogeneity. All glassware and soil handling tools used for setting up the microcosms were cleaned by acid wash followed by deionized water rinse, and autoclaved. A full ladle was then sampled from the buckets, and ~7 g of the sediments (~3.5 g dry weight) was sub-sampled from the ladle using an autoclaved metal spatula and added to 120 mL microcosms. To these microcosms, either 3.7 mL of distilled water, or 3.5 mL of distilled water and 0.2mL of a solution of nutrients in distilled water were added. The nutrient solution led to an amendment in the sediments of an added 100 mg/kg of N, and an added 100 mg/kg of P per dry weight, with N being added as NH_4NO_3, and P as a 1:1 molar ratio of KH_2PO_4 and K_2HPO_4. The dosage of the added salts was similar to the composition of the Bushnell-Haas medium, a well-known medium for hydrocarbon-degrading bacteria. All systems were capped with PTFE-lined silicone septa (Sigma/Supelco) and crimped with aluminum caps. The "summer" conditions were aerobic and incubated at a temperature of 5°C. The systems were decrimped and opened to the atmosphere on a weekly basis in a biosafety cabinet to allow headspace replenishment of oxygen. The "winter" conditions were anoxic and at a temperature of 0-1°C. The headspace was purged with nitrogen for 20 minutes prior to capping and the samples were not opened until sampling to emulate the anoxic conditions. All systems were briefly mixed (<5 minutes) at 12°C and 120 rpm on a weekly basis. The mixing was not fast enough to observe sediment resuspension.

2.6. Total petroleum hydrocarbons (TPH) extraction and quantification

All samplings were done in triplicates from sacrificial microcosms. The site with a high starting TPH concentration (Site 2) was sampled monthly for 10 months in summer conditions and 9 months in the winter conditions. The sites with lower starting TPH concentration (Site 1 and Site 3) were sampled monthly for 6 months in the summer conditions. For the winter conditions at Site 1 and 3, microcosms were sampled after 1 month, 2 months, 4 months, and 6 months. The sampling was more infrequent in the winter conditions at Site 1 and Site 3 given that the levels were low and slow degradation was seen after 2 months. The retention time of each fraction was assessed by spiking n-hexanes standards of varying lengths using the same GC-FID method as for the analytes and noting the retention times of the hydrocarbons. The extraction protocol was an optimized method of the MA. 400-HYD 1.1 protocol by the Centre d'expertise en analyse Environmentale du Quebec (99). Briefly, sediment aliquots were removed and kept at -80°C for future analyses. The sediments were then chemically dried on ice using anhydrous magnesium sulfate. 40 mL hexane and 50 µL of 5-α androstane (a chemical surrogate standard) were then added to the systems. They were sonicated and then shaken overnight. Polar substances were removed using silica gel, and silica residues were removed from the mixture using a glass column with a glass wool plug. The extracts were blown-down using a N_2 evaporator and quantified using an Agilent 5890 Gas-chromatography flame ionization detector (GC-FID) fitted with a DB-1 column (30 m x 530 µm x 5 µm). The concentrations were measured using a standard curve with known concentrations of Kerosene. The detailed extraction protocol as well as the GC-FID conditions can be found in the SI.

2.7. Degradation Rate Kinetics

Average rate constants were calculated assuming a pseudo-first order rate constant. For all sites the rates from time 0 to 1 month as well as from 0 to 4 months were calculated. For Site 2, the rate from 0 to 8 months was also calculated. Average rate constants were done by calculating rates using all possible combination between the triplicates (giving a total of 9 rate constants) and averaging the values.

2.8. DNA extraction

Eppendorf tubes containing sediment slurries collected at day 0, month 1 and month 6 of the microcosm experiment were removed in duplicate for all conditions from the -80°C freezer and thawed on ice. The excess water was then removed to extract the DNA from sediments at approximately water holding capacity, by centrifuging the Eppendorf tubes at 16000 g for 5 mins and collecting the overlaying water. The DNA extractions were done using the DNeasy PowerSoil Pro Kit (Qiagen), but some modifications to the regular protocol were done due to the difficulty in extracting from the sediments, as suggested in the manual and by Qiagen Technical support services. Namely, 0.125 g of sediments were extracted, the samples were incubated in a water bath at 65°C for 10 minutes after addition of the CD1 solution, and the cells were lysed by bead-beating using a Precelys 24 homogenizer with a beating protocol of 3*30 seconds homogenization cycles at a speed of 5000 rpm. These extra steps were done to maximize the lysis of the cells. To ensure maximum concentration, the elution with C6 was done with a volume of 50 µL.

2.9. 16S qPCR analysis

qPCR analysis of the 16S gene was done to assess changes in total bacteria using biological duplicates and analytical duplicates. The 16S standard was made by amplifying the full 16S gene from environmental DNA using the primer combination Ba27F and Ba1492R (44). The

PCR product was then gel-purified using the QiAquick gel extraction kit (Qiagen). The copy numbers in the gene standard were then estimated by using the PicoGreen method. A shorter sub-section of the 16S gene was amplified in the samples and in the standard (10^{0}-10^{-5}) in the qPCR using the general bacterial primers Ba519F and Ba907R as previously used by others (47). All the reactions were done using a CFX connect real-time PCR detection system (BioRad). No template controls were run, the specificity of each reaction was verified after each run through a melting curve analysis between 65 °C and 95 °C, and the efficiency of the reaction was calculated to be 92%. Primer sequences mastermix composition and thermocycling parameters can be found in the SI.

2.10 Sequencing

For sequencing, the DNA and cDNA were sent to the Genomic analysis platform of Laval University in Quebec City Canada. For all samples, the V3- V4 region of the 16S gene was amplified using primers Bact341F (3'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTCC TACGGGNGGCWGCAG-5') and Bact805R (3'-GTGACTGGAGTTCAGACGTGTGCTCTTC CGATCTGACTACHVGGGTATCTAATCC-5'). The amplified sequences were sequenced on an Illumina MiSeq.

2.11 Bioinformatic analyses

The reads were analysed using qiime2 (48). The reads were trimmed at 240bp both forwards and backwards, after which the quality of the sequences decreased significantly. Denoising was done using dada2, and the reads were then classified using a naïve Bayes classifier trained against the Greengenes database containing the full 16S genes.

3. Results and discussion

All the P-values and statistical significances in this section refer to the Student's T-tests, and differences are considered significant for $P<0.05$.

3.1 Sediment characterization results

The hydrocarbons present were predominantly F3 hence carbon length C16-C34 (>95%), with highly negligible F2 (2-4%) levels.

The dynamic light scattering experiments showed that the key size fractions in a clay suspension in pond water were around 438 +/- 2.3 nm, clearly in the sizes expected for clay particles, and the suspension had a ζ-potential of -19.19 +/- 1.29 mV. There was a clear impact of diluting the pond water on the ζ-potential of the sediment solutions, as shown in SI Figure 2.

The metal composition of the sediments is listed in Table 2 below.

Table 2: Total metals in the digested sediment samples.

Metal	**Site 1**	**Site 2**	**Site 3**	**Environmental health quality guidelines (SQGe)**
Arsenic (mg/kg)	10	4	11	
Iron (mg/kg)	34000	34000	32000	
Calcium (mg/kg)	8200	24000	7600	
Magnesium (mg/kg)	24000	68000	43000	
Chromium (mg/kg)	**110**	**210**	**150**	**87** (100)
Zinc (mg/kg)	120	120	130	
Aluminium (mg/kg)	25000	21000	21000	
Potassium (mg/kg)	7200	10000	8000	
Sodium (mg/kg)	510	620	690	
Manganese (mg/kg)	690	680	980	
Titanium (mg/kg)	1500	1700	1200	
Nickel (mg/kg)	**150**	**470**	**320**	**50** (100)

The metals that were at a much higher level than the environmental soil quality guidelines established by the CCME were chromium and nickel. The levels for both nickel and chromium were highest at Site 2.

Some key physiochemical parameters as well as the starting 16S gene copies at the 3 sites can be found in Table 3 below. Tests marked with a * were done by Bureau Veritas.

Table 3 : Chemical and microbiological properties.

Test	**Site 1**	**Site 2**	**Site 3**
Chlorides (mg/kg)*	120	94	160
Nitrates N-NO3 (mg/kg)*	<1.0	<1.0	<1.0
Total Kjeldahl Nitrogen (mg/kg)*	740	280	1800
Orthophosphate P (mg/kg)*	<1.0	2.1	1.4
Total Phosphorus (mg/kg)*	1100	100	1600
Sulfates (SO4) (mg/kg)*	150	130	81
Total organic carbon (TOC) (% g/g)*	0.70	0.79	1.6
16S gene copies in original soil (copies/kg)	$2.12x10^{10}\pm 3.17x10^{9}$	$1.30x10^{9}\pm 6.25x10^{8}$	$1.39x10^{12}\pm 1.63x10^{11}$

A few notable differences can be seen in this table. Firstly, the total organic carbon levels are double at Site 3 compared to Site 1 and 2. The levels of total Phosphorus are lower in Site 2 than Site 1 and 3. The levels of Kjeldahl Nitrogen are lowest at Site 2, followed by Site 1, then Site 3.

The levels of bacteria differ greatly at each site, and every difference was highly statistically significant, with P value Site 1-Site 2: $1.73x10^{-5}$, Site 1- Site 3: $2.75x10^{-6,}$ Site 2-Site 3: $2.52x10^{-6}$. These results are at first unexpected, as Site 1 is deeper than Site 2 and 3, and site 2 and Site 3

are at the same depth. But Site 2 has nonetheless significantly lower levels than both Site 1 and 3.

3.2 Hydrocarbon degradation extents and rates

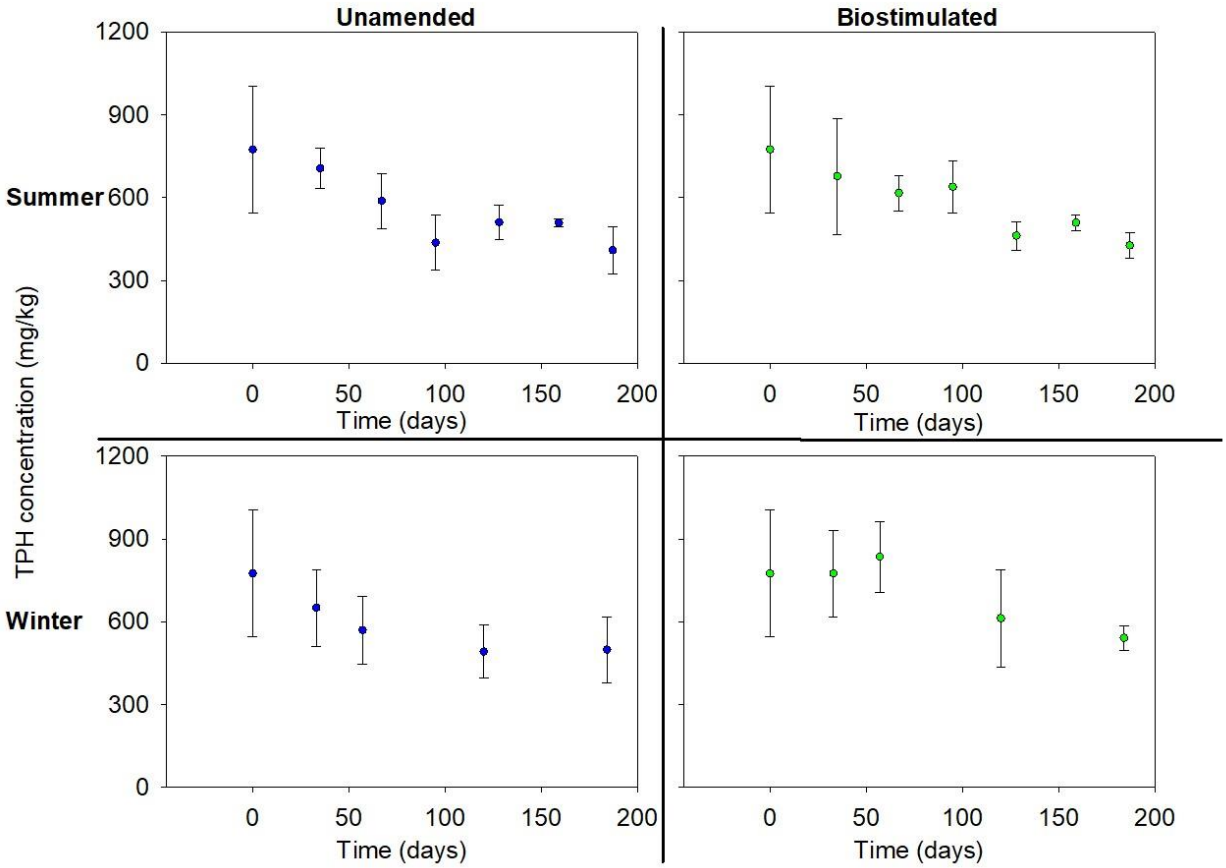


Figure 8: Degradation behavior for Site 1 under various conditions tested. Data points represent average of 3 measurements, and the error bars show the standard deviation. Statistically significant decreases from original values are marked by *.

There is no statistically significant TPH degradation at Site 1 using P=0.05 as a threshold. While we do see a decreasing trend of the TPH levels across all amendment, the heterogeneity in the sample makes the final extents less than significant. The only amendment that comes close to

statistical significance is “Summer unamended”. The starting values for Site 1 were 774.7 ± 228 mg/kg, and the final levels for “Summer unamended” were 445 ± 29 mg/kg. This constitutes a difference with a P-value of 0.068. The final levels in “Summer biostimulated” are much less significant, with a P-value of 0.135.

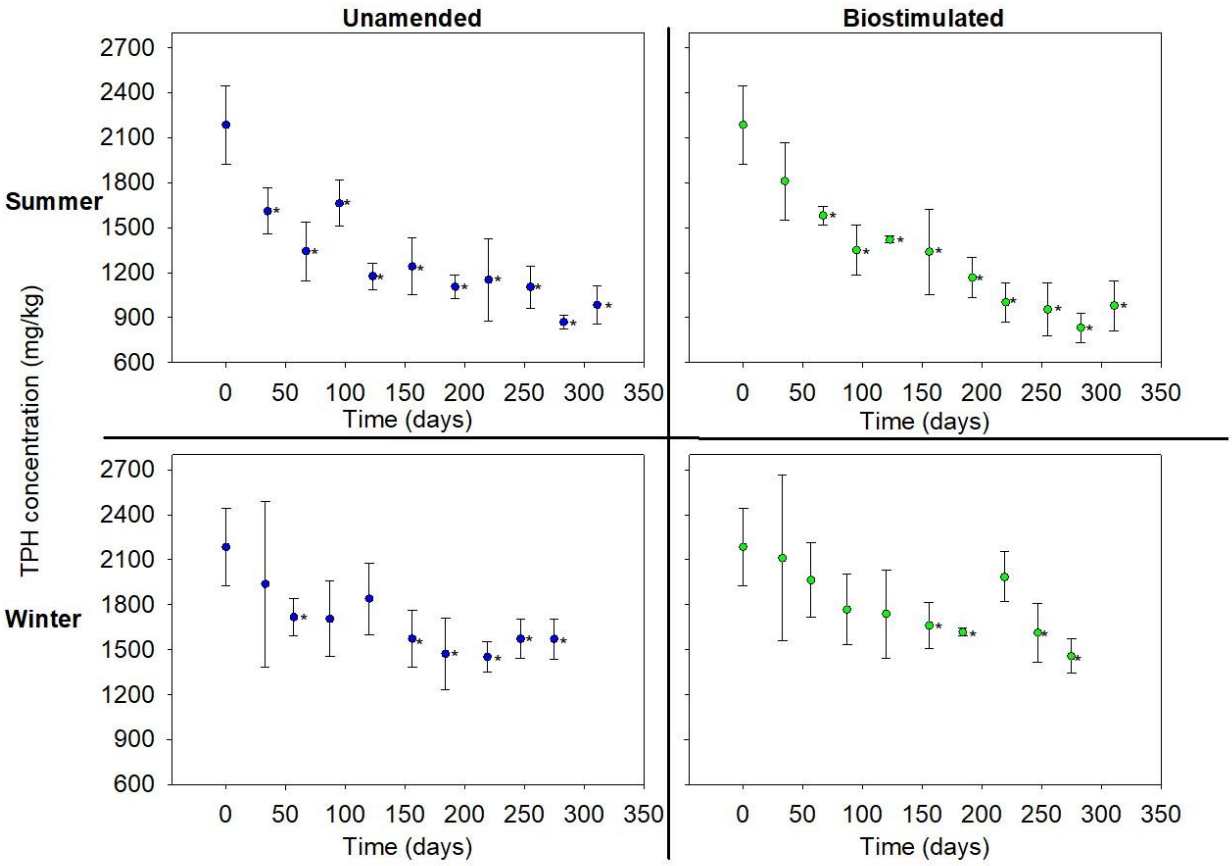


Figure 9: Degradation behavior for Site 2 under various conditions tested. The values of triplicate measurements were averaged and plotted against time elapsed. Statistically significant decreases from original values are marked by *.

At Site 2, we see significantly larger degradation in the summer than in the winter. At both summer conditions the degradation is 55 ± 7% and 55 ± 8% in the unamended and biostimulated scenarios respectively, whilst in the winter conditions the degradation is 26 ± 8 % in the

unamended condition and 33 ± 8% in the biostimulated condition. In both the summer and the winter, adding nutrients causes no statistical difference in hydrocarbon degradation extents.

For the summer, the degradation starts being significant after 1 month in the unamended systems, and after 2 months in the biostimulated systems. There is a start of a plateau forming at the endpoints for both unamended and biostimulated systems, but longer sampling may be necessary to determine whether it is a local minimum or a real plateau.

In the winter in the unamended systems, the degradation starts being significant after 2 months. the 3rd and 4th month are not significant, and from the 5th month, every point is significant. In the biostimulated systems, the first significant point occurs at 5 months, and every other point after that is statistically significant, except for the 7th month.

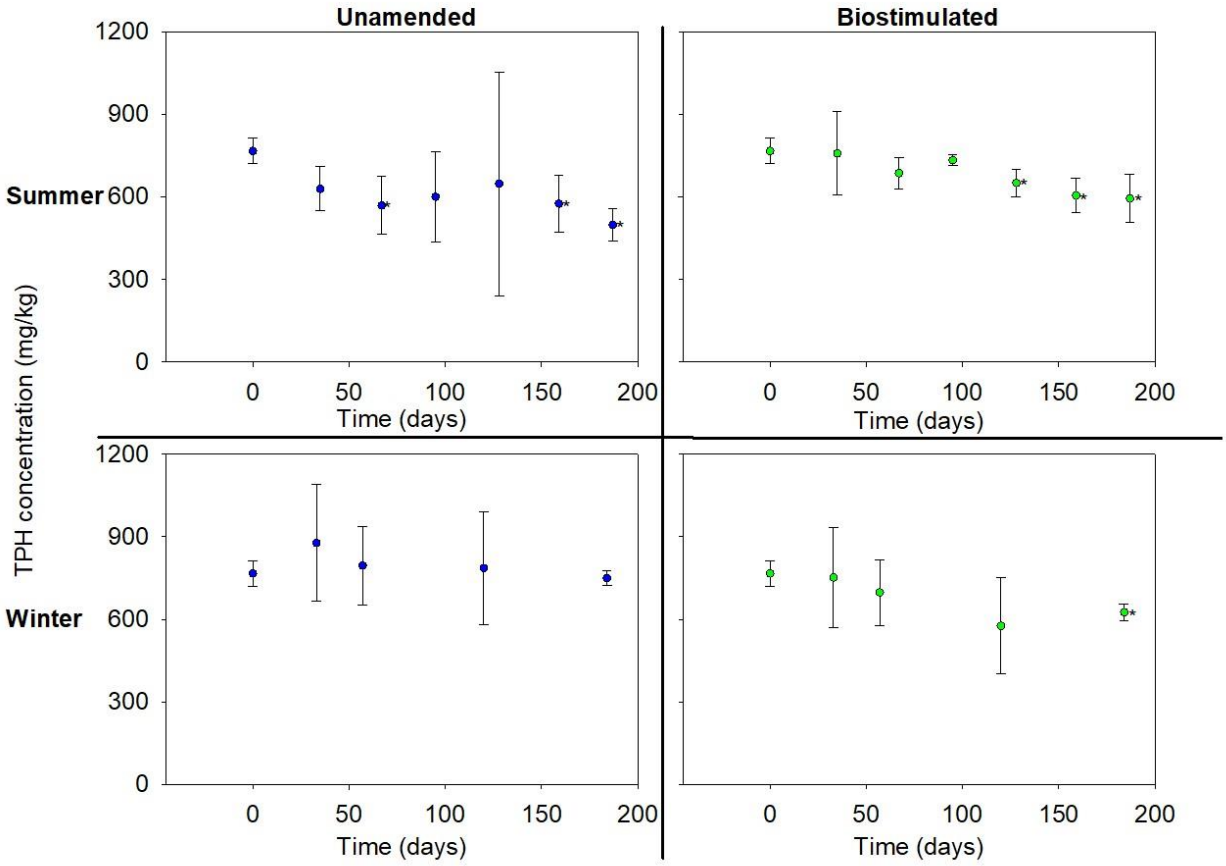


Figure 10: Degradation behavior for Site 3 under various conditions tested. The values of triplicate measurements were averaged and plotted against time elapsed. Statistically significant decreases from original values are marked by *.

At Site 3 (Figure 10), there is degradation in the summer conditions, with 35 ± 8% in the unamended and 22 ± 10% in the biostimulated conditions. In the unamended systems, the first point with statistically significant degradation occurs after 2 months. Month 3-4 are not significant, and month 5-6 are. In the biostimulated conditions, the levels constituting a stastically significant decrease occur from month 4 to month 6.

Both curves in the summer look biphasic. Both have little to no degradation in the first 4 months, followed by degradation in the last 2 months. There is no statistically significant decrease in the winter, except for the 6th month in the biostimulated systems.

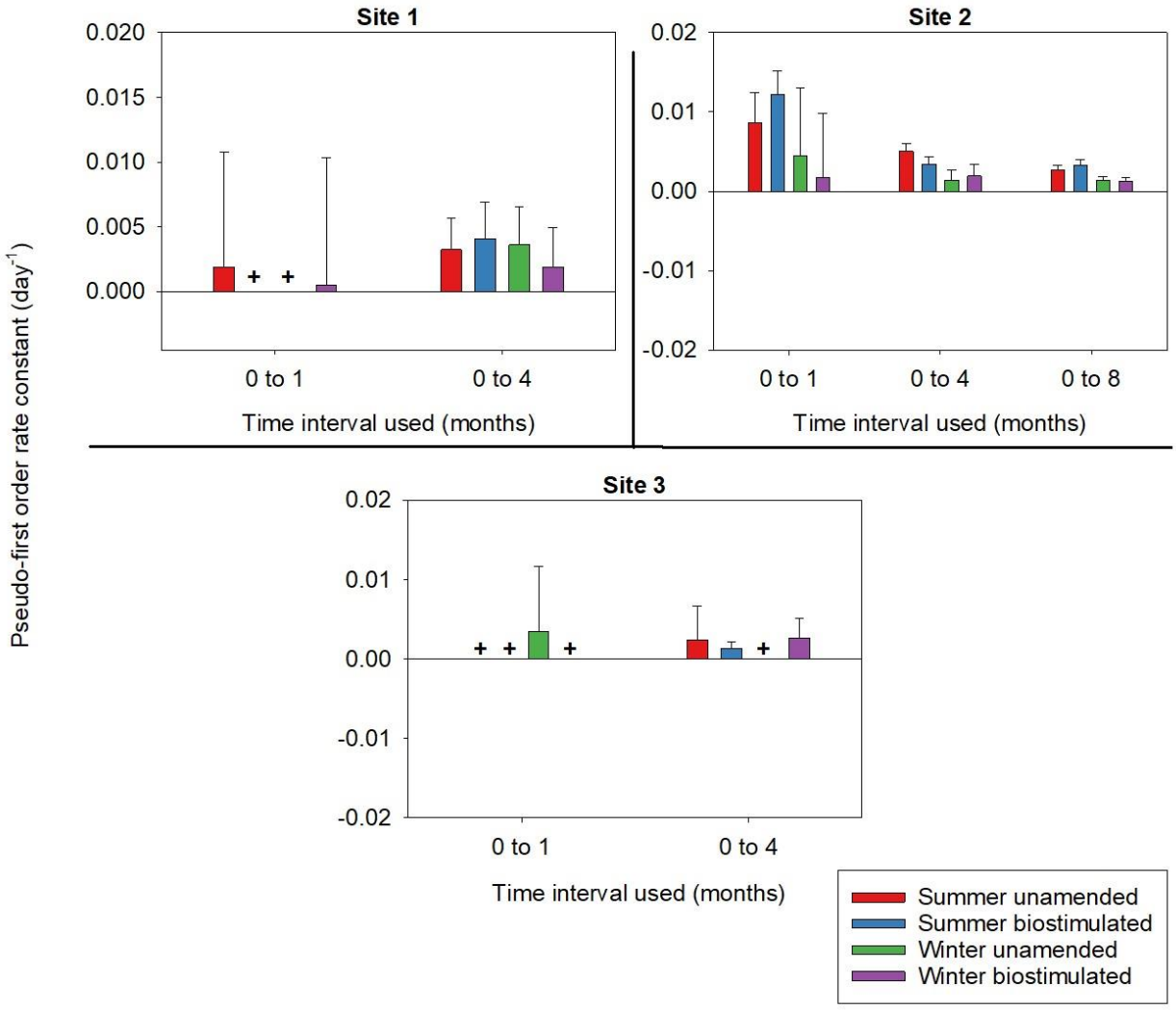


Figure 11: Pseudo-first order rate constants. Pseudo-first order rate constants were calculated, and the time interval used to calculate the constants can be seen as the x-axis. **"+"** indicates no degradation.

As seen in Figure 11, the rates either average to 0 or very low at Site 1 and 3, owing to the high heterogeneity of the original samples and the slow degradation rates. For Site 2, we notice that the degradation rates generally decrease over time. Using for instance the case of the summer unamended: The pseudo first order rate constants calculated using as a time interval the 1st month the first 4 months and the first 8 months, were $0.0087 \pm 0.0038 day^{-1}$ 0.0050 ± 0.00094 day^{-1} and 0.0027 ± 0.00060 day^{-1} respectively (Figure 11).

3.3: 16S gene abundance in various treatments

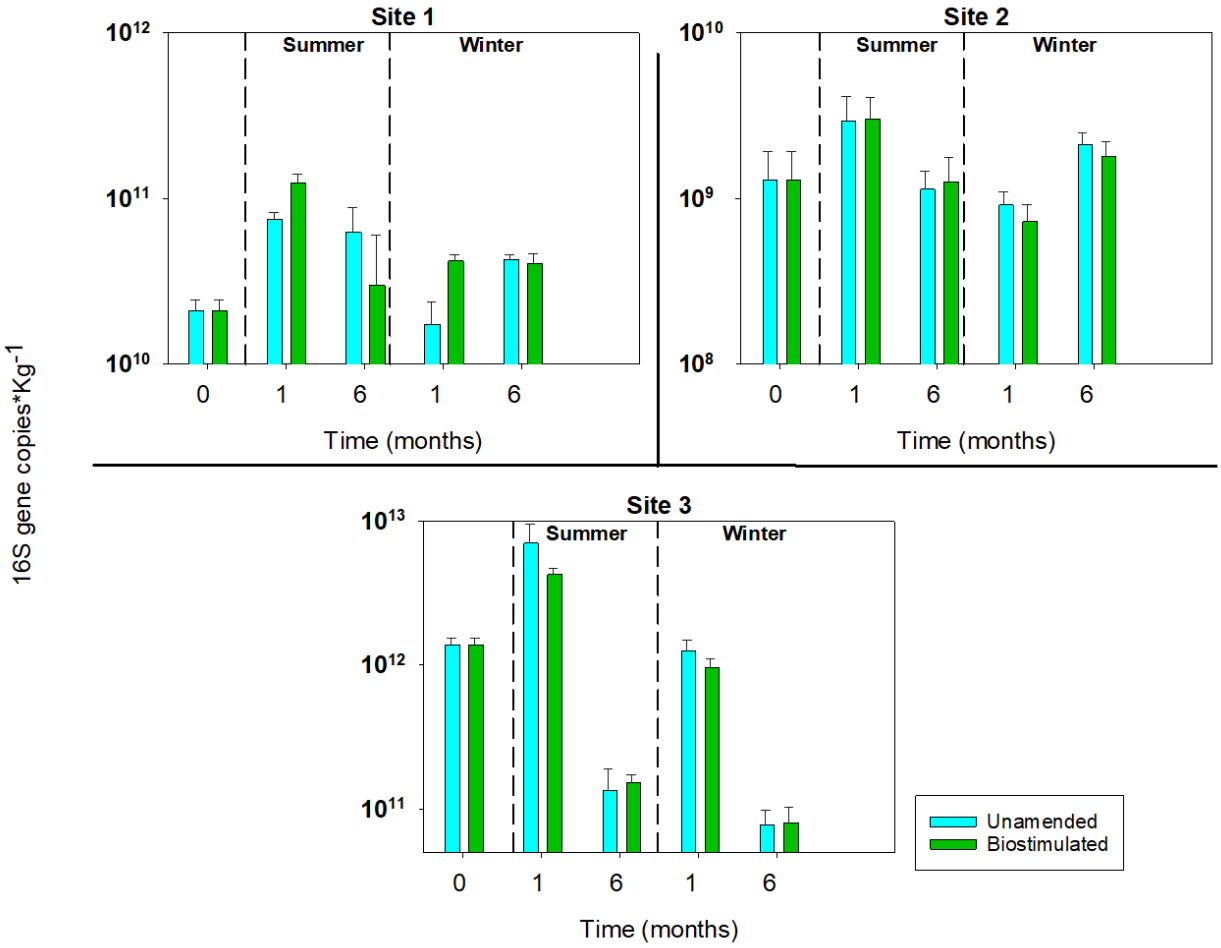


Figure 12: 16s rRNA gene qPCR. Average 16s rRNA gene copy numbers per kg are plotted against time. The levels were measured and plotted in the original sediment ("0"), after 1 month of the microcosm experiment, and after 6 months of the microcosm experiments.

Adding nutrients did not statistically influence the levels of 16S copies at either Site 2 or 3.

For Site 1 adding nutrients statistically increased the gene levels at month 1 for both the summer and the winter conditions. At month 6, there was no longer any statistical difference between unamended and biostimulated systems.

For Site 1-3 in the summer using average values for unamended and biostimulated, the levels had statistically significant increases the 1st month. After 6 months, the levels went back to the same

levels as at time 0 for Site 1 and Site 2. For Site 3, the levels became statistically lower than at time 0.

Using average values for unamended and biostimulated in the winter, at Site 3 the values after both 1 and 6 months were statistically lower than the starting values. For Site 1 and 2, the values did not change the first month but were significantly higher than original values after 6 months.

Comparing between the average levels in the summer and in the winter, at month 1, the levels in the summer were statistically higher than in the winter at all sites. At month 6, this was still the case at Site 2 and 3, but at Site 1, the levels had no statistical difference.

3.4 Metals may inhibit bacterial degradation of hydrocarbons in freshwater sediments

Lower levels of bacteria (16s qPCR) in the original sediments were seen at Site 2 than at Site 1 and 3. The levels of nitrogen Kjeldahl and total Phosphorus were also lower at Site 2. The results from the degradability experiments at Site 2 (Figure 9) showed that adding nutrients at the site had no impact on the degradation of hydrocarbons in both summer and winter conditions. Similarly, the results in Figure 12 showed that adding N&P had no impact on the quantity of bacteria at Site 2. This suggests that the original levels of N&P at Site 2 were not limiting bacterial growth, and that is not the reason for the disparity in original bacterial levels at the 3 sites.

Heavy metals such as nickel and chromium have been found to both lead to microbial toxicity and to inhibit degradation of hydrocarbons. For instance, chromium has been shown to inhibit nitrification as well as microbial respiration (101). Whilst the main species causing chromium toxicity is hexavalent chromium and thus most studies studying inhibition look at that species (102), the Canadian Council of Ministers of the Environment sets recommendations for total

chromium levels as well (100). Nickel has also been found to have many impacts on microbes, such as growth inhibition, impacts on cell surfaces, DNA replication, and disrupting several metabolic activities such as nitrogen fixation (103).

Several reasons are possible for the difference in the levels of these metals at the 3 sites. Aluminium sulfate is an efficient chemical coagulant to remove both nickel and chromium from water (104). As such the treatment occurring at the water treatment plant may be leading to an increased concentration of both nickel and chromium in the sludge which is being disposed at Site 2. The concentrations at the 3 sites may also naturally vary.

The starting TPH concentrations at Sites 1 and 3 are lower than the concentrations at Site 2. The information available assumes entrance of contaminants occurs close to Site 1. It is possible that the starting TPH concentration at Site 2 was higher than at the other sites because degradation at Site 1 and 3 occurred more readily-in situ due to lower levels of these metals. This is a correlation that the data suggests. But to have a more precise understanding of how these metals are influencing the bacteria, information on metal speciation would be necessary, which can be analytically challenging to gather.

3.5 Impacts of nutrients, total organic carbon, and depth

Adding nutrients did not help degradation in any of the sites or conditions. On the contrary, there seems to be a delay in the onset of degradation occurring at Site 2 under both summer and winter conditions when nutrients were added. The final levels were similar, but it took longer for decreases to become statistically significant when nutrients were added. It is possible that the excess nutrients present in the beginning of the experiment caused inhibition of degradation at the start of the study. The treatability experiment was very long and as such this would not have

any impact on the overall hydrocarbon biodegradation extents. The qPCR results do not suggest such a trend is occurring, as the levels after one month are similar when nutrients and no nutrients were added. But given the qPCR results are only for month 1 and 6 and no point in between, a dynamic understanding of how the populations change over time cannot be concluded, and as such the possibility that nutrients may be inhibitory cannot be negated based on the qPCR results alone. Salinity has been found to inhibit degradation of hydrocarbons in the literature; and adding too many nutrients has also been found to be inhibitory to petroleum bioremediation (14, 19).

Some patterns at Site 3 which has over double the starting original total organic carbon content differ from the other sites. Whilst the microcosm set-up either had no impact or increased the levels of 16S genes in both Site 1 and 2, this was not the case for Site 3 (Figure 10). At Site 3, the levels went down in all cases except for the 1st month in the summer. Site 3 has more bacteria at time 0 (higher 16S) than both Site 1 and 3. The pond is blocked off from Lake de Gras right after Site 3, and the wastewater treatment plant would cause flow in the direction of Site 3. As such, Site 3 receives more inputs than Site 1 and 2, which may be a likely reason for its higher TOC. Setting up the microcosms isolated Site 3 from its surroundings, and it did not receive as many new inputs, as such it was likely unable to maintain the same quantity of bacteria as in-situ, as can be seen by a decrease in the levels of 16S.

This decrease in 16S numbers owing to reduced inputs over time may also be a reason for the biphasic-like kinetics observed at Site 3. Whilst the hydrocarbons may not be the most easily available carbon source for degraders at the start of the experiment, depletion of other carbon sources raises the incentive for the thermodynamic cost that will be solubilization/consumption of these hydrocarbons. As such we saw a period of no degradation whilst the bacteria were being

sustained on other sources, then a period of slow degradation when bacteria able to eat hydrocarbons started targeting the contaminants. Still given not all bacteria are hydrocarbon degraders, we still see a decrease in metabolism as seen by the 16S levels.

Site 3 had more degradation than the deeper Site 1 even though the original TPH concentrations were similar. This was expected given there were more microbes at Site 3 in the original sediments. Surprisingly, adding nutrients led to an increase in 16S numbers in month 1 for Site 1 for both the summer and the winter. The sediments at Site 1 would have been the most anoxic in the pond, due to their depth. As such, the oxygenation that would have occurred during the vigorous mixing while microcosms were set-up may have caused the biggest disturbance to Site 1, and a rapidly adapting community may have used the added nutrient source more readily. Another unique difference for Site 1 suggests that the impact of set-up drastically changed the microbial environment at month 1 but this disappeared over time. The original community at Site 1 was more anoxic than at the other two sites due to its depth. Whilst the aerobic summer systems had higher 16S levels than the anoxic winter systems at Site 1, this was no longer the case after 6 months, the anoxic winter systems had just as many 16S genes as the aerobic summer systems. The community developed under anoxic conditions in-situ at Site 1 and as such likely returned to original functioning over time after the original disturbance.

But if the microbes at Site 1 did use the extra nutrient source at month 1, why was there no significant hydrocarbon degradation? Were there limitations in bioavailability or biodegradability of the remaining hydrocarbons?

3.6 Biodegradability, bioavailability, and residual hydrocarbons

There is a decrease in rate occurring over time at Site 2 as visible in Figure 9 and Figure 11. This may be due to bioavailability limitations, limited biodegradability of the remaining compounds, or likely both. The final concentrations in the treatability experiments for Site 2 were similar to the starting concentrations of Site 1 and Site 3. At Site 1 and 3 we see a much slower degradation. As such, it is possible that more easily biodegradable hydrocarbon components are degraded until around 700-1000ppm, and that the rest is less easily degradable.

Another possibility may be that there are bioavailability limitations. Whilst the clays were thoroughly mixed prior to set-up, the <5mins mixing occurring weekly was at a slow rpm and no resuspension of clays occurred, thus unlikely to overcome any bioavailability limitations. We saw a reduction of the reaction rate over time for Site 2 in Figure 11. This could be due to any sorbed hydrocarbons having been resuspended during microcosm set-up resorbing to the sediments over time.

A possibility to remedy this could be to lower salinity in the overlaying water. As can be seen in SI Figure 2, diluting the pond water with deionized water and thus reducing the conductivity/salinity increases the colloidal stability of the clay suspensions, as reflected in the change in ζ-potential of -19.19 ± 1.29 mV for pure pond water to -26.46 ± 2.10 mV in deionized water. In a real site with a constant water influx that would disturb the sediments, a lower salinity would lead to a less packed sediment bed. This may lead to overcoming bioavailability limitations.

3.7 Community analysis

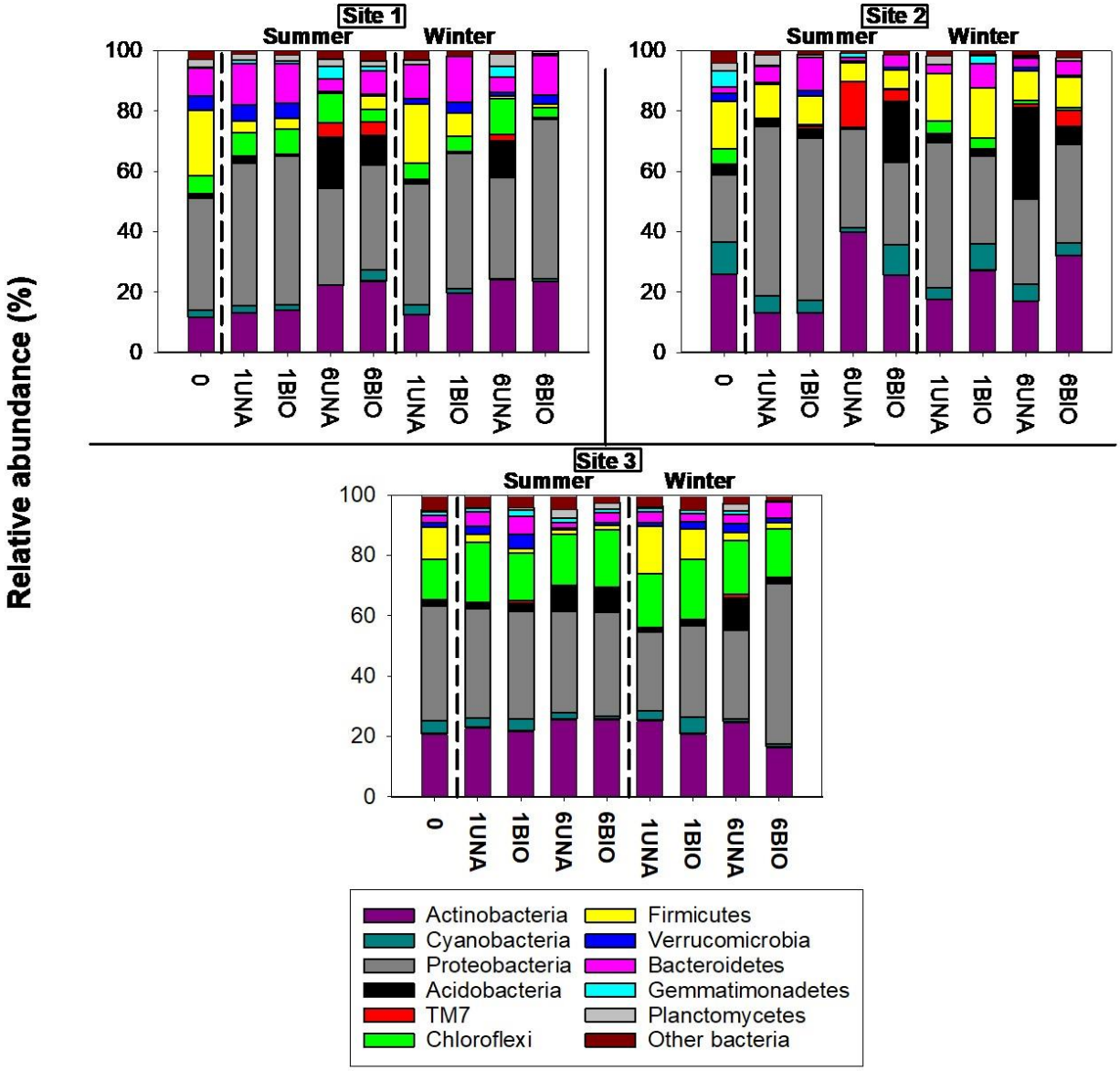


Figure 13: Most represented phyla. The most representative phyla are represented (above 2% of total in any of the samples). The number stands for the month, "UNA" stands for unamended, and "BIO" stands for biostimulated.

Actinobacteria and Proteobacteria are the most abundant phyla for all the systems. Proteobacteria is the most abundant Phylum for all conditions at Site 1 and 3. At Site 2, it is Proteobacteria

except at time 0 as well as in both the unamended and the biostimulated conditions in the summer at month 6 where it is Actinobacteria.

For Site 2 In the summer, there is a statistically significant increase in Proteobacteria between month 0 and month 1, from an average of 22.5 ± 4.70% at time 0 to an average of 55.0 ± 4.02% at month 1 (P=0.00087). The levels then returned to original levels, with the levels at month 6 being 30 ± 4.01%. The level at 6 months has no statistically significant difference compared to the original value at day 0, and the levels at month 6 are statistically lower than at month 1 (P=0.00012). As such, the levels of Proteobacteria seem to correlate with active degradation. The microcosm set-up stimulated the degradation by the addition of the water, resuspension of the sediments, and increased oxygen access, and Proteobacteria are higher at month 1 than original values. The degradation was then slower at month 6, which is correlated with lower Proteobacteria. Proteobacteria phyla is a well-known phylum for hydrocarbon degraders (64, 65) and seems to be correlated with hydrocarbon degradation activity in this study as well.

For Site 2 in the winter: Surprisingly, level of Proteobacteria were elevated compared to day 0 levels in the unamended systems which had a value of 48.2 ± 0.18% (P=0.0162), but not in the biostimulated systems which had levels 29.1 ± 5.23%. Given Proteobacteria is related with systems under high degradation in this study, the systems in the biostimulated systems being lower than in the unamended systems may be due to the added salinity when adding nutrients in these semi-frozen conditions having some inhibitory effect as mentioned previously (14, 19).

Attributing specific degradation pattern to community switches in Site 1 and 3 may be more difficult given the trends in hydrocarbon degradation are less clear. A clear trend that can be seen is that Chloroflexi is more abundant in Site 3 than the others.

4. Conclusion

This study investigated the role of biostimulation in three sediments in a water management pond for the wastewater treatment plant of a mine in sub-artic Canada. Degradation was observed both at 5°C under aerobic conditions and at 0-1°C under anoxic conditions in microcosm studies. There were correlations between levels of nickel and chromium and bacterial inhibition, and nutrients did not increase hydrocarbon biodegradation extents at any of the 3 sites. Proteobacteria was found to be an important phylum for degradation of F3 hydrocarbons in these conditions. The levels of hydrocarbons were concluded to plateau around 700-1000ppm, where either bioavailability or biodegradability limitations slowed down the bioremediation. There has not been a lot of work done in understanding hydrocarbon bioremediation in cold weather freshwater sediments. This research along with future projects investigating this will help gain an a priori understanding of bioremediation in cold-weather freshwater sediments and help inform on-site treatment.

5. Supplementary information

5.1 Hydrocarbon extraction and GC-FID parameters

3.05 grams of the sediment slurry were removed from the microcosms for storage and stored in sterile Eppendorf tubes at -80°C for future analyses. Then, anhydrous magnesium sulfate (Fisher chemicals) was gradually added to the sample on ice and the mixture was mixed with an ethanol-washed spatula. This was done until the sediments were dry, as seen by the sediments becoming a dry powder. The drying step was essential, and it was determined during extraction trials that excluding this step led to low hydrocarbon levels, likely due to the high level of interaction between water and the charged clayey-sediments, leading to low solvent intrusion into the clays and thus poor contact time with the hydrocarbons. 40 mL of hexane and 50 µL of 5-α androstane (a chemical surrogate standard) were then added to the serum vials. The vials were then crimped again and sonicated 2 * 10 minutes, ensuring that the temperature of the water never exceeded 30°C. The vials were then shaken at 18 °C and 180 rpm in a vertical shaker overnight (14 hours). The vials were then opened, and 1.5 g of silica was added to the microcosms. The vials were placed on the shaker at the same settings for 20 minutes. The systems were decanted, then 10mL of hexane was added to the empty microcosms and the systems were decanted again to collect any remaining hydrocarbons. The extracts were run in glass columns containing a thin glass wool plug to remove any remaining silica/dirt residues. The sample was evaporated down to 2 mL using a N_2 evaporator, and 1 mL was loaded into auto-sampler tubes and quantified using an Agilent 5890 Gas-chromatography flame ionization detector (GC-FID) fitted with a DB-1 column (30 m x 530 µm x 5 µm). The injection temperature was 280°C in a splitless injection mode with a helium career gas, with a column flow of 10 mL/min a Run time of 37 minutes and a detector temperature of 300°C. The oven program was as follows: Hold at 40°C for 1min, Temperature ramp from 40°C to 280°C at the rate of 15°C/min, Hold at 280°C for 20min.

5.2 qPCR primers mastermix composition and thermocycling conditions

Ba27F	3’-AGAGTTTGATCMTGGCTCAG-5’
Ba1492R	3’-GGYTACCTTGTTACGACTT-5’
Ba519F	3’-CAGCMGCCGCGGTAATWC-5’
Ba907R	3’-CCGTCAATTCMTTTRAGTT-5’

Primer pair Ba27F/Ba1492R: each 20 µL reaction contained 10 µL of iTaq Universal SYBR Green Supermix (BioRad), 5% Dimethyl sulfoxide (Fisher), 500 nM of each primer, and 1 µL of environmental DNA from the site. The thermocycling parameters were denaturation at 95°C for 3 minutes followed by 40 cycles of amplification (20 s at 95 °C, 30 s at 50 °C, and 105s at 72 °C).

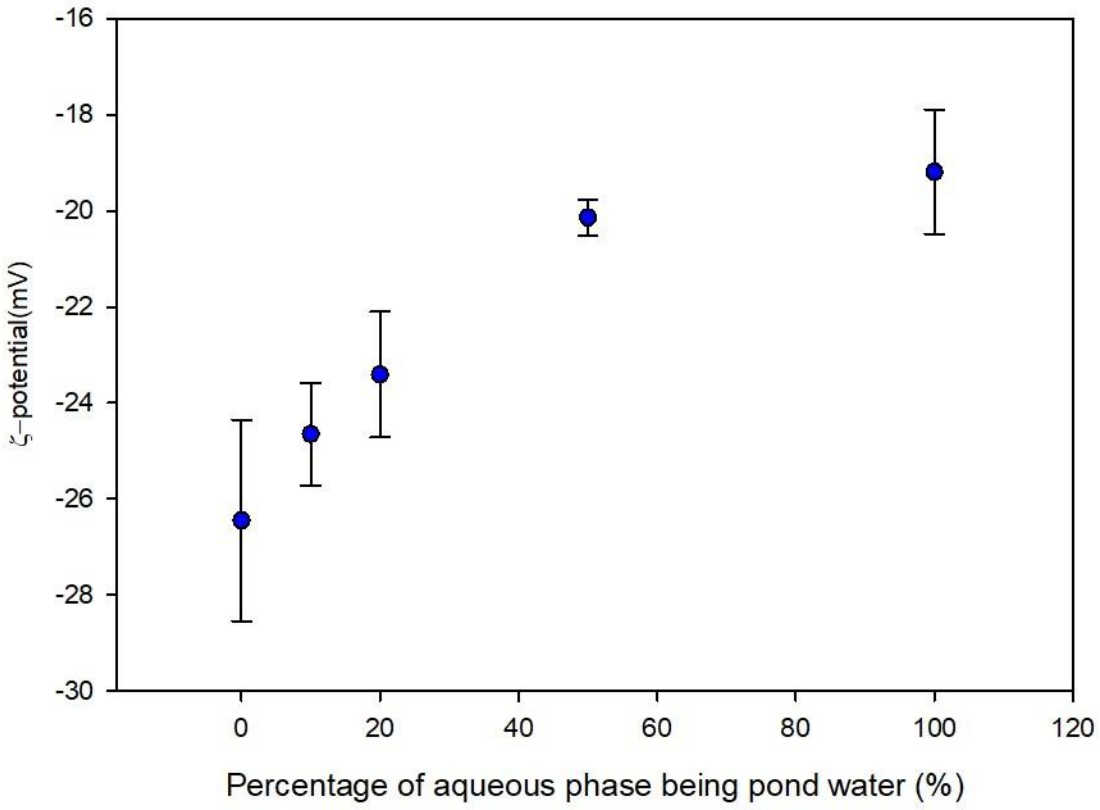
Primer pair Ba519F/Ba907R: Each 20µL reaction contained 10 µL of iTaq Universal SYBR Green Supermix (BioRad), 5% Dimethyl sulfoxide (Fisher), 500 nM of each primer, and 1 µL of sample DNA, or the synthetized standard (10^{0}-10^{-5}). The thermocycling parameters were denaturation at 95°C for 3 minutes followed by 40 cycles of amplification (10 s at 95 °C, 30 s at 50 °C, and 60 s at 72 °C).

5.3: Analytical methods from Bureau Veritas

SI Table 5: Analytical and laboratory methods bureau veritas tests

Test	**Analytical Method**
Chlorides (mg/kg)	MA.300–Ions 1.3 R3 m (73)
Nitrates N-NO3 (mg/kg)	MA.300–Ions 1.3 R3 m (73)
Total Kjeldahl Nitrogen (mg/kg)	MA.300–NTPT 2.0 R2 m (71)
Orthophosphate P (mg/kg)	MA.303–P 1.1 R2 m (105)
Total Phosphorus (mg/kg)	MA.200–Mét. 1.2 R5 m (106)
Sulfates (SO4) (mg/kg)	MA.300–Ions 1.3 R3 m (73)
Total organic carbon (TOC) (% g/g)	MA.310–CS 1.0 R3 m (70)

5.4 ζ-potential of sediment suspensions



SI Figure 2: ζ-potential of 800mg/L sediment nanoparticles solutions in pond water dilutions. The x-axis refers to percentage of the aqueous phase being pond water, with the remaining percentage being deionized water.

Thesis overall discussion and conclusions

This whole thesis focuses on bioremediation of hydrocarbons in northern sites in sub-arctic Canada. The matrix for the first chapter is a soil freshly contaminated by a hydrocarbon source that is primarily size F2 (C10-C16). The second chapter uses sediments with an aged F3 hydrocarbon source (C16-C34). Whilst we saw some relative success in remediating hydrocarbons in both chapters, the timescale of remediation drastically differed.

The relative degradability of different hydrocarbon has been studied in the literature and can vary depending on the sources. For instance: N-alkanes are generally considered most easily degradable, whilst cycloalkanes have been found to be much more resistant to microbes (107). Another study found that for aromatic hydrocarbons, compounds with a larger number of rings (hence heavier) were more difficult to degrade (108). Petroleum mixtures with different size fractions also exhibit different degradability (107). For instance, Walker et Al. found more degradation in lighter petroleum sources. They found less degradation in a light no 2 fuel oil than a heavy no 6 fuel oil, and less degradation in a heavy crude oil then a light crude oil. Our results do show slower degradation occurring in the site at chapter 2, which had a heavier oil than chapter 1. This may be due to the hydrocarbons present at the sites in chapter 2 being less degradable than arctic diesel. It may be due to specific characteristics of the site. It may also be due to bioavailability differences between the two sites, as caused by contaminant aging and the smaller size of the particles in chapter 2. Bioavailability has been shown to impact biodegradation. For instance, using a northern clayey soil, a study found that degradation occurred more readily in macroaggregates(>2 mm) than mesoaggregates (0.25-2 mm), and that the macroaggregates had an order of magnitude higher quantity of pores that could be accessible for bacteria (> 4 μm) (109).

It may be argued that whilst having an already contaminated sediment for chapter 2 gave a more realistic view of on-site treatability, the study conditions of chapter 1 may be more realistic. Chapter 1's treatability study occurred over 52 days. The incubation temperatures selected from surveying environment Canada data corresponded to 2-4 months period. As such the in-situ remediation timelines corresponded with our treatability studies. Instead, the temperatures were kept constant for up to 9 months in chapter 2. This enabled us to see the impact of long-term incubation at these constant temperatures, as may perhaps be observed by several years of a treatability project. But the results from chapter 1 suggest that microbiology and soil properties are heavily impacted by changes in soil temperature in hydrocarbon degradation projects. As such, repeating the experiment in chapter 2 still considering the same temperature and conditions and the passage of several years but with realistic temperature cycles (e.g., 2-4 months summer followed by fall then winter) may yield different results. Whilst that is the case, this simplification and constant temperature incubation allowed a large-scale understanding of what was occurring over a long period of time in these systems. Whilst this is unlikely to give a perfect representation of what is occurring at the site, it can give valuable insights for site-scale up of the treatability, which was a key purpose of the second chapter of this thesis.

Microcosms tend to overestimate hydrocarbon biodegradation rates. Small quantities of soil/sediments lead to optimized oxygen and nutrient distribution. The small quantities also limit the quantity of soil/sediment packing and the bioavailability limitations associated with it. For instance, a study comparing bioremediation treatability between slurry reactors and a biopile in a crude oil-contaminated clayey soil from a sub-arctic site found that the same endpoints that were attained in 30 days in the slurry reactors were attained in 285 days in the biopile (20). While the difference for unsaturated microcosms such as used in chapter 1 may not be as dramatic, using

the smaller volumes still may lead to overestimations of biodegradation. Although using small volumes enables many variables/hypotheses to be studied simultaneously, there remains a need to test the situations that were successful in microcosms at a larger soil/sediment volume. Doing larger-scale pilot-scale experiments prior to on-site treatments may help assess how realistic the microcosms were and extrapolate whether the conclusions would hold-up at a real site. Similarly, it may be important to understand how all the microbiological measurements done in this study differ when scale-up occurs.

It's important to situate the two chapters within their environmental surroundings. Firstly, to understand how characteristics specific to each site may influence the patterns seen in these studies. For instance, for chapter 2. The three sites were incubated using the same experimental parameters. But the freshwater pond receives many inputs (e.g., mine water, sludge) that may cause differences in the motion of the water at each site. Given the small size of the clayey sediments and their colloidal stability, differences in water motion and level of disturbance may cause different levels of suspension and resulting bioavailability in the three sites. Similarly in chapter 1 the site in the different temperatures may have extra differences that could cause deviation from the trends seen in the chapter. For instance, does the site receive more visitors (both human and animal) in the summer, which could cause more disturbance to the soil? Gathering more data and investigating how unique factors at each site influence study variables may help gain a more accurate understanding of how our findings will translate to in situ treatability projects.

Another reason why it is important to situate the experiments within their surroundings at the real sites is the following. Guidelines for safe hydrocarbon level are general, but a certain contamination level may be relatively dangerous at one site, and relatively harmless at another.

This may be due to several reasons. For instance, the chemical composition of the hydrocarbons may be different, and some specific compounds have been associated with larger dangers than others (2). The chemical parameters of the soil/sediment may also impact the danger of the contamination. The degree to which a soil/sediment associates with the hydrocarbon source and also to which hydrocarbons the soil/sediments associate will influence both the bioavailability and mobility of the contaminants. While a lower bioavailability will often mean a lower degradability, it may also decrease the extent to which the contaminant will biomagnify in the food chain. The sensitive receptors that may be impacted by the contaminants is also an important consideration when designing future studies to investigate how the results of this thesis may be influenced by the site. The site at chapter 1 is in a soil in a village. The sites in chapter 2 are in a freshwater pond in a remote industrial site. There will be more immediate sinks for the soil in chapter 1, and perhaps one extra study that could be done is to monitor the air in the microcosms to understand how the degradation may influence what will be volatilized and how this may impact the biota surrounding the site. The sediments in chapter 2 are upstream of Lake de Gras, which has several biota, for instance trout (110). It may be important to further study the mobility of the contaminant in water and how they transiently associate to the clays to understand contaminant travel and the biomagnification that may occur because of it.

We saw significant bioremediation in both chapters. But while we know that the levels go down, we do not know what is left as well as what the transformation products/metabolites are. Being able to answer this would aid the interpretation of many of the results in this thesis. For instance, it may help give a larger understanding as to why in chapter 1 there was a plateauing in hydrocarbon biodegradation occurring at 7°C when nutrients were added (system DWNP). Was it that the metabolites produced from the start of the degradation are good alternative carbon

sources for bacteria to feed on? Or perhaps, the remaining hydrocarbons were more largely composed of hydrocarbons we know are difficult to degrade. Similarly, being able to characterize the hydrocarbons remaining after a plateau has been reached in the treatability studies in chapter 2 could help give a better understanding for why the plateau is occurring. In turn, this may help identify ways to drive the levels even further down. Currently, answering these questions for such a complex mixture as are petroleum hydrocarbons is analytically challenging.

In chapter 1, we analyzed the dynamics of both cDNA and DNA for the 16S and alkB gene, as well as the community dynamics of total and active microbes. This enabled us to build theories to attempt to gain an a-priori understanding of why bioremediation was successful in some situations and not others. In chapter 2, we did not conduct such extensive analyses, and whilst some microbiological understanding was gained regarding differences occurring at the sites, it was nowhere as dynamic and complete. Key reasons why such extensive analyses were not conducted were the following: the sediment matrix was difficult to handle and steps to extract TPH and DNA both required optimization and significantly more effort than at chapter 1, so RNA was not attempted. This is a limitation of many analyte extractions in environmental samples, the chemical properties of specific matrices may make extractions more difficult. Secondly, there is less RNA (used to make cDNA) than DNA present in soils and sediments. As such it may only be feasible to analyze cDNA in metabolically active soils and for a limited number of genes. The soil from chapter 1 was metabolically active and had enough RNA, whilst some sediments in chapter 2 (e.g., Site 2) had limited microbiota. Applying more biochemical methods could give increased information and help build stronger theories and a priori understanding of why treatability differs at different sites and under different conditions. For

example soil metaproteomics may be a useful way to further an understanding of how different factors will change the metabolism (111). But again, analytical challenges may mean that such methods can not be easily applied to all sites and situations.

Surprisingly, although the two chapters presented in this study studied very contrasting sites, there are some common conclusions. Firstly, if nutrients are not necessary, adding some will not cause any enhancement in degradation and may even cause some inhibition. While degradation is still possible in the coldest temperatures we studied, namely -5°C for chapter 1 and 0-1°C for chapter 2, temperature has a role in influencing hydrocarbon bioremediation. As such bioremediation treatability plans at a site should be assessed separately for different temperatures. Proteobacteria was concluded to be a very important phyla for degradation at both sites. The information presented in this thesis is important for implementing site treatments in real sites in sub-arctic Canada, but also asks important questions on parameters that influence bioremediation. This is useful as it serves to increase the knowledge base on cold climate hydrocarbon bioremediation, which will help gain an a priori understanding on a cold site's treatability potential.

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