Molecular characterization of the hydrocarbon biodegradation process on Canadian Arctic beaches

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List of abbreviations

ANI: average nucleotide identity

ASV: amplicon sequence variant

BIOS: Baffin Island Oil Spill

CAA: Canadian Arctic Archipelago

db-RDA: Distance-based redundancy analysis

DCM: dichloromethane

DMSP: dimethylsulfoniopropionate

DNRA: dissimilative nitrate reduction to ammonium

eDNA: extracellular/environmental DNA

Faith's PD: Faith's phylogenetic diversity

GTDB: Genome Taxonomy Database

HFO: heavy fuel oil

HPM: hits per million genes

iDNA: internal DNA

IMO: International Maritime Organization

ITOSS: In situ Treatment of Oiled Sediment Shorelines

LNG: liquefied natural gas

LSFO: low sulfur fuel oil

MAG: metagenome-assembled genome

MAH: monocyclic aromatic hydrocarbons

MAP: monoammonium phosphate

MARPOL: International Convention for the Prevention of Pollution from Ships

MGO: marine gas oils

ML: maximum likelihood

MPRI: Multi-partner Research Initiative

NMDS: Non-metric multidimensional scaling

NWP: Northwest Passage

PAH: polycyclic aromatic hydrocarbon

PHCs: petroleum hydrocarbons

rTCA cycle: reverse tricarboxylic acid cycle

SECA: sulfur emission control area

SVOCs: semi-volatile organic compounds

TPH: total petroleum hydrocarbons

ULSFO: Ultra Low Sulfur Fuel Oil

VLSFO: very low sulfur fuel oil

VOCs: volatile organic compounds

Abstract

The warming effect of climate change is causing a decrease in sea ice across the Arctic Ocean, especially in the Canadian high Arctic. This is expected to cause an increase in shipping traffic through the Northwest Passage. This comes with the risk of environmental damage to the region with one of the largest concerns being a hydrocarbon spill from a passing ship. Due to the remoteness of the Canadian high Arctic, the response in case of such a spill will be slow and the spilled hydrocarbons might reach the shoreline. Simpler remediation strategies could be preferrable due to the limited resources available for a cleanup in this area. One of said strategies is bioremediation using the native microbial communities that inhabit these beaches. Limited research has been carried out to understand the hydrocarbon biodegradative capabilities of Arctic microbes on shorelines. This limits our understanding of the microbial community and the metabolic processes these microbes are carrying out in response to the presence of hydrocarbons in their environment. This is crucial information that needs to be obtained to understand whether bioremediation will be an effective cleanup strategy. This thesis aims to narrow this knowledge gap by utilizing state-of-the-art molecular techniques to describe the potential and actualized *in situ* hydrocarbon biodegradation capabilities of Arctic shoreline microorganisms.

Initially, I first performed a metagenomic survey of the baseline microbial communities inhabiting 9 Arctic beaches from 4 regions of the Canadian Arctic archipelago to understand the genomic potential available in those beaches before any spill has occurred. The presence of known hydrocarbon-degrading taxa as well as genes involved in hydrocarbon degradation pathways, especially those associated with the degradation of short, medium, and long-chain alkanes was identified. I then compared metagenome-assembled genomes from the survey with genomes of isolates obtained from the same beaches that can grow using hydrocarbons as a source of carbon.

To further corroborate the results of the survey, *in situ* mesocosm experiments consisting of hydrophobic netting covered with three fuels commonly used by the shipping industry: Marine diesel, Bunker C, and Ultra Low Sulfur Fuel Oil (ULSFO) were performed. These nettings were deployed in Assistance Bay (Cornwallis Island, Nunavut) for a month. I then performed 16S rRNA gene amplicon, metagenomic, and metatranscriptomic sequencing on the netting to characterize how the native microbial community of this beach responds when fuels are added to the beach. A shift in the community composition towards taxa commonly associated with hydrocarbon degradation as well as the presence of multiple genes these taxa are using to metabolize aliphatic and aromatic hydrocarbons was observed. Analysis of the remaining fuel showed that 14 – 78% of the compounds were biodegraded, with Marine diesel having the highest degradation and Bunker C having the lowest.

Finally, a second round of *in situ* mesocosm experiments was performed to determine whether allowing biodegradation to occur for a whole year along with the addition of fertilizers would further stimulate the Assistance Bay beach microbiota and lead to higher Marine diesel and ULSFO biodegradation. After a year, I observed more defined differences in the microbial communities of the fuel treatments compared to the controls, but no noticeable differences between the communities of the two fuels, suggesting that similar microbes can metabolize both kinds of fuel. There was no effect of the addition of fertilizers for the microbial composition or the biodegradation performance. The increased duration of the experiment also did not result in more of the fuel being biodegraded, with similar percentages observed after a year (33 – 72%) which could have been caused by the decrease in metabolic activity of most microbes under the sub-zero temperatures experienced in the prolonged Arctic winter.

Résumé

L'effet de réchauffement du changement climatique entraîne une diminution de la glace de mer dans l'océan Arctique, particulièrement dans le Haut-Arctique canadien. Cela pourrait causer une augmentation du trafique maritime au travers du passage du Nord-Ouest. Ce trafique s'accompagne d'un risque de dommages environnementaux pour la région, dont l'une des principales préoccupations étant la possibilité d'un déversement d'hydrocarbures après un accident de navire. En raison de l'éloignement du Haut-Arctique canadien, la réponse en cas de déversement sera lente et il y a de fortes chances que les hydrocarbures déversés atteindront le littoral. L'utilisation des stratégies de remédiation les plus simples pourraient être privilégiée en raison des ressources limitées disponibles pour un nettoyage de la zone. Une de ces stratégies est la bioremédiation, qui fait appel aux communautés microbiennes indigènes qui habitent les plages de l'Arctique canadien. Peu de recherches ont été menées pour comprendre les capacités de biodégradation des hydrocarbures des micro-organismes arctiques sur les littoraux. Cela limite notre compréhension des changements dans les communautés microbiennes et des processus métaboliques que ces micro-organismes mettent en œuvre en réponse de la présence d'hydrocarbures dans leur environnement. Ce sont des informations cruciales qui doivent être obtenues afin de comprendre si la bioremédiation sera une stratégie de nettoyage valable. Cette thèse vise à combler ce manque de connaissances en utilisant des techniques moléculaires de pointe pour décrire les capacités potentielles et réelles de dégradation des hydrocarbures *in situ* des micro-organismes du littoral de l'Arctique.

J'ai d'abord réalisé une étude métagénomique des communautés microbiennes de référence de 9 plages arctiques de 4 régions de l'archipel arctique canadien afin de comprendre le potentiel génomique actuellement disponible sur ces plages avant qu'un déversement ne se produise. J'ai constaté la présence de taxons connus pouvant dégrader les hydrocarbures ainsi que des gènes impliqués dans les voies de dégradation des hydrocarbures, en particulier ceux

associés à la dégradation des alcanes à chaîne courte, moyenne et longue. J'ai également comparé les génomes assemblés de métagénomes provenant de l'étude avec les génomes des isolats obtenus sur les mêmes plages arctiques pouvant se développer en utilisant des hydrocarbures comme source de carbone.

Pour corroborer davantage les résultats de l'étude métagénomique, j'ai effectué une expérience de mésocosme *in situ* composée d'un filet hydrophobe recouvert de trois carburants couramment utilisés par l'industrie maritime : le diesel marin, le Bunker C et le mazout à très faible teneur en soufre (ULSFO en anglais). Ces filets ont été déployés sur le littoral de la baie d'Assistance (Île Cornwallis, Nunavut) pendant un mois. J'ai ensuite procédé au séquençage d'amplicon du gène d'ARNr 16S, à la métagénomique et à la métatranscriptomique sur les filets afin de caractériser comment les communautés microbiennes indigènes de cette plage répondent à l'ajout de carburants sur la plage. J'ai observé un changement dans la composition de la communauté vers des taxons communément associés à la dégradation des hydrocarbures, ainsi que la présence de multiples gènes que ces taxons utilisent pour métaboliser les hydrocarbures aliphatiques et aromatiques. L'analyse du carburante restant montre que 14 à 78% des composés étaient biodégradés, dont le diesel marin ayant la plus forte dégradation et du Bunker C ayant la plus bas.

Finalement, j'ai effectué une deuxième série d'expériences de mésocosme *in situ* pour déterminer si le fait de laisser la biodégradation se produire pendant une année entière avec l'ajout d'engrais stimulerait davantage le microbiote de la baie d'Assistance et conduirait à une biodégradation plus importante du diesel marin et de l'ULSFO. Après un an, j'ai observé des différences plus marquées dans les communautés microbiennes des traitements de carburant par rapport aux traitements témoins, mais aucune différence notable entre les communautés des deux carburants n'a été observé. Ce qui suggère que des micro-organismes similaires peuvent métaboliser les deux types de carburant. L'ajout d'engrais n'a eu aucun effet sur la composition

microbienne ou sur les performances de biodégradation. L'augmentation de la durée de l'expérience n'a pas entraîné une augmentation de la proportion de carburant qui a été dégradé avec des pourcentages similaires observés après un an (33 à 72%). Ceci pourrait être dû à la diminution de l'activité métabolique de la plupart des micro-organismes sous les températures inférieures à zéro enregistrées au cours de l'hiver arctique prolongé.

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Thank you, everyone. I hope I have made you all proud.

Contribution to original knowledge

This thesis contributes to the current knowledge of oil remediation and biodegradation in Arctic environments. Specifically:

1. In Chapter 2, I described the baseline metagenomes of 9 tidal beaches in the Canadian high Arctic. This study described:
    a. The presence of key genes and complete pathways for the hydrocarbon degradation in the absence of hydrocarbons in these environments.
    b. The first instance where state-of-the-art molecular methods are used to describe the hydrocarbon metabolic capacity of Arctic shorelines and provides a reference of the current state of these ecosystems before any contamination due to an oil spill has ever occurred.
2. In Chapter 3, I performed the first *in situ* experiment evaluating the biodegradability of the new generation of low sulfur fuel oils under Arctic conditions and compare their biodegradative potential against current and legacy fuels. For this study:
    a. The first metagenomic and metatranscriptomic description of the hydrocarbon biodegradation process on high Arctic beach sediments was performed.
    b. Evidence of the limited capabilities of the beach microbiome to degrade polycyclic aromatic hydrocarbons (PAH) was provided. This explains why the low sulfur fuels, which contain a reduced amount of these types of compounds, are more efficiently degraded than the PAH-rich legacy fuels.
3. In Chapter 4, I performed a follow-up study to the *in situ* experiment of Chapter 3 in which I attempt to improve the biodegradative capacity of the shoreline microbes by increasing the incubation time to a year and by adding N and P fertilizers to supplement the nutrient-poor conditions present on these beaches. This study:

a. Described for the first time the long-term biodegradation process of the tested ultra-low sulfur fuel oil and how after a year, other processes such as physical removal due to tidal action have higher effect than biodegradation.
b. Contributes to our knowledge of the reduced effectiveness of the current cleanup techniques available to oil response teams in Arctic marine and shoreline environments.

Contribution of Authors

1. In Chapter 1, I am the first author of the literature review. Mira Okshevsky and I conceived and designed the structure of the review. Madison Ellis contributed with information for the section "Factors affecting biodegradation and bioremediation on shorelines in Arctic environments". Ya-Jou Chen contributed with information for the "Exxon Valdez, 1989" and "The microbial community and function associated with hydrocarbon degradation on Arctic environments" sections. Lyle Whyte provided guidance during the writing process.

2. In Chapter 2, I am the first author of the published manuscript. I conceived the study in along with Lyle Whyte. I designed the study and its methodology. Lyle Whyte, Charles Greer, and I collected the sediment samples. I carried out the DNA extractions and library preparation, performed and interpreted the data analysis, and wrote the manuscript. Antoine-Olivier Lirette contributed with the genome sequences of the *Rhodococcus* isolates used in the pangenome. All the authors reviewed and revised the manuscript.

3. In Chapter 3, I am the first author of the manuscript. Ianina Altshuler, Madison Ellis, Mira Okshevsky, and I conceived the study, designed the field experimental design, and deployed and collected the *in situ* mesocosms. I performed the nucleic acid extractions and the DNA, RNA, and 16S rRNA gene amplicon library preparations. I performed the data processing and analysis of the 16S rRNA gene, metagenomic, metatranscriptomic, and hydrocarbon datasets. I wrote the manuscript. Charles Greer and Lyle Whyte provided guidance during the manuscript writing process. Lyle Whyte also helped to oversee the experimental design.

4. In Chapter 4, I am the first author of the manuscript. Ya-Jou Chen and I conceived the study and designed the field experiment. Ya-Jou Chen, Nastasia Freyria, Antoine-Olivier Lirette, and I deployed and recovered the *in situ* mesocosms. I carried out the nucleic acid extractions and the 16S rRNA gene amplicon library preparation. I performed the data processing and analysis of the 16S rRNA gene amplicons and hydrocarbon datasets. I wrote the manuscript. Charles

Greer and Lyle Whyte provided guidance during the manuscript writing process. Lyle Whyte also helped to oversee the experimental design.

Introduction

In this thesis, I evaluated the *in situ* hydrocarbon biodegradation potential of the microbiota of beach sediments from shorelines in the NWP. The overall goal of this thesis is to characterize the hydrocarbon biodegradation process at a molecular level under environmental conditions characteristic of high Arctic beach sediment in order to understand if and under which conditions bioremediation could be used as a feasible cleanup strategy in case of an oil spill reaching a shoreline in the NWP. Throughout this thesis, I focus on beaches located around the Inuit community of Resolute, Nunavut, particularly on the remote beach known as Assistance Bay located 17 km away from Resolute. This work is also complemented with samples from other locations throughout the NWP to place a larger regional perspective on my results. This thesis takes advantage of state-of-the-art molecular biology tools, namely 16S rRNA gene amplicon sequencing, metagenomics, and metatranscriptomics, to characterize the current microbiome of shorelines in the high Arctic and to describe how these microbes would respond if an oil spill washed onto a beach located along the NWP.

The specific questions my research aims to answer with the three research chapters of this thesis, respectively, are as follows:

1. What is the composition of the baseline community of the sediment from a NWP beach? Does the microbiome of NWP shorelines have the molecular potential to degrade hydrocarbons? What are the molecular mechanisms that putative hydrocarbon-degrading microbes inhabiting high Arctic beach sediments perform in the absence of a large source of hydrocarbons in their environment?
2. Can various types of fuels used by the shipping industry be biodegraded *in situ* when introduced onto a NWP shoreline? Are the new LSFOs more biodegradable than current and legacy fuels under high Arctic environmental conditions? Which molecular

pathways are expressed by NWP beach sediment microbes in their natural environment in the presence of hydrocarbons?

3. Is it possible to improve the biodegradative capacity of the microbiome of a NWP beach by increasing the contact time with shipping fuels? Does the use of fertilizer additions further stimulate the hydrocarbon bioremediation potential of high Arctic microorganisms? What are the changes in the native microbial community from a NWP shoreline that occur after a year of contact with a simulated fuel spill?

In Chapter 1, I provide a literature review of the historical perspective of the state-of-the-art of bioremediation as a cleanup strategy for hydrocarbon spills on Arctic and cold marine and shoreline environments. A description of recent advancements in molecular biology that have helped researchers understand how microorganisms grow and degrade hydrocarbons is also provided. In Chapter 2, a metagenomic survey was used to understand the baseline molecular potential of the native microbial community of 9 high Arctic beaches to degrade hydrocarbons. In Chapter 3, the hydrocarbon biodegradation capability of the microorganisms inhabiting a NWP shoreline was evaluated with an in situ mesocosm experiment. In Chapter 4, I build upon the results of Chapter 3 to understand how the biodegradation of shipping fuel can be improved with a long-term in situ biostimulation experiment.

Connecting text

The following literature review was published by Elsevier in:

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Chapter 1. Hydrocarbon bioremediation on Arctic shorelines: historic perspective and roadway to the future

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1.1. Abstract

Climate change has become one of the greatest concerns of the past few decades. In particular, global warming is a growing threat to the Canadian high Arctic and other polar regions. By the middle of this century, an increase in the annual mean temperature of 1.8°C – 2.7°C for the Canadian North is predicted. Rising temperatures lead to a significant decrease of the sea ice area covered in the Northwest Passage. As a consequence, a surge of maritime activity in that region increases the risk of hydrocarbon pollution due to accidental fuel spills. In this review, we focus on bioremediation approaches on Arctic shorelines. We summarize historical experimental spill studies conducted at Svalbard, Baffin Island, and the Kerguelen Archipelago, and review contemporary studies that used modern omics techniques in various environments. We discuss how omics approaches can facilitate our understanding of Arctic shoreline bioremediation and identify promising research areas that should be further explored. We conclude that specific environmental conditions strongly alter bioremediation outcomes in Arctic environments and future studies must therefore focus on correlating these diverse parameters with the efficacy of hydrocarbon biodegradation.

Keywords: biostimulation, polar environment, hydrocarbon spill

1.2. Introduction

1.2.1. Climate change and its effects on marine transportation

Climate change is the most important environmental issue of our times which has disproportionately affected and will continue to affect Canada more than the rest of the world (Bush and Lemmen, 2019). Between 1948 and 2016, while the rest of the world experienced a mean temperature increase of 0.8°C, the average temperature in Canada increased by 1.7°C (Bush and Lemmen, 2019; Vincent et al., 2015). The changes are even greater for Arctic regions which are warming at almost three times the global rate (Bush and Lemmen, 2019; Vincent et al., 2015). It has been projected that towards the end of the century, temperatures will be drastically increased by 1.8°C – 6.3°C for Canada and 2.1°C – 7.8°C for the north in particular (Bush and Lemmen, 2019). These observed and projected increases in temperature have a great impact on sea ice coverage. Reductions in summer sea ice area coverage between 6% - 15% per decade were observed over the past 60 years for different regions of the Canadian Arctic (Tivy et al., 2011) and it is expected that the Canadian Arctic Archipelago will be ice free during the summer months by 2075 (Laliberté et al., 2016).

This decrease in the sea ice area has led to higher shipping activity in Canadian Arctic waters. The total distance travelled by ships in this region has almost tripled between 1990 and 2015 (Dawson et al., 2018), mostly due to observed increases since 2007 (Pizzolato et al., 2016). The Northwest Passage (NWP) is a sea route connecting the Pacific and Atlantic Oceans through the Canadian Arctic Archipelago. The NWP is a very attractive route for the transport industry as it presents a ~30% reduction in distance travelled, compared to the Northern Sea Route following the Northern coast of Russia (Smith and Stephenson, 2013). For the southern route of the NWP, there is an increase of shipping activity of ~2.5 transit equivalents per year (Pizzolato et al., 2016). Expected increases in shipping across the Arctic may pose a danger of collisions and shipwrecks (Bush and Lemmen, 2019). This is mainly due to the fact that the

northern part of the Canadian Arctic Archipelago and Greenland will be the source of thick and highly ridged multi-year sea ice that can potentially drift southwards into the channels of the Northwest Passage (Haas and Howell, 2015). Other risks, such as the rise of storm frequency bringing increases in wave heights and wind speeds in the Arctic should also be considered (Liu et al., 2016; Wang et al., 2015). An increased risk of shipwrecks and collisions increases the likelihood of a hull rupture leading to accidental fuel release. This ultimately increases possibility that fuel will wash up on Arctic beaches leading to hydrocarbon contamination of coastal ecosystems and nearby Indigenous communities.

It is estimated that 22% – 47% of oil discharges into the marine environment worldwide are caused by ship spills (Chilvers et al., 2021; National Research Council, 2003). General shipping activities are responsible for 72% of light oil spills, 60% of intermediate oil spills, and 35% of crude and heavy oil spills (Chilvers et al., 2021). Given that the NWP is a remote route in a highly sensitive Arctic ecosystem with few communities throughout the area and sparse spill response infrastructure and manpower (Transport Canada, 2014), the feasibility of oil spill response strategies must be carefully considered. Particular importance should be given to the development of remediation strategies for shoreline cleanup as stranded oil on Arctic beaches and in intertidal sediments can persist for prolonged periods of time if no remediation method is applied (Lindeberg et al., 2018; Prince et al., 2002; Wang et al., 1995).

In this review, we first present an overview of the conventional approaches to hydrocarbon remediation. Secondly, we outline the main biodegradation pathways and the characteristics of high Arctic shorelines that can influence bioremediation. Next, we present the details of several experimental studies on bioremediation in cold marine shorelines, followed by examples conducted during actual high-latitude oil spills. We then provide an overview of how omics technologies can be incorporated to remediation strategies. Finally, we conclude by providing recommendations for future research avenues.

1.2.2. Oil spill cleanup strategies

When hydrocarbons come in contact with the marine environment, a fraction of the hydrocarbons are volatilized and/or dissolved (Tarr et al., 2016). The remaining components of the spilled fuel that arrive on the beach can be removed by natural mechanical factors such as wave dispersal and sediment abrasion (Owens, 1985, 1978). Additionally, there are many different methods that can be used by spill responders to remove hydrocarbons from contaminated sediments. These diverse technologies can be separated into four categories: thermal, physicochemical, biological, and chemical (Lim et al., 2016; Ossai et al., 2020).

Thermal remediation methods use high temperatures for the destruction of contaminants or their removal from the soil via desorption (Ossai et al., 2020). There are many variations of these technologies including incineration (Rushton et al., 2007), smoldering (Switzer et al., 2009), pyrolysis (Vidonish et al., 2016), thermal desorption (Ossai et al., 2020), and radio frequency heating (Price et al., 1999). Physicochemical methods aim to remediate soils by separating the contaminants from the soil particles using multiple physical and/or chemical approaches such as solvents (Silva et al., 2005), subcritical fluid extraction (Lagadec et al., 2000), supercritical fluid extraction (Meskar et al., 2018), soil vapour extraction (Al-Maamari et al., 2009), ultrasonic extraction (Flores et al., 2007), and electrokinetic degradation (Kim et al., 2010). Many of these thermal and physicochemical methods have proven to be highly effective at rapidly decreasing hydrocarbon concentrations in sediments in both laboratory and field settings. However, these methods may not be as feasible in the NWP due to the difficult environmental and logistical conditions.

Bioremediation harnesses endemic microbes and may also introduce foreign microorganisms and nutrients in order to stimulate the hydrocarbon degradation. Hydrocarbon biodegradation has been most widely studied in bacteria, but archaea, algae, and fungi are also capable of performing this metabolic process (Al-Nasrawi, 2012; Prince, 2005). Various

bioremediation strategies could be applied to Arctic shorelines including natural attenuation, biostimulation, and bioaugmentation. Natural attenuation is the simplest remediation strategy where endemic microorganisms use the introduced hydrocarbons as a carbon source and transform them into less toxic compounds, usually CO_2 and water (Kuppusamy et al., 2020). Biostimulation aims to improve biodegradation rates through the addition of nutrients that limit microbial metabolism in the contaminated area (Nikolopoulou and Kalogerakis, 2010). Bioaugmentation also aims to accelerate biodegradation rates by introducing previously isolated hydrocarbon-degrading microorganisms into the contaminated environment (Vázquez et al., 2013).

There are two major chemical methods that can be used to treat hydrocarbon contamination: chemical oxidation and application of dispersants. Chemical oxidation uses reactive radicals to degrade hydrocarbons into smaller and less toxic compounds (Lim et al., 2016; Ossai et al., 2020). Dispersants contain surfactants (amphiphilic organic surface active agents) and solvents that reduce the oil-water interfacial tension, allowing hydrocarbons to emulsify by the creation of micelles (Karlapudi et al., 2018; Lopes et al., 2018). These smaller hydrocarbon droplets trapped inside the micelles are more readily available for microorganisms to degrade them (Kumar et al., 2018). While there are success stories with the use of these type of chemicals, issues have also arisen (Kleindienst et al., 2015). Commercial dispersants are not effective on sediments once the hydrocarbons have penetrated into them (Kleindienst et al., 2015; Macías-Zamora et al., 2014). Many of the compounds in chemical dispersants could actually decrease microbial hydrocarbon degradation (Bruheim et al., 1999; Kleindienst et al., 2015). Dispersants can decrease the abundance of known hydrocarbon degraders (Hamdan and Fulmer, 2011; Kleindienst et al., 2015), with the synthetic surfactants being especially toxic as they interact with cell membranes (Kleindienst et al., 2015; Nagell et al., 1974). Some dispersants can also be preferentially biodegraded over the hydrocarbons they aim to help

disperse and degrade (Kleindienst et al., 2015; Lindstrom and Braddock, 2002). Finally, biofilm formation can decrease the efficacy of oil dispersion by chemical dispersants only seven days after oil application, suggesting a narrow window of opportunity to successfully apply dispersants (Omarova et al., 2019).

Given these issues, recent studies have focused on the development of more environmentally-friendly alternatives to chemical dispersants, such as leveraging the biotechnological potential of microbes (Perfumo et al., 2018). Bacteria naturally produce different types of surfactants, commonly known as biosurfactants, in order to increase the bioavailability of different compounds they are able to metabolize (Kurata et al., 2016). Biosurfactants are categorized into low molecular weight glycolipids and high molecular weight polysaccharides, proteins, lipopolysaccharides, and lipoproteins (Ron and Rosenberg, 2002). Promisingly, many marine and polar hydrocarbon degraders have been shown to naturally produce biosurfactants when they are cultured in a medium containing hydrocarbons (Abdulrasheed et al., 2020; Cai et al., 2014; Gesheva et al., 2010; Godfrin et al., 2018; Habib et al., 2020; Malavenda et al., 2015; Trudgeon et al., 2020; Yakimov et al., 1998). Biosurfactants can be more effective than chemical dispersants such as Corexit or SDS while having lower toxicity and higher biodegradability (Cai et al., 2021; Trudgeon et al., 2020). Biosurfactants are able to desorb hydrocarbons bound to particles (Ron and Rosenberg, 2002) and increase phenanthrene desorption in soils treated with freeze-thaw cycles (Yao et al., 2017). This could be a useful quality in Arctic environments that are subject to freeze-thaw events, but there are still issues that must be further studied regarding their application in Arctic environments. Biosurfactants are vulnerable to structural changes at low temperatures (Luong et al., 2018), leading to a decrease in micelle stability and a need to add larger amounts of biosurfactant to achieve the desired results (Zhu et al., 2021). The development of cold active biosurfactants by cold-adapted microorganisms to counter this weakness is proposed and

represents a promising area of current and future research (Habib et al., 2020; Kennicutt et al., 2015; Perfumo et al., 2018).

1.3. Bioremediation in Arctic shorelines

Considering that the time before detecting the presence of a spill and the arrival of a response team to a remote Arctic beach could be weeks, this could lead to hydrocarbons settling on the shoreline which would limit the effectiveness of traditional mechanical removal and containment methods. Given these circumstances, and with the logistical difficulties of carrying out more advanced remediation strategies in Arctic beaches, bioremediation presents itself as one of the most cost-effective and simple alternatives that can be applied to this type of shorelines. We will focus the rest of this article reviewing what is known about the bioremediation process, how Arctic conditions may affect it, and what has been learnt from previous oil spill bioremediation studies.

1.3.1. Bioremediation mechanisms

Crude oil contains a wide range of different hydrocarbons (Fig. 1.1) and requires various microorganisms to be involved in the biodegradation process. A wide range of microorganisms can degrade hydrocarbons under oxygenated and anoxic environments using diverse metabolic pathways (Fig. 1.2; Abbasian et al., 2016; Leahy and Colwell, 1990).

1.3.1.1. Aerobic hydrocarbon degradation

The first step for this type of metabolic processes involves the oxidization of the hydrocarbon by an electron-carrier-dependant oxygenase system that transfers one or two electrons to the contaminant (Abbasian et al., 2016; Bargiela et al., 2017; Sierra-Garcia and de Oliveira, 2013; Torres Pazmiño et al., 2010; Widdel and Musat, 2010a). Alkanes are degraded by monooxygenases which can be rubredoxin-dependent or cytochrome P450-containing enzymes (Abbasian et al., 2016). One of the most studied rubredoxin-dependent enzymes is

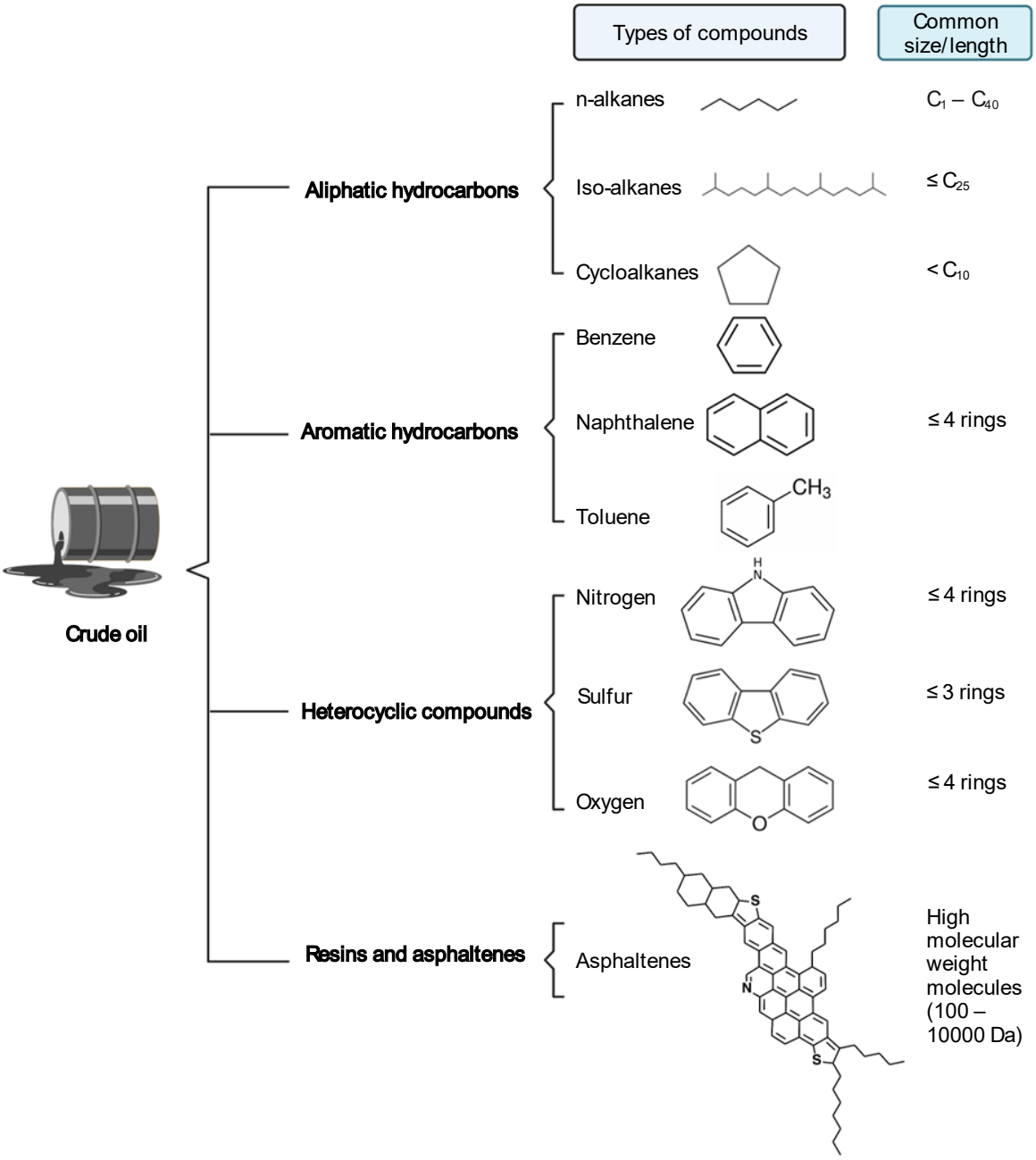

Figure 1.1. Chemical composition of crude oil. Adapted from Mahjoubi et al. (2017) with additional information from Acevedo et al. (2005) and Tissot and Welte (1984). Figure created with BioRender.com.

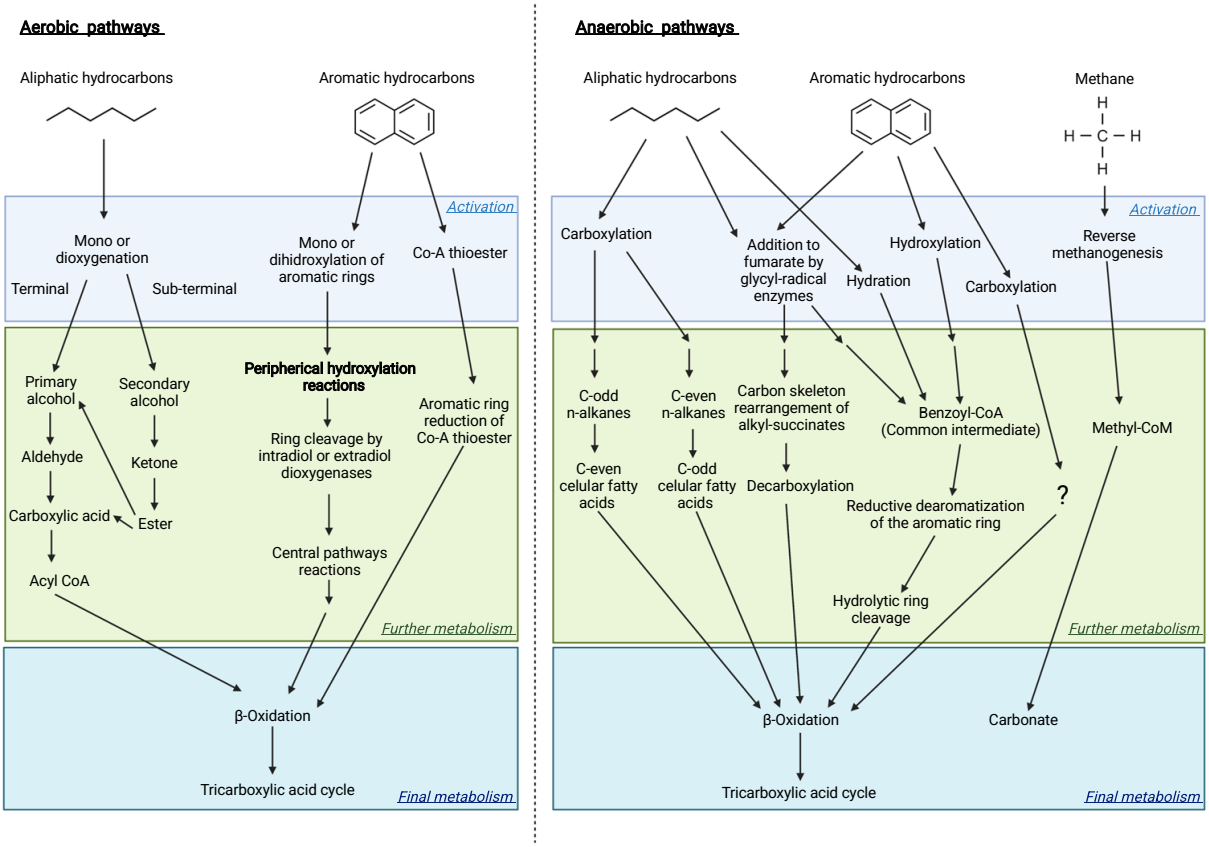

Figure 1.2. Known metabolic hydrocarbon degradation pathways that are carried aerobically and anaerobically by microorganisms. Adapted from Sierra-Garcia and de Oliveira (2013) with additional information from Abbasian et al. (2016), Heider (2007), Kleemann and Meckenstock (2011), and Thauer (2011). Figure created with BioRender.com.

the alkane monooxygenase encoded by the *alkB* gene which catabolizes medium-chain (C_5 – C_{13}) alkanes (van Beilen et al., 2001). Other genes associated with the degradation of long-chain alkanes (up to C_{36}) have been identified such as the *almA* and *ladA* genes (Feng et al., 2007; Li et al., 2008; Throne-Holst et al., 2007; Wang and Shao, 2014; Wentzel et al., 2007). For cycloaliphatic compounds, the first step is regulated by a cytochrome P450-containing system which converts cyclohexane into cyclohexanol (Karande et al., 2016; Salamanca et al., 2015; Trickett et al., 1991). Cyclohexanol is then oxidized into adipic acid by the *chn* gene cluster (Cheng et al., 2002).

Mono and polyaromatic rings are initially activated mainly by a dioxygenase enzyme (Abbasian et al., 2016; Ghosal et al., 2016; Sierra-Garcia and de Oliveira, 2013). The naphthalene dioxygenase, encoded in the *nahAaAbAc* genes, is one of the key enzymes for aromatic hydrocarbon degradation (Resnick et al., 1996). Other variants of ring-hydroxylating dioxygenases (*nag* and *phn* genes for gram-negative bacteria and *nid*, *phd* and *pdo* genes for gram-positive bacteria) have also been described (Elyamine et al., 2021; Laurie and Lloyd-Jones, 1999; Lu et al., 2019; Saito et al., 1999; Zylstra et al., 1997). There are different sections and variations of the sections for the aromatic degradation pathway (Díaz et al., 2013). The reactions in the upper pathway transform the aromatic compound into salicylate and are encoded in the *nahABCDEF* operon (Obayori and Salam, 2010; You et al., 1988).

Salicylate is mainly converted into catechol, a key intermediate which many pathways converge to (Fuchs et al., 2011; Harayama and Timmis, 1989). There are two ways in which catechol can be metabolized depending on the catechol dioxygenase used (Abbasian et al., 2016). The meta-cleavage pathway is the most common of the two cleavage pathways and uses the catechol 2,3-dioxygenase, encoded by *nahH* (Fuchs et al., 2011; Obayori and Salam, 2010; Yen et al., 1988; Yen and Gunsalus, 1985; You et al., 1988). The final products of the meta-cleavage pathway are acetaldehyde and pyruvate (Goyal and Zylstra, 1997). The ortho-cleavage pathway uses the catechol 1,2 dioxygenase, encoded by *catA* (Eulberg et al., 1998; Fuchs et al., 2011; Yen et al., 1988). The final products of this side of the pathway are succinyl-CoA and acetyl-CoA (Yen et al., 1988). Catechol can also be degraded using other intermediaries such as salicylate or gentisate, among others (Fetzner, 2012; Jiménez et al., 2010; Liu et al., 2011; Rogers and Lipscomb, 2019; Zhou et al., 2001). Substituted aromatic compounds such as toluene or xylene are also degraded using catechol as an intermediate (Díaz et al., 2013; Harayama and Rekik, 1990; Law and Boulanger, 2011; Stephanopoulos et al., 1998; Teufel et al., 2010; Worsey and Williams, 1975). There are also other pathways for

toluene/xylene degradation which use different intermediates such as protocatechuate and 3-methylcatechol (Zylstra and Gibson, 1991).

1.3.1.2. Anaerobic hydrocarbon degradation

Even though aerobic hydrocarbon degradation is the most studied group of processes and is the most energetically beneficial (Widdel and Musat, 2010b), oxygen concentrations in the contaminated sediments are rapidly depleted due to increased hydrocarbon degrader metabolic activity. In that scenario, anaerobes take over the degradation process and couple their anaerobic respiration with one of five general degradation pathways: fumarate addition, oxygen-independent hydroxylation, aromatic carboxylation/methylation, alkene/alkyne hydration, and reverse methanogenesis (Abbasian et al., 2016; Bargiela et al., 2017). Alkenes, alkynes, and methane are not common components of fuels and oils so they will not be described here, but they have already been reviewed elsewhere (Abbasian et al., 2016).

Fumarate is added to aromatic and aliphatic hydrocarbons including toluene, xylenes, short and long-chain alkanes and alkenes, and cycloalkanes to form succinates (Heider, 2007; von Netzer et al., 2016, 2013). Denitrifying, sulfate-reducing, ferric iron-reducing, and phototrophic bacteria are capable of performing this pathway and the produced succinates are later converted to CoA-thioesters via β-oxidation-like reactions (Boll et al., 2014; von Netzer et al., 2016). The marker genes for these group of processes are the fumarate-adding enzymes such as benzylsuccinate (for toluene) or alkylsuccinate (for alkanes and alkenes) synthases (Callaghan et al., 2008; von Netzer et al., 2013). They are encoded by the *bssA* and *assA* genes, respectively (Callaghan et al., 2008; Toth and Gieg, 2018; von Netzer et al., 2013). The CoA-thioesters are metabolized to acetyl-CoA via β-oxidation for aliphatic compounds (Wilkes et al., 2002). For aromatic hydrocarbons, the aromatic ring (with benzoyl-CoA as the key intermediate) is cleaved by Birch reduction or by ATP-dependent or independent pathways

after which the dearomatized CoA-thioester is degraded by β-oxidation-like reactions (Boll et al., 2014; Fuchs et al., 2011).

Ethylbenzene, benzene, toluene, and phenol, among others, can be metabolized by denitrifying bacteria using oxygen-independent hydroxylation in which a secondary or tertiary alcohol is formed and then converted to benzoyl-CoA (Boll et al., 2014; Grossi et al., 2007; Rabus et al., 2005). The key enzyme forming the initial alcohol is the ethylbenzene dehydrogenase encoded by the *ebdABC* genes (Rabus et al., 2002). Only metabolites for proposed pathways have been detected but the genes or enzymes have not been described (Grossi et al., 2011).

It has been hypothesized that aromatic hydrocarbons can be anaerobically degraded with the addition of a carboxyl or a methyl group as the initial activation step to produce a carboxylic acid as a common intermediate by sulfate-reducing, ferrous iron-reducing, and nitrate-reducing microorganisms (Boll and Heider, 2010). The general mechanism of the methylation pathway involves the addition of a methyl group into the aromatic ring which is then metabolized by the fumarate addition pathway (Boll and Heider, 2010). In the carboxylation pathway, the aromatic compound is directly converted into the carboxylic acid and is then metabolized by β-oxidation (Bergmann et al., 2011; Kleemann and Meckenstock, 2011).

1.3.2. Factors affecting biodegradation and bioremediation on shorelines in Arctic environments

1.3.2.1. Beach structure and substrate characteristics

Understanding hydrocarbon bioremediation on Arctic beaches requires consideration of the unique features of shoreline environments, which consist of the beach sediment within the intertidal zone. Beach sediment can vary distinctly in physical composition which impacts hydrocarbon biodegradation. Marine tar residues formed by oil-sediment interactions may

facilitate bioremediation if they take the form of microscopic oil-particle aggregates with fine sediments interspersed. Alternatively, marine tar residues may take the form of macroscopic oil-sediment aggregates or asphalt pavement with coarse sediments, which may negatively impact bioremediation (Gustitus and Clement, 2017; Owens et al., 1987a). Beach structure in the Arctic region varies in depth, which influences biodegradation. Rates of hydrocarbon biodegradation are slower at greater depths where nutrient and oxygen levels are lower and oil sequestered in subsurface sediments may therefore remain more toxic and persistent in the long-term (Lindeberg et al., 2018; Tang et al., 2006). Tidal and wave action must also be taken into account as it can cause nutrient washout along shorelines resulting in ineffective treatment (Wrenn et al., 1997). Moreover, wave action differentially affects the shoreline, with the upper intertidal zone likely to experience greater persistence of high oil concentrations (Owens et al., 1987a).

1.3.2.2. Nutrients, oxygen, and salinity

Given that petroleum is carbon-rich, microorganisms must obtain mineral nutrients including nitrogen, phosphorus, and iron from the environment to effectively metabolize hydrocarbons (Atlas, 1988). The importance of nitrogen and phosphorus on shorelines has been widely characterized as these nutrients are often limited both in surface and subsurface sediments (Prince, 1993). Low oxygen concentrations limit aerobic hydrocarbon biodegradation (Atlas, 1995; Van Hamme et al., 2003). While oxygen concentrations may be less rate-limiting on shoreline surfaces, dissolved oxygen concentration within interstitial water varies with depth (Jansson, 1967; Lindeberg et al., 2018). During anerobic degradation, the rate-limiting factor is availability of suitable electron acceptors (Tang et al., 2006). Salinity varies drastically across the Arctic (Supply et al., 2020) and changes in salinity may negatively impact hydrocarbon biodegradation (Cao et al., 2022; Mille et al., 1991; Minai-Tehrani et al., 2009). An increase in salinity causes a decrease in cell surface hydrophobicity which leads to cells

moving further away from the oil phase to the aqueous phase where they would preferentially degrade soluble hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs) over insoluble ones such as alkanes (Cao et al., 2020). These bacteria also produced less biosurfactants as a result of the reduced contact with the oil phase. In contrast, greater biodegradation of crude oil has also been observed in Arctic shoreline sediments where salinity was slightly higher (3.5% from 3.0%; Sharma and Schiewer, 2016).

1.3.2.3. Temperature

Air and water temperature play a critical role in determining hydrocarbon biodegradation potential on Arctic shorelines. The Arctic's climate is one of the world's harshest. Decreased hydrocarbon biodegradation rates are associated with lower temperatures as a consequence of decreased rates of enzymatic activity (Atlas, 1991; Sharma and Schiewer, 2016). Cold-adapted microorganisms can be active at low and even sub-zero temperatures (Aislabie et al., 2006; Margesin and Schinner, 2001; Whyte et al., 1998). However, most hydrocarbon degraders are still considered psychrotolerant instead of psychrophilic having optimal growth temperatures above 15°C (Aislabie et al., 2006). Additionally, microbial activity also depends on thaw conditions that allow for presence of liquid water (Aislabie et al., 2006). Thawing of the nearshore intertidal environment is influenced first by surface snow and ice coverage, then by a subsurface frost-table which can only begin to thaw once the surface is exposed and seawater begins to infiltrate (Owens and Harper, 1977). It is estimated that Arctic beaches on Baffin Island, Canada experienced approximately only 63 ice-free days each year from 1981-2002 (Prince et al., 2002). However, this approximation is likely an underestimate for the current and future climate (Farquharson et al., 2018). Temperature not only affects microbial activity, but also oil properties. At lower temperatures, biodegradation is delayed due to increased oil viscosity and decreased volatilization of toxic short-chain alkanes (Atlas, 1991).

1.3.3. Experimental hydrocarbon bioremediation field studies on cold shorelines

One of the most common approaches for shoreline bioremediation is biostimulation using nitrogen and phosphorus. Three different types of fertilizers are commonly used in biostimulation: inorganic, slow-release, and oleophilic fertilizers. Inorganic fertilizers supply nutrients immediately upon dissolution, whereas slow-release fertilizers are coated such that nutrients are released more slowly over time as the coating breaks down (Nikolopoulou and Kalogerakis, 2010). Oleophilic fertilizers are designed to have strong affinity for oil, thereby delivering nutrients and enhancing oil bioavailability while being less susceptible to wash-out by wave action (Atlas and Bartha, 1973; Gallego et al., 2006).

Determining which fertilizer is appropriate for biostimulation requires consideration of the environment. While studies often highlight the potential advantages of slow-release fertilizers (mainly increased nutrient delivery efficiency and decreased cost due to fewer applications), slow-release fertilizers may be less effective than inorganic nutrient fertilizers at lower temperatures such as those common on Arctic shorelines (<15° C) due to reduced coating permeability (Lee et al., 1993). Determining appropriate quantities and application rates with either fertilizer class is imperative to avoid potential adverse effects such as eutrophication (Macaulay and Rees, 2014).

There are currently a limited number of studies on in situ hydrocarbon bioremediation on Arctic shorelines. Below we review the major studies conducted to date and their main findings, including the studies of the Baffin Island Oil Spill (1980-1984), the *In situ* Treatment of Oiled Sediment Shorelines program (1996-1998), and Svalbard (2002-2003). There have also been studies in other polar and cold regions (Table 1.1). Of these, we will discuss some of the most important studies in the Kerguelen Archipelago (1996-1998) and the spills of the Bahía Paraiso and Exxon Valdez vessels in 1989.

1.3.3.1. Baffin Island Oil Spill, 1980-1984

The Baffin Island Oil Spill (BIOS) was a four-year experimental field project beginning in 1980 where 45,000 L of medium crude oil were released in Cape Hatt, Baffin Island, Canada (72°31′ N, 79°50′ W) to study the short and long term fates of chemically dispersed oil in the water and beached oil cleanup techniques on land (Sergy and Blackall, 1987). The experiments were broadly categorized as nearshore and shoreline studies (Sergy and Blackall, 1987). For the nearshore studies, oil was deposited by wind and wave action on the shoreline. Two thirds of the oil was flushed away by the subsequent four tidal cycles (48 h), and within two years (18 weeks of open water conditions), the original amount of stranded oil was reduced by 70% (Owens et al., 1987a). The majority of this oil removal was attributed to physical processes.

For the shoreline studies, oil was applied to 20 – 40 m^2 in the intertidal and backshore zones of beaches located in the sheltered "Z-lagoon" of Cape Hatt. Various cleanup methods were tested on these plots including dispersant washing, mechanical mixing, chemical solidification, flushing, burning, and natural biodegradation of the released oil (Owens et al., 1987a, 1987b). Within the intertidal zone, waves were the main cause of oil removal. However, oil was still visible four years after the spill (Sergy and Blackall, 1987). In the supratidal zone, experimental plots were treated with a 50% oil-water emulsion and the impact of fertilizers was investigated. Nutrient addition increased oil-degrading bacterial counts after 17 days and respiratory CO_2 production after 23-24 days. Despite a lack of differences among treatments after the first 25 days, after 2 years, enhancement of oil biodegradation in nutrient-added plots was confirmed by significant changes to the alkane/isoprenoid ratio in the residual oil (Sendstad et al., 1982; Swannell et al., 1996), suggesting that nutrient addition was an appropriate cleanup strategy for oil stranded on shorelines.

Table 3.1. Summary of the classic hydrocarbon degradation field studies on cold shorelines and their main findings.

Location	Date	Type of spilled oil	Volume of oil spilled	Bioremediation method used	Percentage oil removed by biodegradation	Duration of the biodegradation process	Observations	References
Arctic								
Gluudneset, Spitsbergen (78°54′ N, 12°04′ E)	Summer 1976	Forcados crude oil (1% C_1-C_4, 1% light naphtha, 12% heavy naphtha, 9% kerosene, 38% gas oil, 39% residue)	200 L in two experimental plots of 10 m^2 each	Commercial fertilizer at 0.1 kg/m^2	74%	7 years	Fertilizer treatments were able to accelerate the initial rate, but not the extent of biodegradation	Environment Canada, n.d.; Sendstad, 1980; Sendstad et al., 1984
Cape Hatt, Baffin Island (72°31′ N, 79°50′ W)	1980 - 1984	Venezuelan Lago Medio sweet medium gravity crude oil	45,000 L on multiple spill scenarios	Commercial fertilizer (Fullgjødsel C) for the shoreline experiments at 6.4 g of N/m^2, 64g of N/m^2, or	12% – 31%	2 years	Tidal action removed most of the oil on the intertidal zone. Enhancement of	Eimhjellen and K. Josefsen, 1984; Sendstad et al., 1982

				64 g of N/m^2 with tilling			biodegradation was observed on the fertilized plots	
Sveagruva, Spitsbergen (77°56′ N, 16°43′ E)	July 1997 - September 1998	IF-30 intermediate fuel oil (19% saturates, 63% aromatics, 12% resins, 6% asphaltenes, 3% waxes)	5550 L in 3 sites of 120 - 429 m^2	Soluble and slow-release (Inipol SP1) fertilizers and tilling	(a) 54% – 61% (b) 88%	(a) 2 months (b) 1 year	Fertilizer treatments stimulated biodegradation rates compared to tilled and unfertilized plots on the same beach, but had lower removal rates compared to other sites on different beaches where sediment relocation was	Environment Canada, n.d.; Garrett et al., 2003; Grossman et al., 2000; Owens et al., 2003; Prince et al., 2003

							the only applied treatment	
Kapp Wijk, Spitsbergen (78°36′ N, 15°10′ E)	July - September 2002 and 2003	Kerosene (190 – 250 °C bp, <0.3% aromatics) amended with heavier alkanes (3.5% hexadecane, 1.7% heptadecane, 2.2% octadecane 1.1% nonadecane, 1.1% eicosane and 1.7% pristane by weight)	7 L/m^2 on PVC tube enclosures	Oleophilic fertilizers (Inipol EAP 22 and Inipol$^+$)	17%	2.5 months	Oleophilic fertilizers stimulated microbial growth and biodegradation rates. The fertilizers might be shaping the microbial communities more than the oil itself	Røberg et al., 2011, 2007
Antarctica								

Davis Station, Pridz Bay (68°30′ S, 78°20′ E)	Summer 1992	Special Antarctic Blend (96% light volatile C_{10} – C_{14} hydrocarbons, 4% heavy waxes, resins, and tars)	1 L on a ~0.2 m^2 plot	No treatment (natural attenuation)	No biodegradation detected	2 months	No evidence of biodegradation was detected and losses were attributed to other natural processes (evaporation and dispersion)	Green et al., 1992; Payne et al., 2014
Several locations around the Kerguelen Archipelago	Not specified	Arabian light crude oil (51% saturates, 39% aromatics, 6% resins, 3% asphaltenes, 5% waxes)	100 mL per plot on containment plots made of PVC pipes of 15 cm in diameter	No treatment (natural attenuation)	0.8% – 40%	1 month	Biodegradation was observed on a third of the studied sites. This could be due to environmental factors such as varying sediment grain size among beaches	Delille and Delille, 2000; Environment Canada, n.d.

Anse sablonneuse, Kerguelen Archipelago (49°19′ S, 69°42′ E)	February 1997 - August 1999	Arabian light crude oil	2 L for each 1 m^2 plot	Fish compost (alone or amended with nutrients and cationic or non-ionic surfactants) and Inipol EAP 22	(a) 51% for Inipol and 88% for fish compost (b) 97% for Inipol and 92% for fish compost	(a) 3 months (b) 6 months	Fish compost fertilizers increased biodegradation rates the most, followed by Inipol. Treatments improved initial biodegradation rates, but not the total amount of degradation	Delille et al., 2002; Pelletier et al., 2004
Temperate environments								
Sheltered cove on the eastern coast of Nova	1985 - 1987	Scotian Shelf condensate (SCC; high proportion of low molecular	200 mL in 7 L of wet sand buried inside Nitrex bags	9.5 g of agricultural fertilizer (AF; 10:1:0 N:P:K) or	(a) 59% for Inipol and 96% for AF using SCC	(a) 6 months (b) 2.3 years	Inipol reduced biodegradation rates, while agricultural	Lee and Levy, 1989

Scotia, Canada		weight hydrocarbons) or Hibernia crude oil (HCO; high wax content)		20 mL of Inipol EAP 22	(b) 90% for Inipol and 96% for AF using HCO		fertilizers improved them	
Long Cove (LC; sand beach) and Clam Harbour (CH; salt marsh) on the eastern coast of Nova Scotia, Canada	June - December 1989	Terra Nova crude oil (1.4% $C_1 - C_4$, 5.7% light naphtha, 15.7% heavy naphtha, 14.1% kerosene, 17.2% diesel, 29.5% gas oil, 16.4% residue)	0.3% - 3% oil in 7 L of wet sand buried inside Nitrex bags	0.34 - 1.36 g/L of agricultural fertilizer (10:1:0 N:P:K)	For LC: (a) 95% for the 0.3% oil (b) 98% for the 3% oil For CH: (c) 92% for the 0.3% oil (d) 4% for the 3% oil	(a) 15 days (b) 6 months (c) 2 months (d) 6 months	For LC, fertilizers improved overall biodegradation for both oil concentrations. For CH, the fertilizer stimulated almost complete biodegradation in the 0.3% oil treatment. For	ExxonMobil, n.d.; Lee and Levy, 1991

							the higher concentration samples, there was almost no biodegradation observed, which was attributed to the anoxic nature of the salt marsh	
Fowler Beach, Delaware Bay	Summer 1994	Weathered Bonny Light crude oil (35.3 API gravity)	2040 L into 36 m^2 randomized blocks	Sodium nitrate (~0.82 mg/L) and sodium tripolyphosphate dissolved in seawater applied daily. A bacterial inoculum (30 L of 1.9 x 10^5 mL	(a) 94% for alkanes and 88% for aromatics for the nutrient and inoculum treatments (b) 97% for alkanes and	(a) 2 months (b) 3.3 months	The nutrient amendment treatment provided the best biodegradation rates overall. However, it was suggested that nutrient	MacNaughton et al., 1999; Venosa et al., 2000, 1996

				of alkane degraders and 2.5 x 10^4 mL of aromatic degraders) was also added to some treatments	94% for aromatics for the nutrient treatment 88% for alkanes and 76% for aromatics for the inoculum treatment		amendments might not be cost effective. Bioaugmentation was not a feasible strategy either as nutrient amendments alone already reached the presumed carrying capacity of hydrocarbon degraders on the studied beach	

Hyogo prefecture, Japan (35°39′ N, 134°41′ E)	June - September 2000	Weathered Arabian light crude oil	1 g of oil mousse (~3% w/v) mixed with 30 g of dry sand on acrylic vessels deployed in the intertidal and subtidal zones	Synthetic fertilizers: Super IB at 0.9 g, 3 g, or 15 g and Linstar 30 at 0.2 g, 0.6 g, or 3 g	40% for alkanes, 75% for naphthalenes, and 50 for phenanthrenes	3 months	Fertilizer treatments increased biodegradation rates during the initial phase of the experiment with an estimated 10-day lag for the unfertilized oil controls. However, the extent of degradation was not affected by the addition of fertilizers at the end of he experiment	Maki et al., 2003

							where oil composition was indistinguishable between experiments and biodegradation had ceased almost entirely.	
Spills in high-latitude environments								
Bahía Paraiso, Antarctic Archipelago (64°46′ S, 64°05′ W)	January 28, 1989	Diesel fuel arctic (mixture of diesel and jet fuel with high concentrations of semi-volatile aromatic hydrocarbons and a range of $C_8 – C_{30}$	680,000 L	No treatment (natural attenuation)	No biodegradation detected	1 month	The primary cause for fuel removal were physical processes in combination with the deployed clean up and containment	Karl, 1992; Kennicutt, II, 1990; Kennicutt, II et al., 1991

		aliphatic compounds) and a smaller proportion of lube oil					measurements. Biodegradation was only observed at low and almost negligible rates	
Exxon Valdez, Gulf of Alaska (60°50' N, 146°51' W)	March 24, 1989	Alaskan North Slope, primarily Prudhoe Bay crude oil (3.9% $C_1 - C_4$, 7.5% light naphtha, 17% heavy naphtha, 13.1% kerosene, 15.4% diesel, 26.5% gas oil, 16.6% residue)	41.6 million L	Slow-release (Customblen) and oleophilic (Inipol EAP 22) fertilizers for an approximate total of 48,600 kg of N and 5000 kg of P added	(a) 70% for site KN-135 (b) 63% for Snug Harbor	(a) 1 month (b) 3.5 months	Biodegradation was determined to be the largest contributor to the cleanup strategy removing around 50% of the spilled oil. Oleophilic fertilizers provided nutrients and also helped to	Bragg et al., 1994; ExxonMobil, n.d.; Prince and Bragg, 1997; Pritchard and Costa, 1991; Wolfe et al., 1994

							thin out the oil slick for a higher surface area for microbial degradation	

1.3.3.2. The *In situ* Treatment of Oiled Sediment Shorelines program, 1996-1998

The *In situ* Treatment of Oiled Sediment Shorelines (ITOSS) program was an international collaboration of oil spill response and research agencies aimed at investigating the effectiveness of various bioremediation treatments (Sergy et al., 2003, 1997). The main component of the ITOSS program was the Spitsbergen trials: a 400-day experiment carried out in Spitsbergen, Svalbard (77°56′ N, 16°43′ E) between July 1997 and September 1998 (Guénette et al., 2003). A total of 5,500 L of intermediate fuel oil (IF-30) was applied to the upper intertidal zone in 3 120 – 429 m^2 sites to approximate oil loadings of 5 – 10 L/m^2. Oil was also applied to the supratidal zone for one of the sites (Guénette et al., 2003; Sergy et al., 2003). After the initial stabilization period of 10 days, various treatments were applied to plots within the sites: sediment relocation from the upper intertidal and supratidal zones to the surf zone, mixing by tilling to a depth of 20 cm, biostimulation using soluble (ammonium nitrate, superphosphate, ferrous sulphate, and yeast extract) and slow-release (Inipol SP1) fertilizers, biostimulation combined with tilling, and natural attenuation (Guénette et al., 2003; Owens et al., 2003; Sergy et al., 2003).

Fertilizers did not leach into the offshore water (Prince et al., 2003). Sediment relocation appeared to be the most effective treatment as only very low oil amounts could be detected just 5 days after the treatment (Owens et al., 2003). Toxicity of the released oil was reduced to background levels after 10 – 61 days (Lee et al., 2003). It was estimated that sediment relocation to the intertidal zone accelerated oil removal by at least a year. However, this promising technique did not change the overall amount of oil removed (Owens et al., 2003; Sergy et al., 2003).

Biostimulation was also effective, with clear evidence of increased biodegradation rates in the fertilized plots observed compared to the tilled and untreated plots (Garrett et al., 2003; Grossman et al., 2000; Prince et al., 2003). However, oil removal was much slower in the

biostimulation plots compared to sediment relocation plots (Owens et al., 2003). For the natural attenuation potential, wave action reduced oil concentrations rapidly for the first 10 days before the treatments were applied (Sergy et al., 2003) with oil losses between 30 – 92% (Owens et al., 2003). The authors ultimately suggested a two-stage strategy where natural removal processes combined with biostimulation are allowed to act for a few weeks and relocation is then applied to areas where the natural processes appear to not be efficient enough (Owens et al., 2003; Sergy et al., 2003).

1.3.3.3. Svalbard, 2002-2003

A group of Norwegian researchers (Røberg et al., 2011, 2007) carried out field trials at Kapp Wijk, Svalbard (78°36′ N, 15°10′ E) between July – September in 2002 and 2003 to examine microbial response to hydrocarbon contamination using a kerosene-type fuel supplemented with heavier alkanes combined with the addition of the oleophilic fertilizers Inipol EAP 22 and Inipol+ (Røberg et al., 2007). The fuel was added inside PVC tube enclosures inserted into the intertidal sediment. The enclosures were open in both ends to allow tidal water to enter through the bottom and percolate vertically inside the tube, replicating the tidal oscillations occurring on the surrounding beach.

For both years, most of the fuel was quickly lost within the first 2 days (98% loss for 2002 and 94% loss for 2003). Oleophilic fertilizers increased kerosene retention, with 96% and 76% loss detected after 2 days in 2002 and 2003, respectively. The removal rates for these samples increased on subsequent days and hydrocarbon losses from the fertilized samples equaled those of the unfertilized treatment after 30 – 80 days. No changes in the heptadecane/pristane ratio were observed for the unfertilized samples suggesting that the observed losses were due to abiotic processes. For the fertilized samples there was a decrease in the heptadecane/pristane ratio with the largest changes observed after day 43. The

researchers estimated that approximately 17% of the released fuel was removed by biodegradation (Røberg et al., 2007).

A larger proportion of culturable hydrocarbon-degrading microorganisms (dominated by Gammaproteobacteria) was observed after the addition of the modified kerosene and became the majority of the culturable organisms after the first 2 weeks of the study (Røberg et al., 2007). These numbers were, however, only a small proportion of the total cell counts determined for these sediments. 16S rRNA gene-based fingerprinting showed that the community composition of the treatment enclosures quickly changed compared to the untreated controls (Røberg et al., 2011). The authors concluded that the use the of oleophilic fertilizer Inipol+ led to a higher microbial stimulation and hydrocarbon biodegradation rates. Almost identical patterns for viable counts and 16S community composition in the treatments with fertilizer amendments were observed for control enclosures in which only the fertilizer was added suggesting that the stimulation may have been caused solely by the fertilizer (Røberg et al., 2011, 2007).

1.3.3.4. Kerguelen Archipelago, 1996-1998

Mesocosm experiments were carried out in the Kerguelen Archipelago (a group of sub-Antarctic islands) involving burying PVC pipes into beach sediments and applying 100 mL of Arabian light crude oil for 5 or 90 days on nine relatively sheltered beaches exposed to different wave energies (Delille and Delille, 2000). For three of the beaches, increases in hydrocarbon-degrading microorganisms (MPN counts) as well as changes in oil composition (n-C_{17}/pristane and n-C_{18}/phytane ratios) were observed, suggesting that biodegradation was occurring. For two out of the three sites where biodegradation was observed, the residual oil concentrations were much higher than for the other beaches. Given that those beaches contained coarser grained and more porous sediment, it was suggested that sediment structure allowed for a higher oil retention which later allowed for higher biodegradation rates. In contrast, on the finer

sediment beaches, oil was only briefly retained on the surface and was easily removed and resuspended due to tidal action. This is consistent with previous studies suggesting that clay-oil aggregates can be formed and easily removed by water motion (Owens et al., 1994). The researchers acknowledged limitations with extrapolating bioremediation results based on generalized environmental conditions as site-specific conditions could drastically affect bioremediation.

Delille et al. (2002) performed a second year-long field trial on Anse sablonneuse, a beach in Grande Terre, Kerguelen Archipelago (49°19′ S, 69°42′ E) in February 1997. Mesocosms comprised 1 m2 wooden enclosures containing 2.85 L/m^2 of light Arabian crude oil with three different types of fish compost and Inipol EAP 22 used as the nutrient amendment treatments. Nutrients were added at two different time points (10 and 21 days after oil application) at a proportion of 5% of the added oil. Fish compost was added alone (F1) or with the addition of urea and phosphate and either a cationic (F2) or a lipidic neutral (F3) surfactant (Pelletier et al., 2004). Hydrocarbon-degrading bacteria MPN counts increased in the contaminated plots and became the dominant bacteria in the microbial community (up to 95%) a month after the oiling event with their estimated peak abundance observed after 6 months. These bacteria remained present, to a lesser extent, at the experimental plots even after a year and there were observable differences between the control and experimental plots after 2.5 years (Delille et al., 2002).

A correlation between bacterial growth and biodegradation rates was detected between sampling points at 10 and 90 days, further evidencing the effect of microbial metabolism on fuel removal (Delille et al., 2002; Pelletier et al., 2004). The n-C_{18}/hopane ratio was lower over the first 90 days for the F3 fertilizer, compared to the F2 fertilizer. This was attributed to the fact that non-ionic surfactants have a higher affinity for oil and are able to increase the oil/water interface which could lead to more bacterial colonization. After 90 days, n-C_{18}/hopane ratios

were the lowest in the fish compost treatments, had intermediate values for the Inipol treatment, and were highest for the unfertilized plots. After 177 days the n-C_{18}/hopane ratios for all treatments reached similar values (Pelletier et al., 2004). The n-C_{12}/iso-C_{12} ratio was also used to detect biodegradation in the early stages of the bioremediation process where the n-C_{18}/hopane ratio might not be sensitive enough due to hydrocarbon evaporation occurring early on after the spill event. n-C_{12}/iso-C_{12} ratios decreased slightly, but significantly, between days 10 and 19, suggesting that microorganisms are already active few days after the oil application (Pelletier et al., 2004). The authors noted persistent, but decreasing, toxicity on the sediments that could be caused by the degrading bacteria being unable to degrade the more toxic compounds in the oil (Delille et al., 2002). This could be an issue when trying to apply bioremediation as a cleanup strategy as toxic hydrocarbons could remain for longer periods of time.

1.3.4. The story of two spills: historical high-latitude spills and bioremediation field studies

While the experimental studies above provided insight into various in situ bioremediation strategies, disastrous real life oil spills can also provide valuable knowledge that cannot be replicated in smaller scale experimental spills. The year 1989 was a very important, yet unfortunate, year in the field of hydrocarbon bioremediation: the Bahía Paraiso crashed in the Antarctic Archipelago and the Exxon Valdez ran aground in the Gulf of Alaska. We will now review these two spills as case studies in which the natural microbial population was not metabolically capable of mineralizing the released hydrocarbons (for the Bahía Paraiso), and a contrasting case in which bioremediation was effective in a high-latitude environment (for the Exxon Valdez).

1.3.4.1. Bahía Paraiso, 1989

On January 28, the Argentinian ship Bahía Paraiso ran aground in Arthur Harbor (64°46′ S, 64°05′ W), near Anvers Island in the Antarctic Archipelago (Karl, 1992). The ship spilled approximately 680,000 liters of mostly diesel fuel arctic (Kennicutt, II, 1990; Kennicutt, II et al., 1991).

Radiorespiration experiments were performed on subtidal and intertidal sediment samples collected from areas affected by the spill to evaluate microbial hydrocarbon degradation potential (Karl, 1992). Microcosms containing 100 – 400 nM of ^{14}C-hexadecane were incubated in a time series ranging from 26 – 138 hours. Low but detectable mineralization rates were detected from all samples, including those from control sites. Based on the estimated concentration of alkanes present in the studied sediments, Karl (1992) suggested that biodegradation would be a negligible cause of fuel loss during this spill.

A year after the spill, most of the fuel was not detectable and, when detectable, hydrocarbon composition showed that it was heavily degraded and consisted mostly of more complex PAHs, such as alkylated phenanthrenes, at approximately 1 order of magnitude less compared the previous year. Authors determined that on-site clean up and containment measurements plus the relatively light composition of the fuel allowed for a reduced environmental impact and removal mostly due to evaporation and dilution rather than by biodegradation or photooxidation (Kennicutt, II et al., 1991), corroborating the initial radiorespiration results.

1.3.4.2. Exxon Valdez, 1989

The Exxon Valdez oil spill is one of the worst oil spills in human history (Atlas and Hazen, 2011; Skinner et al., 1989). 41.6 million L of Alaskan North Slope heavy oil were spilled into Prince William Sound, Alaska (60°50’ N, 146°51’ W) on March 24, 1989 when the Exxon Valdez oil tanker ran aground (Prince and Bragg, 1997; Skinner et al., 1989). A severe storm

struck Prince William Sound right after the spill occurred with wind speeds of around 80 km/h causing a considerable amount of oil to be carried to the shoreline (Atlas and Hazen, 2011). The remote location exacerbated the difficulty of a prompt response. Consequently, the oil spread to approximately 782 km of the shoreline of Prince William Sound (Page et al., 1999). This environmental disaster caused the death of much wildlife and irreversible environmental impacts. Various remediation approaches, including physical, chemical, and biological cleanup were applied to shorelines.

The Exxon Valdez cleanup was the first large-scale bioremediation effort that utilized fertilizers demonstrating in situ success (Pritchard et al., 1992; Pritchard and Costa, 1991). The slow-release (Customblen) and oleophilic (Inipol EAP 22) fertilizers were further proven to be effective on field test plots (Bragg et al., 1994; Prince and Bragg, 1997; Pritchard et al., 1992; Pritchard and Costa, 1991). The goal of using two types of fertilizers was that Inipol EAP 22 would adhere to the oil film on the surface and stimulate degradation while Customblen would slowly release nutrients that would penetrate into the subsurface (Prince and Bragg, 1997). The beach sediments treated with the nutrient amendments appeared visually cleaner than the untreated sediments within 10 days. It was estimated that fertilizer addition enhanced biodegradation rate 8.6 times, compared to the control (Bragg et al., 1994). It was also determined that up to 18-fold higher mineralization rates, measured by ^{14}C-hexadecane and ^{14}C-phenantrene radiorespiration assays, occurred in the fertilized parts of the beach compared to untreated sections (Bragg et al., 1994; Lindstrom et al., 1991). A total of 48,600 kg of N were applied on the shoreline of Alaska between 1989 and 1991 (Prince and Bragg, 1997; Pritchard et al., 1992). In 1992, the amount of oiled shoreline had decreased to 1.3% (Bragg et al., 1994). 20% of the oil was lost by evaporation and/or photolysis, 14% of the oil was recovered or incinerated, less than 1% of the oil was estimated to be dispersed in the water column, and 50% was biodegraded either after it beached or in the water column (Wolfe et al.,

1994). 13% and 2% of the oil remained in subtidal sediments or on the beaches, respectively, mostly as weathered oil residuals (Wolfe et al., 1994). Mathematical models and studies on the effects of the fertilizers on the environment showed that there was no eutrophication or adverse toxic effects as a result of the nutrient amendments (Bragg et al., 1994; Coffin et al., 1997; Lung et al., 1993). Additionally, it was determined that the modest delayed (0.5 – 1.5 years after the spill) eutrophication of the contaminated shoreline was caused by the high mortality of the dominant algal and gastropod grazer species due to the oil toxicity which caused colonization by opportunistic algae (Peterson, 2001; Peterson et al., 2003). These encouraging results showed that nutrient addition is a useful bioremediation approach, particularly in the nutrient-limited conditions.

Unfortunately, follow-up studies 26 years after the Exxon Valdez oil spill showed that 0.6% of the oil persisted in intertidal and subtidal sediments and, notably, remained unchanged from the 2001-2015 period (Lindeberg et al., 2018). This persistence highlights not only the inherent challenges of oil spill cleanup, made even more difficult in the remote and harsh climate of the Canadian high Arctic, but also the need for improved long-term oil spill cleanup strategies.

1.3.5. The microbial community and function associated with hydrocarbon degradation on Arctic environments

While the reviewed studies provided valuable insights as to how hydrocarbon bioremediation occurs in cold environments, these studies are several decades old and do not include modern monitoring techniques that could help us better understand how biodegradation is occurring and, potentially, how to accelerate the process. Multi-omics approaches such as genomics, transcriptomics, and proteomics have expanded the horizon of microbial research, especially in environmental microbiology (Franzosa et al., 2015; Jansson and Baker, 2016). Recent advances in high-throughput sequencing allow us to reveal hydrocarbon degradation potential

quickly, cheaply, and with only a small sample of sediment using the detailed knowledge of hydrocarbon degradation enzymes and the identity of the genes that encode them (Chakraborty et al., 2012; Kumari and Kumar, 2021). Applying this advanced technology is particularly important in Arctic environments where the hydrocarbon degradation process happens at extremely slow rates. A potential application of this technology is to detect changes in the microbial community and functional genes in real-time during the early stages of biodegradation, when it is still too soon to observe significant rates of biodegradation from oil chemistry or from field observations. Additionally, prediction of biodegradation potential for a sampled microbial community is currently being explored with public databases and tools designed to provide information, including environmental conditions, in which biodegradation has already been proven (Caspi et al., 2007; Karthikeyan et al., 2020; Wicker et al., 2016). Omics tools can also be used to complement other degradation monitoring techniques such as measuring decreases in oxygen and nutrient concentrations to provide a holistic understanding of the bioremediation process in an approach known as systems biology (Atlas and Hazen, 2011; Chakraborty et al., 2012; Ellis et al., 2022; Zhou et al., 2011).

Recently, advanced omics techniques have been used to study marine oil spills (Table 1.2). Yergeau et al. (2012) demonstrated that biopile treatment of hydrocarbon contaminated Arctic soils can facilitate the growth of potential hydrocarbon degraders (*Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Caulobacter*). The result of metagenomic analyses indicated that alkB-related sequences were mostly linked to Gammaproteobacteria. Reverse-transcriptase real-time PCR confirmed that *Pseudomonas* and *Rhodococcus* expressed *alkB* genes in Arctic biopile soils. *Pseudomonas* and *Rhodococcus* were also identified as important genera in the degradation of a heavy marine fuel oil on Arctic shorelines via 16S rRNA gene and metagenomic sequencing (Ellis et al., 2022). Garneau et al. (2016) showed that sea-ice and sub-ice microcosms can degrade 94% and 48% of hydrocarbons respectively after incubation

Table 1.4. Application of multi-omics approaches for the study of hydrocarbon bioremediation in marine and Arctic environments.

Omics approaches	Location	Environment	Description	Citation
16S rRNA gene sequencing, metagenomics	The Gulf of Mexico (Deepwater Horizon oil spill)	Marine sediment	The sediments collected from the northern Gulf of Mexico were incubated with Louisiana sweet crude oil and Corexit dispersant for 180 days. The abundances of Alteromonadales, Methylococcales, Oceanospirillales, Rhodanobacterales and *Bacteriovorax* were positively correlated to the level of hydrocarbon degradation	Bacosa et al., 2018
Metagenome, metatranscriptome and single-cell sequencing	The Gulf of Mexico (Deepwater Horizon oil spill)	Seawater	Seawater samples from the Deepwater Horizon oil spill were sequenced (metagenomes and metatranscriptomes) showing that genes associated with aliphatic hydrocarbon degradation were significantly increased. Single-cell sequencing data suggested that Oceanospirillales harboured genes associated with n-alkane and cycloalkane degradation	Mason et al., 2012
Metagenomics and metabolomics	The Gulf of Mexico (Deepwater Horizon oil spill)	Marine sediment	Metagenomic analysis of sediment cores from the area surrounding the Deepwater Horizon oil spill showed that Deltaproteobacteria and anaerobic functional genes were more abundant in the sediments closest to the oil spill area. The metabolomic analysis also showed that more putative metabolites were present in the samples most proximate to the oil spill site	Kimes et al., 2013
16S rRNA gene sequencing, metagenomics	The Gulf of Mexico (Deepwater Horizon oil spill)	Marine sediment	16S rRNA gene sequencing of sediment samples from 64 sites in the area of the Deepwater Horizon oil spill showed that an uncultured gammaproteobacterium and a *Colwellia* species were the dominant taxa in the highly-contaminated sites. Metagenomic data suggested that genes associated with the aliphatic and simple aromatic hydrocarbon degradation pathway were the most predominant hydrocarbon degradation genes	Mason et al., 2014

Metagenomics, qPCR	CFS-Alert, Nunavut, Canada	Arctic soils	Hydrocarbon degradation genes were primarily associated with Gammaproteobacteria at one month and then with Alphaproteobacteria and Actinobacteria at one year.	Greer and Juck, 2017; Yergeau et al., 2012
Automated ribosomal intergenic spacer analysis (ARISA) fingerprinting, Ion Torrent 16S rRNA gene sequencing	Resolute, Nunavut, Canada	Sea ice and sub-ice seawater	Microcosms were amended with Arabian Light oil for 15 days at below zero degrees. The microbial diversity decreased, and the abundance of *Colwellia* and *Moritella* increased significantly in the oil-contaminated microcosms	Garneau et al., 2016
16S rRNA gene sequencing, metagenomics	Norwegian fjords	Arctic and temperate seawater	Microcosms using seawater from two Norwegian fjords were treated with chemically dispersed oil at 0 – 2 °C. Larger presence of certain microbes in the Arctic seawater allowed for faster alkane degradation compared to the temperate seawater microcosms. Similar degradation rates were observed for aromatic compounds	Ribicic et al., 2018
16S rRNA gene sequencing, qPCR	Nuup Kangerlua, Greenland	Arctic seawater and sea ice	*In situ* microcosms consisting of an adsorbent netting covered with a thin layer of marine gas oil were deployed in ice-covered seawater and sea ice. *Oleispira antarctica* was the main alkane degrader and *Colwellia*, *Peredibacter*, *Bacterivorax*, and *Arcobacter* were the main PAH degraders during the first 31 days. After this time, the degradation was taken over by bacteria from the class Flavobacteriia	Vergeynst et al., 2019a
16S and 18S rRNA gene sequencing, ddPCR	Kangerluarsoruseq, Greenland	Arctic seawater	*In situ* netting microcosms were deployed using three different types of oil (marine gas oil, troll blend crude oil, and intermediate fuel oil). Alkane-degrading bacteria from the genus *Oleispira* dominated the oil-degrading proportion of the community during the first 24 days. After 112 days, several Gammaproteobacteria genera such as	Vergeynst et al., 2019b

			Alkanindiges and *Cycloclasticus* dominated the community and degraded long-chain and branched alkanes, and PAHs	
16S rRNA gene sequencing	Utqiaġvik, Alaska	Arctic seawater	Incubation mesocosm experiments using nutrient supplemented seawater from the Chukchi Sea were carried out to characterize the biodegradation of Alaska North Slope crude oil and/or Corexit 9500 dispersant over 30 days. It was observed that the microbial community was shaped differentially for the mesocosms which contained only oil or only dispersant. In the mesocosms that contained both oil and dispersant, the microbiome first appeared most similar to the dispersant-only community and then transitioned into the oil-only community	Gofstein et al., 2020
16S rRNA gene sequencing, metagenomics	Resolute, Nunavut, Canada	Arctic beach sediments	Microcosm experiments containing beach sediments incubated with ultra low sulfur fuel oil treated with a nutrient fertilizer over 55 days. Metagenomics showed that nutrient-amended microcosms had increased abundances of key hydrocarbon biodegradation genes (*alkB* and CYP153) and implicated several potential oil-degrading genera that have not previously been detected on high Arctic shorelines (*Nocardiodes, Sphingorhabdus, Sulfitobacter, Psychrobacter, Marinobacter, Novosphingobium, Sphingobium, Alicyclobacillus, Hyphomonas* and *Sphingopyxi*)	Ellis et al., 2022

for 15 days at -1.7°C. Bacteroidetes was most dominant bacterial group in sea-ice microcosms, while Epsilonproteobacteria dominated in sub-ice microcosms. Ribicic et al. (2018) found that bacteria from the genus *Oleispira* were responsible for the initial n-alkane breakdown in cold seawater microcosm experiments, while aromatic hydrocarbons were mainly degraded by *Cycloclasticus*.

While this new wave of studies have increased our knowledge of the underlying mechanisms occurring during the biodegradation process, there are still some aspects of the omics and systems biology approaches that need to be addressed. There are tools that allow for metagenome sequencing to be performed in situ within a few days in remote locations such as the MinION sequencer which has been tested in Arctic samples (Goordial et al., 2017; Maggiori et al., 2021) and with hydrocarbon-contaminated sediments (unpublished data). However, most of the pipelines that can be used to process metagenomic datasets rely on large online databases and the use of high-processing power computing which is not something available at a remote spill site (Chakraborty et al., 2012). The development of portable databases and bioinformatics pipelines that can be run on computers with limited processing power such as laptops is needed. Additionally, available prediction tools rely on environmental data from previous studies to generate their predictions. Given the limited amounts of studies using omics techniques in Arctic sites, it is possible that the predictive power of these databases might be low when applied to Arctic samples. Finally, while the costs of certain omics techniques such as 16S rRNA gene sequencing have made this tool more accessible, other more advanced alternatives such as metatranscriptomics or metaproteomics are still expensive.

1.4. Conclusions: Lessons learned and the roadway to the future

While still considered a secondary cleanup technique (Environment and Climate Change Canada, 2016; EPPR, 2017), bioremediation has become a crucial part of cleanup strategies after the proven success during events such as the Exxon Valdez spill (Atlas and Hazen, 2011;

Prince, 2005). In many remote Arctic regions such as the NWP, the logistics and costs of deploying a large scale clean up response on shorelines remains a significant challenge (Canadian Coast Guard, 2018; EPPR, 2017; McDonald and Knox, 2014; Transport Canada, 2014). This makes strategies involving reduced human intervention and equipment, such as those which rely on bioremediation and tidal action, potentially the most feasible alternatives (Environment and Climate Change Canada, 2016; Yang et al., 2009) leading to a need to reliably understand how the bioremediation process works in the Arctic (Lee et al., 2015). Hydrocarbon bioremediation on Arctic and cold shorelines has been studied for almost half a century and has several limitations. First, this technique appears to improve the rate, but not the extent, of microbial hydrocarbon degradation. This makes this strategy heavily dependent on the metabolic potential and activity of the microbial community of the spill site. Second, the efficiency of bioremediation does rely on environmental factors. Arctic shorelines are oligotrophic, particularly low in nitrogen and phosphorous, and have extremely cold air and water temperatures (Ardyna et al., 2011; Cosme et al., 2015; Ellis et al., 2022; Henley et al., 2020; Pettersen and Song, 2017). These conditions cause Arctic microorganisms to have slow metabolisms, causing the cleanup process to be relatively slow. Third, with only 2 – 4 ice-free and above 0°C months when biodegradation mostly occurs, this increases the timeline of a bioremediation strategy to a scale of years, depending on the timing of the spill.

Nonetheless, the limited number of studies have taught us valuable lessons on how to overcome some of these issues in order to develop optimized bioremediation approaches specifically for Arctic beach environments. Fertilizers are key for any bioremediation attempt in the Arctic to be successful. Studies have found success with multiple types of fertilizers so there does not appear to be a particular type that could be preferentially used. Most studies do agree that multiple rounds of fertilization (or the use of slow release fertilizers) should be applied to keep nutrient levels optimal. Additionally, even though oleophilic fertilizers

adequately stimulate hydrocarbon-biodegradative microorganisms, they are also a more readily consumable carbon source that leads to a reduction of the overall hydrocarbon degradation rates. Frequent sediment mixing vastly improves removal rates by allowing the aeration and input of additional nutrients into the sediment that will further stimulate microbial metabolism. It can also facilitate physical removal by tidal action as well as leading to faster biodegradation rates that are observed in seawater. Dispersants can potentially improve biodegradation by emulsifying hydrocarbons which can boost microbial colonization of the increased oil-water surface. Similar to mixing, it can also help to physically remove hydrocarbons into water. Special attention should be paid to biosurfactants in upcoming years as they present a cheaper and less toxic alternative to chemical dispersants, the latter being particularly important when dealing with fragile Arctic ecosystems.

Dominant microbial taxa are distinct in different hydrocarbon-contaminated environments and are largely determined by the environmental settings. Similarly, beach environments are unique and complicated ecosystems and can be influenced by sediment properties, tidal waves, and concentrations of nutrients, oxygen, and salinity (Boufadel et al., 2019). Thus, it is likely that the microbial communities will respond to hydrocarbon and nutrient amendments differently on various Canadian Arctic beaches compared to other Arctic environments. Future studies of bioremediation on Canadian Arctic shorelines should focus on setting up well-designed in situ micro- and mesocosm experiments or ex situ experiments that better simulate and document actual hydrodynamic and environmental conditions coupled with advanced omics approaches to increase the understanding of detailed metabolic mechanisms on the Arctic shoreline. These studies will allow stakeholders to prepare tailored responses in terms of the types of fertilizers and dispersants to be used in these environments as well as the creation of personalized bioinformatic pipelines and databases that will help to monitor biodegradation during future spills. However, a future in which a bioremediation approach can

be specifically tailored to the unique complement of microorganisms in the environment will only be possible with diligent curation of high-quality metadata for each data set produced (Lee et al., 2015). Finally, future studies will need to understand how microbial populations will respond to climate change in the Arctic to understand how the increases of temperature, a longer ice-free season, and changes in nutrient concentrations and salinity could positively or negatively affect the biodegradation process.

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Connecting text

Our knowledge of how hydrocarbon bioremediation occurs in Arctic and cold environments mostly comes from studies that do not utilize the state-of-the-art omics tools that are currently available as shown in Chapter 1. This limits our capacity of understanding exactly which members of the microbial community are performing hydrocarbon biodegradation and which genes they are utilizing. Additionally, not much is known about the microbial communities of beaches across the NWP, which limits our ability to identify preparedness priorities in this high-risk region. In this next chapter, I performed a metagenomic survey of beaches across the NWP and the Canadian high Arctic with the goal to provide baseline values of the microbiome composition of sediment from the studied beaches. Additionally, I showed that genes associated with hydrocarbon biodegradation are already present in these presumably pristine beaches.

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Supplementary material can be found in the following appendices:

Appendix 2.1. Supplementary figures (Figures S2.1-S2.8), supplementary tables (Tables S2.2, S2.4 and S2.6), and legends for supplementary tables in Appendix 2.2 (Tables S2.1, S2.3, S2.5, S2.7-S2.10).

Appendix 2.2. Supplementary tables (Tables S2.1, S2.3, S2.5, S2.7-S2.10).

Chapter 2. Metagenomic survey reveals hydrocarbon biodegradation potential of Canadian high Arctic beaches

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2.1. Abstract

2.1.1. Background

Decreasing sea ice coverage across the Arctic Ocean due to climate change is expected to increase shipping activity through previously inaccessible shipping routes, including the Northwest Passage (NWP). Changing weather conditions typically encountered in the Arctic will still pose a risk for ships which could lead to an accident and the uncontrolled release of hydrocarbons onto NWP shorelines. We performed a metagenomic survey to characterize the microbial communities of various NWP shorelines and to determine whether there is a metabolic potential for hydrocarbon degradation in these microbiomes.

2.1.2. Results

We observed taxonomic and functional gene evidence supporting the potential of NWP beach microbes to degrade various types of hydrocarbons. The metagenomic and metagenome-assembled genome (MAG) taxonomy showed that known hydrocarbon-degrading taxa are

present in these beaches. Additionally, we detected the presence of biomarker genes of aerobic and anaerobic degradation pathways of alkane and aromatic hydrocarbons along with complete degradation pathways for aerobic alkane degradation. Alkane degradation genes were present in all samples and were also more abundant (33.8 ± 34.5 hits per million genes, HPM) than their aromatic hydrocarbon counterparts (11.7 ± 12.3 HPM). Due to the ubiquity of MAGs from the genus *Rhodococcus* (23.8% of the MAGs), we compared our MAGs with *Rhodococcus* genomes from NWP isolates obtained using hydrocarbons as the carbon source to corroborate our results and to develop a pangenome of Arctic *Rhodococcus.* Our analysis revealed that the biodegradation of alkanes is part of the core pangenome of this genus. We also detected nitrogen and sulfur pathways as additional energy sources and electron donors as well as carbon pathways providing alternative carbon sources. These pathways occur in the absence of hydrocarbons allowing microbes to survive in these nutrient-poor beaches.

2.1.3. Conclusions

Our metagenomic analyses detected the genetic potential for hydrocarbon biodegradation in these NWP shoreline microbiomes. Alkane metabolism was the most prevalent type of hydrocarbon degradation observed in these tidal beach ecosystems. Our results indicate that bioremediation could be used as a cleanup strategy, but the addition of adequate amounts of N and P fertilizers, should be considered to help bacteria overcome the oligotrophic nature of NWP shorelines.

Keywords: Hydrocarbon bioremediation, baseline survey, Northwest Passage, Arctic marine shoreline

2.2. Background

The continued reduction in sea ice area in the Canadian Arctic Ocean [1] is predicted to allow for open waters between July and October for most regions by the end of the century [2]. One of these regions is the Northwest Passage (NWP) which connects the Atlantic and Pacific Oceans through the Canadian high Arctic (Fig. 2.1). The predicted increase in shipping traffic through the NWP in the coming decades [3] will present environmental risks for the indigenous human populations and the marine and terrestrial environments of the area [4, 5]. This also increases the possibility of an accident leading to a hydrocarbon spill due to the movement of drifting sea ice from the northernmost part of the Canadian Arctic Archipelago and Greenland into the NWP [6] or a rise in storm frequencies [7]. Released hydrocarbons can also be entrapped in and under drifting sea ice which can then be transported away from the spill location [8, 9]. These factors increase the likelihood of marine hydrocarbon spills reaching NWP shorelines. Spill response in the Arctic by governments, industry, and other stakeholders will most likely be limited and slow due to the lack of equipment and resources, high costs, and poor accessibility in highly remote regions [8–10]. For these reasons, simpler remediation options should be considered due to their feasibility and lower cost.

One such remediation option is microbially-mediated hydrocarbon degradation, also known as bioremediation [11, 12]. Indeed, it has been observed that microorganisms capable of biodegrading hydrocarbons are ubiquitous in marine Arctic environments [13]. Previous experimental spills such as the Baffin Island Oil Spill (BIOS) project (1980 – 1984) [14, 15], the *In situ* Treatment of Oiled Sediment Shorelines (ITOSS) program in Svalbard (1996 – 1998) [16–19], as well as real-world cleanup efforts following the *Exxon Valdez* oil spill (1989) [20, 21] have shown that bioremediation can be used to clean hydrocarbons released on Arctic and subarctic marine beach ecosystems.

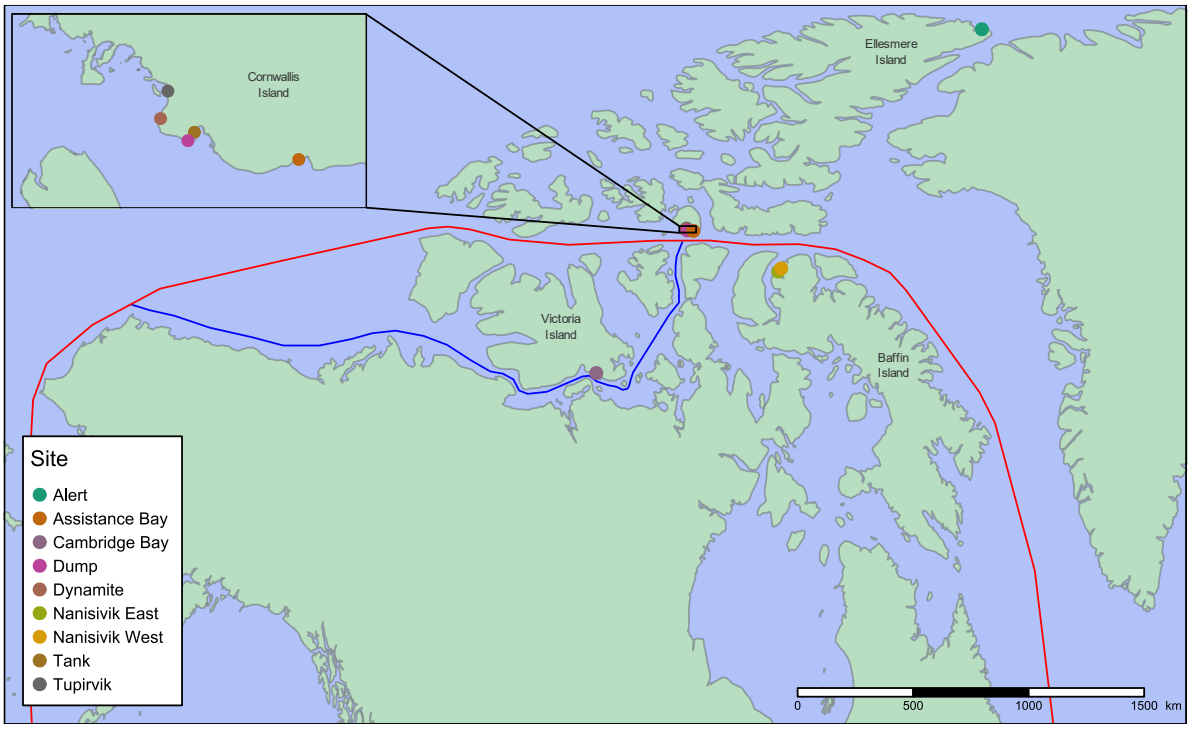

Fig. 2.1. Map of the Canadian high Arctic with the locations of the study sites. Lines show approximate current (blue) and future (red) routes that could be used by the shipping industry to transit the NWP.

Despite their efficacy, bioremediation treatments will only be effective in the Arctic if the native microbiota already contains microorganisms capable of hydrocarbon degradation under the extreme environmental conditions encountered in polar environments. For example, at the *Bahía Paraiso* spill in the Antarctic Archipelago (1989), biodegradation was a negligible as determined by the low mineralization rates in hydrocarbon radiorespiration experiments [22, 23]. The cold and sub-zero temperatures present during most of the year, no sunlight during the winter months, and the highly oligotrophic nature of the Arctic marine environment can slow down the biodegradative activity of the microbial communities [10, 24–26]. For example, on some experimental plots from the BIOS project, oil remains in beach sediments almost 40 years later [27, 28]. Hydrocarbon biodegradation can also be affected by increased salinity on the upper intertidal and supratidal zones of a shoreline due to the salinity tolerance of different

types of microorganisms [29, 30]. Likewise, sediment heterogeneity makes it complicated to compare results between beaches [26]. Accordingly, stakeholders and response teams should have a baseline awareness of which Arctic regions have microbial communities with hydrocarbon degradation potential and which do not.

Previous studies have used conventional microbiological methods such as plate counts to detect changes in oil-degrading microorganism abundances or CO_2 production indicating respiration [15, 31, 32]. Others used indirect methods such as changes in the oil composition relative to specific hydrocarbon chemical markers such as hopane, pristane, or phytane, among others [19, 33, 34]. More recent studies have taken advantage of advancements in molecular microbiology to help detect the presence and explain the activity of hydrocarbon-degrading microorganisms. For example, 16S rRNA gene, metagenomic, metatranscriptomic, and single-cell sequencing were all used by multiple research groups during the Deepwater Horizon oil spill to help describe the changes in the microbial communities in the water column, sediment, and shoreline and show how microbiomes responded to the spill at the functional level [35–37]. These techniques have also been applied to understand the microbial ecology of hydrocarbon biodegradation in Arctic soils [38–41], Arctic seawater and sea ice [42–46], Arctic beach sediments [47–49], and Arctic deep-sea sediments [50–52].

While these studies have increased our understanding of the ecology of hydrocarbon spills in Arctic terrestrial and marine environments, there is a scarcity of research on the interface connecting both areas, the shoreline. In this study, we performed a metagenomic survey of the microbial communities of 8 high Arctic beaches located along the NWP and an additional high Arctic beach that was impacted by a diesel spill. We aimed to understand the hydrocarbon biodegradation potential of NWP beach sediments from both natural and human-impacted shorelines. We also described the overall community composition to provide an overview of scenarios where bioremediation could be used as one of the main cleanup

strategies in the case of a spill in one of these types of beaches due to the delayed and limited response expected under Arctic conditions, as mentioned above [8]. We also compared the results obtained from our metagenomes with microcosm experiments and genome sequences of isolates from some of the same sites of this study grown on hydrocarbons as the sole source of carbon [48, 49, 53]. The corroboration of our metagenomic survey results with the proven metabolic capacity of these isolates serves as evidence that metagenome sequencing can be used as an initial surveying tool to determine the feasibility of bioremediation as a cleanup strategy. Finally, we described other metabolic processes detected in these metagenomes to provide a better understanding of the microbial ecology of these shorelines, which can further help to determine if there are certain environmental conditions that could be limiting the hydrocarbon degradation potential of these microorganisms. Our results serve as a baseline description of the microbial communities of these sites that have not experienced any documented spills which could be used to focus contingency plans to the most vulnerable shorelines as well as to determine remediation endpoints [54].

2.3. Methods

2.3.1. Sampling sites: description and sample collection

The 9 sites used in this study were spread across four regions in the Canadian high Arctic: Cambridge Bay, Resolute, Nanisivik, and Alert (Fig. 2.1; Table S2.1). These sites were selected to represent both natural and human-impacted beaches around the NWP. The hamlet of Cambridge Bay on Victoria Island is one of the most frequented stopover sites for vessels around the current NWP route (blue line on Fig. 2.1); beach sediment was sampled by the docks (sample referred to hereafter as “Cambridge Bay”). The hamlet of Resolute on Cornwallis Island is expected to be a central stopover hub in the future once the ice on the most optimal route of the NWP has disappeared (red line on Fig. 2.1). For this reason, we sampled 5 beaches

around Resolute. (1) “Dump beach”, near where the waste from the hamlet is deposited and later incinerated; (2) “Dynamite beach”, in close proximity to an abandoned dynamite storage site in the relatively pristine Allen Bay; (3) “Tank farm”, adjacent to the fuel storage tanks that supply the Resolute community year-round; (4) Tupirvik, a territorial park on Allen Bay where local hunters often launch their boats; (5) Assistance Bay, an uninhabited beach approximately 17 km away from Resolute, was selected to represent a pristine location facing the NWP that is unlikely to be experiencing any kind of hydrocarbon contamination from anthropogenic sources. We additionally sampled two beaches on Baffin Island near the docks of the former company mining town of Nanisivik. The town is being converted into a refueling station for the Canadian Navy and government ships following an extensive decontamination project to remove metal and fuel contaminants left behind by the mining operations. These beaches are located east and west of the docks. Finally, while not directly on the NWP, we sampled a beach at the Canadian Forces Station – Alert on Ellesmere Island, which is adjacent to areas that experienced diesel spills in 2006 and 2007 [38, 40]. A trench and pond were constructed shortly after a pipeline break to prevent the fuel from reaching the shoreline, but it has not been determined if any fuel was able to go past these barriers.

Between July and August 2018 (Table S2.1), beach sediment samples from the upper 5 cm of the intertidal zone of all beaches were collected aseptically into sterile Whirl-Pak bags and stored at -20 ºC until processed. To evaluate the stability of the microbial communities with time, the sites around Resolute were also sampled in July 2019 (Table S2.1). For Assistance Bay, beach sediment samples were collected only in July 2019 from the upper 5 cm of both the intertidal and supratidal zones of the beach to evaluate how sediment heterogeneity within the same beach affects the microbial community of these two zones.

2.3.2. Physicochemical and hydrocarbon analyses

Salinity and dissolved oxygen were measured *in situ* on the pore water of the beach sediments using a YSI probe (Xylem) for the 2019 samples from the Resolute region. Nitrate, nitrite, ammonia, and phosphate were measured using the same pore water with CHEMetrics test kits (K-6933, K-7003, K-1503, K-8503, respectively) using a CHEMetrics V-2000 photometer. A sub-sample of the collected sediments from 2018 and from the 2019 Assistance Bay intertidal sediment were analyzed by SGS Canada Inc. to quantify petroleum hydrocarbons (PHCs), semi-volatile organic compounds (SVOCs) and volatile organic compounds (VOCs). PHCs were quantified using the Canadian Council of Ministers of the Environment Reference Method for the Canada-wide Standard for Petroleum Hydrocarbons in Soil – Tier 1. SVOCs were quantified using the USEPA methods 3541 and 8270D. VOCs were quantified using the USEPA methods 5035A, 5030B, 8260C.

2.3.3. DNA extraction, library preparation, and sequencing

We extracted internal DNA (iDNA) from intact viable cells and from extracellular/environmental DNA (eDNA) separately from the collected beach sediments. We used this approach to account for the fact that shorelines are an active environment receiving microbial inputs from both land and sea which leads to the possibility that we could detect eDNA from different sources that is not an active part of the shoreline microbiome [55–57]. For this we followed the methods described elsewhere [58, 59]. Briefly, the beach sediment was suspended in a sodium phosphate buffer and cells were separated from the eDNA by shaking and centrifuging the solution so that cells and the remaining sediment particles were collected in a pellet and eDNA was obtained from the supernatant. The pellet was resuspended in sodium phosphate buffer and lysed using a PowerBead tube (Qiagen). iDNA and eDNA were recovered from their respective supernatants using silica beads in a guanidine hydrochloride solution. Libraries were prepared using the Nextera XT DNA Library Prep Kit

(Illumina) and indexed using the Nextera XT Index Kit v2 (Illumina) following the manufacturer's instructions. The indexed libraries were sequenced by Genome Québec in an Illumina HiSeq 4000 platform using a PE100 flow cell.

2.3.4. Bioinformatics and statistical analyses

Reads were first trimmed with Trimmomatic v0.11.5 [60] to remove low quality bases and sequencing adapters. iDNA and eDNA metagenomes were separately assembled with metaSPAdes v3.14.1 [61]. Reads were mapped to the assembled metagenomes with BBMap v38.87 [62]. Metagenomes were annotated and classified using MetaErg v1.2.0 [63]. We first tested for differences between the viable and potentially active (iDNA) and the inactive transient (eDNA) communities of the beaches based on the 16S rRNA gene sequences identified by MetaErg after removing reads classified as chloroplast or mitochondria (Fig. S2.1). The Shannon index was calculated for each metagenome using the phyloseq package v1.40.0 [64] and, after assessing the normality of the dataset with a Shapiro-Wilk test, a paired *t*-test was used to test differences in Shannon indexes between the two types of metagenomes (Fig. S2.2). 16S rRNA gene counts were transformed using relative abundances to account for read depth, then Bray-Curtis dissimilarities were calculated with phyloseq and visualized using non-metric multidimensional scaling (NMDS). PERMANOVA and PERMDISP were calculated with the vegan package v2.6-4 [65] to test for differences in community composition between iDNA and eDNA (Fig. S2.3). Alpha level for all tests was 0.05 and were performed in R v4.2.2.

Since we observed no statistical differences in Shannon index or Bray-Curtis dissimilarities between the iDNA and eDNA metagenomes (Fig. S2.2 and S2.3), we merged the iDNA and eDNA metagenomes of each sample into a total DNA metagenome. The total DNA metagenomes contained 40,735,858 ± 14,414,163 paired reads per sample. After combining the two datasets for each sample, reads were co-assembled using metaSPAdes.

Reads were mapped to the co-assembled metagenomes with BBMap, the metagenomes were annotated and classified with MetaErg. We used the 16S rRNA gene sequences annotated from the co-assembled dataset by MetaErg to determine differences in alpha diversity and community composition among the beaches. We tested differences in Shannon index using a paired Wilcoxon signed ranked test for differences between years and ANOVA to test differences among regions. Differences in community composition among regions and between years were analyzed with PERMANOVA and PERMDISP based on Bray-Curtis dissimilarities. Given that we obtained a significant result for the region PERMANOVA, we then tested for pairwise differences in community composition among regions using a pairwise PERMANOVA [66]. To further corroborate the community composition stability between years, we compared the Bray-Curtis dissimilarities for all pairs of sites collected in 2018 and 2019 (e.g., Tank farm – 2018 vs Tupirvik – 2018, etc. and Dynamite beach – 2019 vs Dump beach – 2019, etc.) and with the Bray-Curtis dissimilarities between years for all samples (e.g., Tupirvik – 2018 vs Tupirvik – 2019, etc.) using a Kruskal-Wallis rank sum test. As we observed the presence of aromatic and anaerobic degradation genes only for a few samples (see Results below), we tested for differences in the overall community composition of these samples with a PERMANOVA against those which did not contain these types of genes.

Genome binning was performed with MetaBAT2 v2.12.1 [67] after which the quality of the produced metagenome-assembled genomes (MAGs) was improved with RefineM [68]. Overall bin statistics were estimated with CheckM [68] and MAG completeness and contamination was determined with CheckM2 [69]. MAGs were classified with GTDB-Tk v2.1.0 [70] using the Genome Taxonomy Database (GTDB) r207 and individually annotated with MetaErg. A phylogenomic tree of the final MAG collection was created by first obtaining the aligned and concatenated amino acid sequences of single-copy core genes of the anvi'o (v7.1) Bacteria_71 collection [71]. The phylogenomic tree was then inferred by maximum

likelihood using FastTree [72] within anvi'o and the resulting tree was manually midpoint rooted.

To further understand the microbial ecology in the absence of hydrocarbons, we manually explored the KEGG annotations obtained from MetaErg to detect the presence of nitrogen, sulfur, and carbon metabolisms in the MAGs (Table S2.2). We used CANT-HYD (--cut_nc) [73] to identify the presence of 37 marker genes involved in aerobic and anaerobic degradation pathways of a wide variety of aliphatic and aromatic hydrocarbons. To complement the CANT-HYD results, if we obtained a CANT-HYD hit for a given hydrocarbon degradation gene, we looked at the KEGG annotation to determine if the rest of the genes in the respective degradation pathway were present in selected high-quality MAGs. The MAG1 and MAG12 cell diagram used to exemplify the genomic potential of the NWP beach microbiomes was created with Biorender.com.

Because we observed a high abundance of MAGs assigned to *Rhodococcus*, we created a pangenome of Arctic *Rhodococcus* to determine the ubiquity of hydrocarbon degradation genes in genomes from this genus. The pangenome was created with anvi'o using *Rhodococcus* MAGs obtained in this study along with *Rhodococcus* genomes from NWP beach isolates capable of fuel oil degradation [53]. The genome sequences of these *Rhodococcus* isolates are available on NCBI under the BioProject accession number PRJNA945214.

2.4. Results

2.4.1. Taxonomic composition of metagenomes and MAGs

Based on average relative abundances of the 16S rRNA gene sequences extracted from the metagenomes of all samples, the microbial communities of the studied NWP beaches are dominated by *Pseudomonadota* (43.4% ± 14.45), *Actinomycetota* (36.3% ± 27.9), and *Bacteroidota* (13.4% ± 8.1); fewer than 1% of 16S rRNA gene reads were classified as *Archaea*

(Fig. 2.2). Similar patterns were observed for the taxonomic classification of the metagenomic reads based on the MetaErg annotation (Fig. S2.4). The 20 most abundant genera all belonged to the same three dominant phyla (Fig. S2.5, Table S2.3) with *Rhodococcus* having the highest average relative abundance among samples (24.7% ± 20.3).

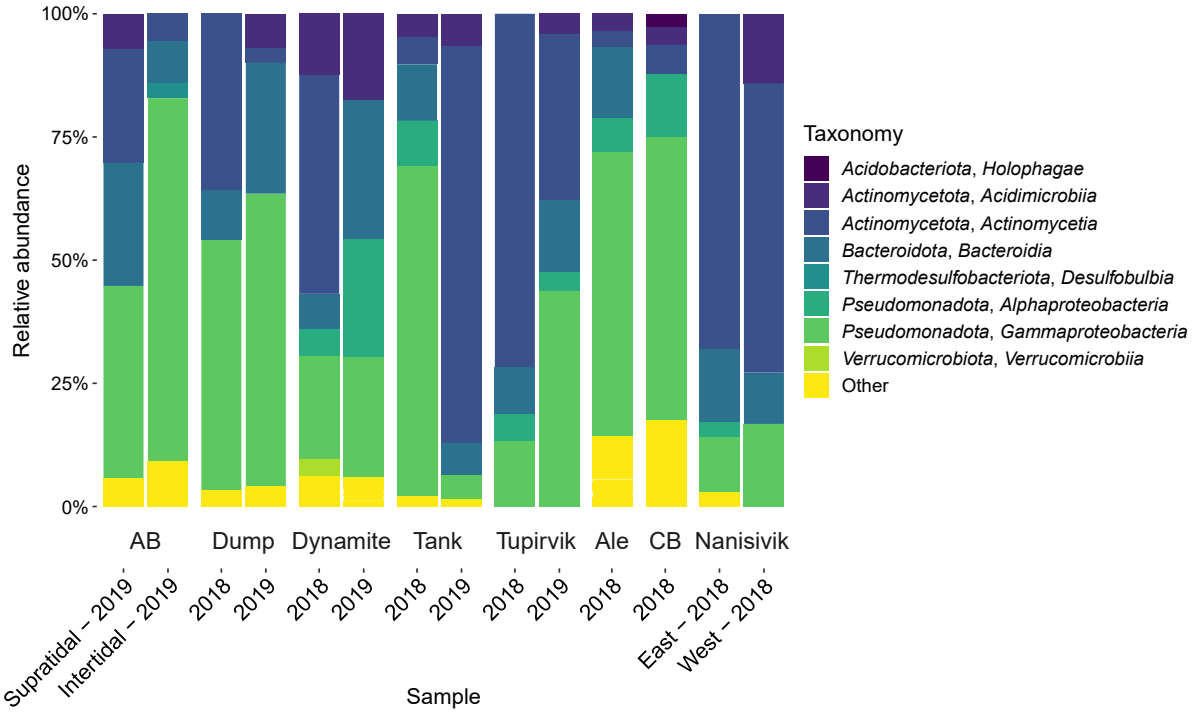

Fig. 2.2 Taxonomy of the microbial communities of the studied NWP beaches. The taxonomic ranks included were Phylum and Class, respectively. Relative abundances were calculated using metagenome-extracted 16S rRNA gene sequences. Taxa with a relative abundance lower than 2.5% were pooled into "Other". AB: Assistance Bay; Ale: Alert; CB: Cambridge Bay.

We recovered 82 bins which recruited 48.9% of the total metagenomic reads. From these, we obtained 19 high-quality (>90% completeness and <5% contamination) and 23 medium-quality (>50% completeness and <10% contamination) MAGs (Table S2.4). The MAGs were classified into 5 different phyla (Fig. 2.3A), with most of them belonging to *Pseudomonadota* (59.5%, 25 MAGs) and *Actinomycetota* (23.8%, 10 MAGs). We assessed

genome novelty of these MAGs based on the taxonomic classification rank assigned with the GTDB (Fig. 2.3B; Table S2.4). Most of the MAGs (71.4%, 30 MAGs) were unclassified at the species level and 4.8% (2 MAGs) further showed novelty at higher taxonomic ranks: 1 at the genus level (MAG26; family *Porticoccaceae*) and 1 at the family level (MAG38; order *Woeseiales*).

2.4.2. Beach communities are stable over time and among NWP regions

We observed no statistical differences in the Shannon index (Wilcoxon signed rank test: V = 1, p = 0.25; Fig. S2.6) or Bray-Curtis dissimilarities (PERMANOVA: pseudo-F = 0.616, R^2 = 0.093, p = 0.857; Kruskal-Wallis: H = 1.550, p = 0.461; Fig. S2.7) for the beaches in the Resolute region sampled in 2018 and 2019. For differences among the studied regions (Resolute, Alert, Nanisivik, and Cambridge Bay), we detected no statistical differences in the Shannon index (ANOVA: F = 2.529, p = 0.116; Fig. S2.8). PERMANOVA did detect statistically significant differences in community composition among regions (PERMANOVA: pseudo-F = 1.496, R^2 = 0.310, P = 0.012), but after pairwise comparisons were performed with a pairwise PERMANOVA, the statistical differences were no longer observed (Table S2.5; Fig. S2.9). We observed a higher proportion of *Gammaproteobacteria* in the intertidal zone of the Assistance Bay sediment compared to the supratidal zone sediment of the same beach. On the other hand, there was a higher proportion of *Actinomycetia* and *Bacteroidia* 16S rRNA gene reads in the supratidal zone compared to the intertidal zone (Fig. 2.2).

2.4.3. NWP beach microbiomes contain genes from multiple pathways associated with hydrocarbon degradation

We were able to detect 15 out of the 37 hydrocarbon degradation marker genes analyzed by CANT-HYD in our metagenomes (Fig. 2.4) with the most prevalent genes being associated with the aerobic degradation of alkanes. Genes associated with aerobic alkane degradation were

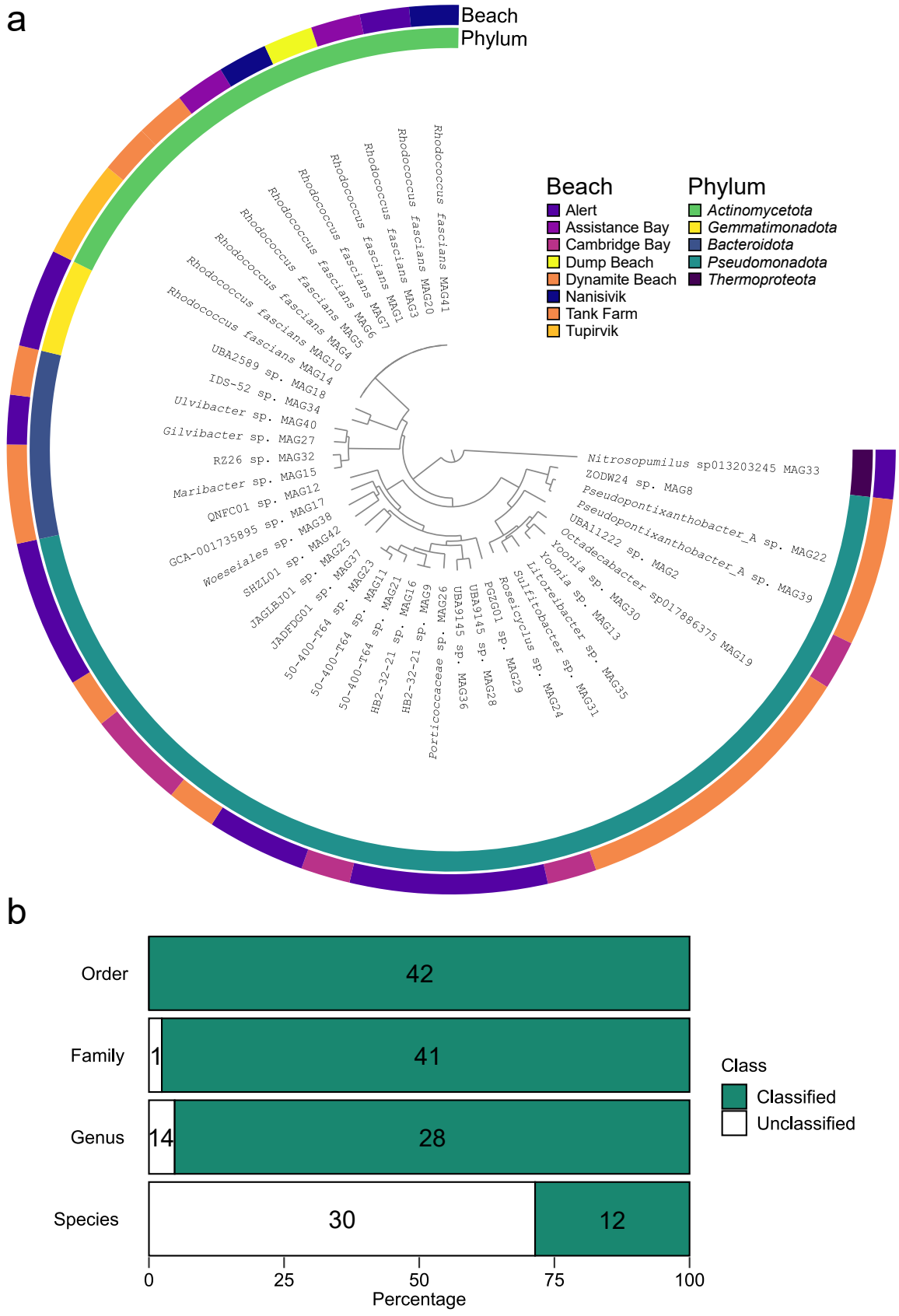
a
Beach
Phylum
Beach
Alert
Assistance Bay
Cambridge Bay
Dump Beach
Dynamite Beach
Nanisivik
Tank Farm
Tupirvik
Phylum
Actinomycetota
Gemmatimonadota
Bacteroidota
Pseudomonadota
Thermoproteota
Rhodococcus fascians MAG41
Rhodococcus fascians MAG20
Rhodococcus fascians MAG3
Rhodococcus fascians MAG1
Rhodococcus fascians MAG7
Rhodococcus fascians MAG6
Rhodococcus fascians MAG5
Rhodococcus fascians MAG4
Rhodococcus fascians MAG10
Rhodococcus fascians MAG14
UBA2589 sp. MAG18
IDS-52 sp. MAG34
Ulvibacter sp. MAG40
Gilvibacter sp. MAG27
RZ26 sp. MAG32
Maribacter sp. MAG15
QNFC01 sp. MAG12
GCA-001735895 sp. MAG17
Woeseiales sp. MAG38
SHZL01 sp. MAG42
JAGLBJ01 sp. MAG25
JADFDG01 sp. MAG37
50-400-R64 sp. MAG23
50-400-R64 sp. MAG11
50-400-R64 sp. MAG21
HB2-32-21 sp. MAG16
HB2-32-21 sp. MAG9
Porticoccaceae sp. MAG29
UBA9145 sp. MAG36
UBA9145 sp. MAG28
PGZG01 sp. MAG29
Roseicyclus sp. MAG24
Sulfitobacter sp. MAG31
Litorcibacter sp. MAG35
Yoonia sp. MAG13
Octadecabacter sp017886375 MAG19
UBA11222 sp. MAG2
Pseudopontixanthobacter_A sp. MAG39
Pseudopontixanthobacter_A sp. MAG22
ZODW24 sp. MAG8
Nitrosopumilus sp013203245 MAG33
b
Order
Family
Genus
Species
42
1
41
14
28
30
12
0
25
50
75
100
Percentage
Class
Classified
Unclassified

Fig. 2.3 Taxonomy and novelty of NWP metagenome-assembled genomes (MAGs). **A** Maximum-likelihood phylogenomic tree of the medium- and high-quality MAGs obtained from the sampled Arctic beaches. Samples are grouped by beach and phylum. **B** Percentage of MAG taxonomic novelty at various ranks based on their GTDB-Tk classification. Numbers on the boxes represent the number of MAGs classified/unclassified for a given rank.

highly prevalent with alkB (associated with the degradation of C5 – C22 hydrocarbons [74, 75]), cyp153 (associated with the degradation of C5 – C10 hydrocarbons [75]), and ladAα (associated with the degradation of C15 – C36 hydrocarbons [76]) being present in all 9 beaches. Genes coding for the large (prmA) and small (prmC) subunits of the propane monooxygenase were also present in 7 beaches (77.8%). Genes associated with the degradation of mono- (MAH alpha/beta and tmoA/E) and polycyclic (nboB/C and non-ndoB) aromatic hydrocarbons were much less abundant with 62.5% of the sampled beaches (5 beaches) containing at least one gene from these pathways. Anaerobic hydrocarbon degradation genes were much less prevalent as they were only found in the Alert beach sediment. We also detected hydrocarbon degradation genes in 23 (54.7%) of our MAGs and 12 (30%) of our low-quality bins (Fig. 2.5). Most of these contained one or more aerobic alkane degradation genes with cyp153 and alkB being the most abundant and present in 22 (52.3%) and 8 (20%) of the MAGs and bins, respectively. Aerobic aromatic degradation genes were detected in 6 (7.3%) of the MAGs and bins, whereas genes involved in the anaerobic degradation pathway of alkanes (ahyA) and ethylbenzene (ebdA) were only present in MAG12. We observed statistical differences in the community composition of samples where we detected aromatic and anaerobic degradation genes (Dynamite beach – 2019, Tank farm – 2018, Alert – 2018, Cambridge Bay – 2018, Nanisivik East – 2018) compared to the samples that did not contain these genes (PERMANOVA: pseudo-F = 1.8141, R2 = 0.131, P = 0.043).

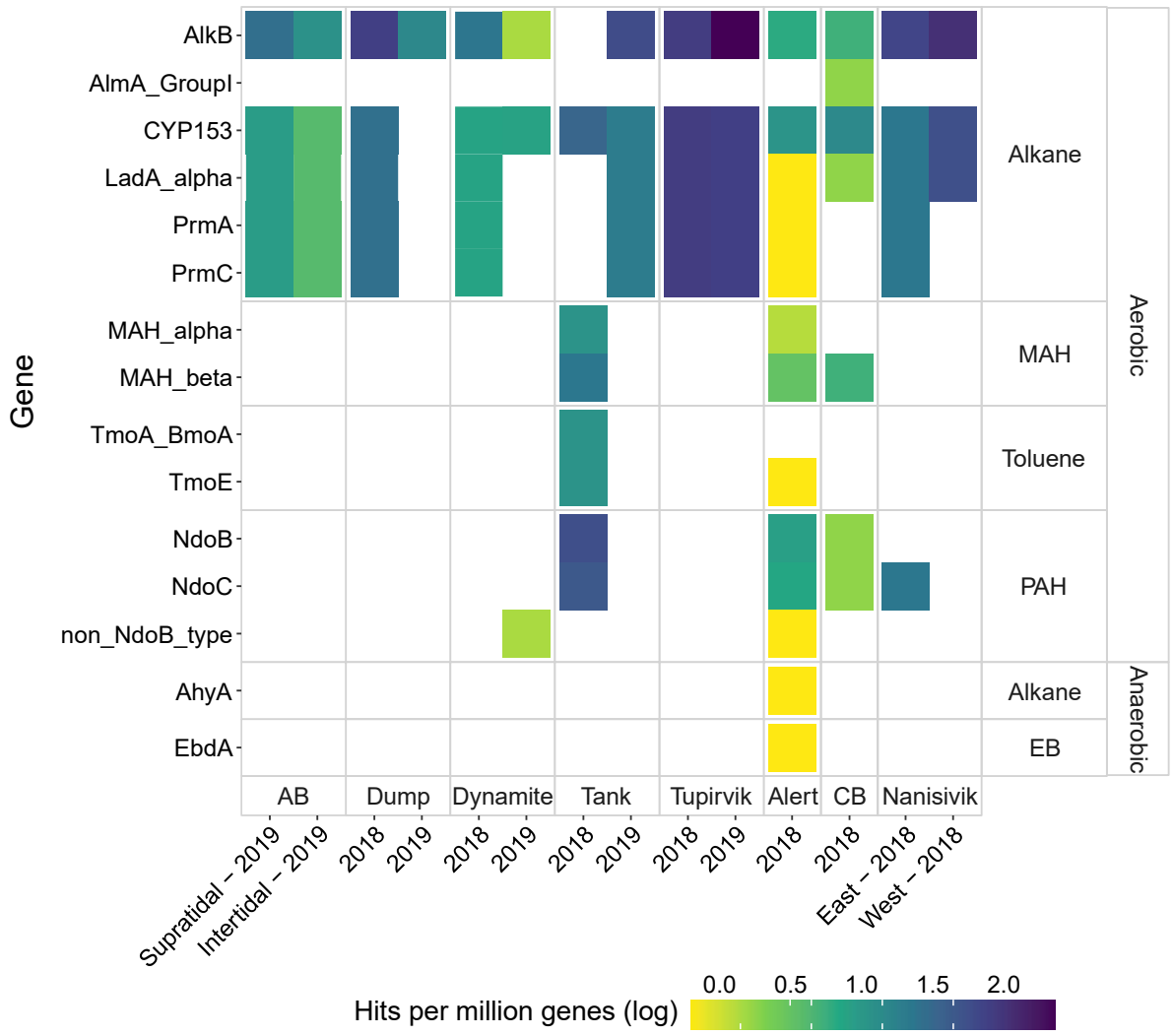

Fig. 2.4 Abundances of CANT-HYD marker hydrocarbon degradation genes present in the NWP metagenomes. CANT-HYD gene counts were normalized to hits per million coding genes per sample and then transformed using the natural logarithm. AB: Assistance Bay; CB: Cambridge Bay; MAH: monocyclic aromatic hydrocarbon; PAH: polycyclic aromatic hydrocarbon; EB: ethylbenzene.

2.4.4. High-quality MAGs reveal the functional potential of NWP beaches

Taxonomic classification showed that the genus *Rhodococcus* were highly prevalent in most beaches with an average relative abundance of 26.1% ± 22.4 of 16S rRNA gene reads, 30.3% ± 27.8 overall metagenome reads, and 10 (23.8%) of the MAGs were classified as *Rhodococcus*

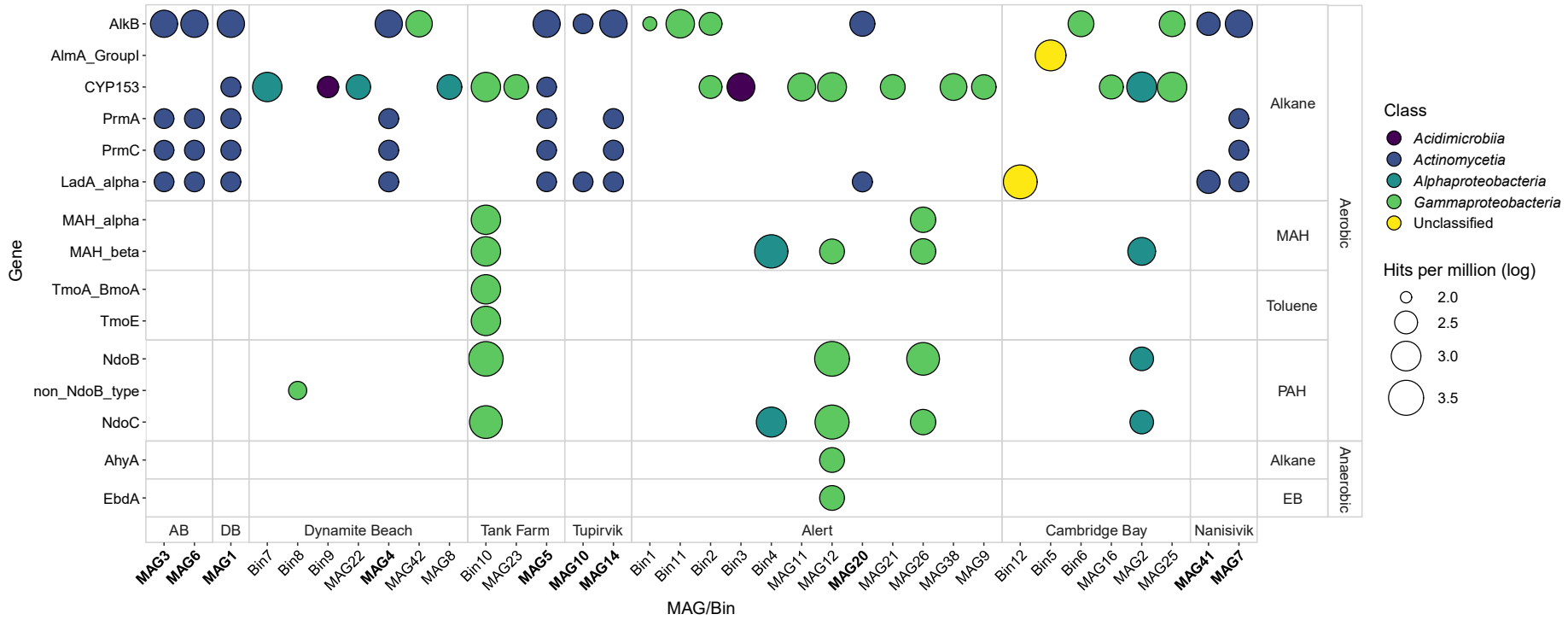

Fig. 2.5 Abundances of CANT-HYD marker hydrocarbon degradation genes in the recovered MAGs and selected bins. CANT-HYD gene counts were normalized to hits per million coding genes per sample and then transformed using the natural logarithm. AB: Assistance Bay; DB: Dump beach; MAH: monocyclic aromatic hydrocarbon; PAH: polycyclic aromatic hydrocarbon; EB: ethylbenzene. MAGs with their name highlighted in bold were classified as *Rhodococcus fascians*. The detailed taxonomy of each MAG/bin is listed in Table S2.4.

fascians with 5 of them having 100% completion and less than 0.5% contamination. Additionally, we also observed the presence of 13 (30.9%) MAGs that belonged to various taxa within the phylum *Pseudomonadota* with 6 (14.3%) having over 90% completion and less than 1% contamination. Therefore, we studied these MAGs in more detail to look for the presence of a larger variety of the genes comprising these degradative pathways. One of the limitations of the CANT-HYD pipeline is that it detects only one or a few key marker genes per pathway [73], usually associated with the first enzyme involved in the pathway, but it cannot determine whether the full biodegradative pathway is present. By complementing the CANT-HYD results with the KEGG annotations obtained from MetaErg, we were able to reconstruct hydrocarbon degradation pathways with a higher resolution for MAG1, classified as *R. fascians* and representing the highly prevalent *Rhodococcus* clade, and MAG12, one of the *Pseudomonadota* MAGs classified to the putatively novel genus QNFC01 of the family *Immundisolibacteraceae* (Fig. 2.6). Through this method, we observed that MAG1 and MAG12 encoded a complete pathway for alkane degradation. The CANT-HYD results showed that MAG1 contains three copies of *alkB* and one copy each of *cyp153*, *ladAα*, *prmA*, and *prmC*, while MAG12 contains two copies of *cyp153*. Alkanes are converted to fatty acids followed by β-oxidation (ko00071) and the resulting acetyl-CoA molecules are further metabolized via the TCA cycle (ko00020). MAG12 also contained a copy of the putative anaerobic alkane hydroxylase *ahyA* for which there is still not a concrete metabolic pathway defined after the hydroxylation step [77].

The CANT-HYD results showed no MAH or PAH genes in MAG1, but we did detect one copy each of the MAH beta subunit and *ebdA*, 7 copies of *ndoB*, 6 copies of *ndoC* in MAG12. While we did not obtain complete MAH and PAH degradation pathways, we detected a larger number of genes from the KEGG annotations compared to the standalone CANT-HYD results. The KEGG annotations detected the presence of genes involved in the initial activation

of the aromatic rings (*phyA*, *tmoF*, and *xylA*) as well as genes belonging to three intermediate pathways: the catechol ortho- (*catA*) and meta-cleavage (*catE*), and the protocatechuate (*pcaGH*) pathways [78]. Additionally, we observed the presence of a gene (*bbsB*) involved in an intermediary step of the anaerobic degradation of toluene [79].

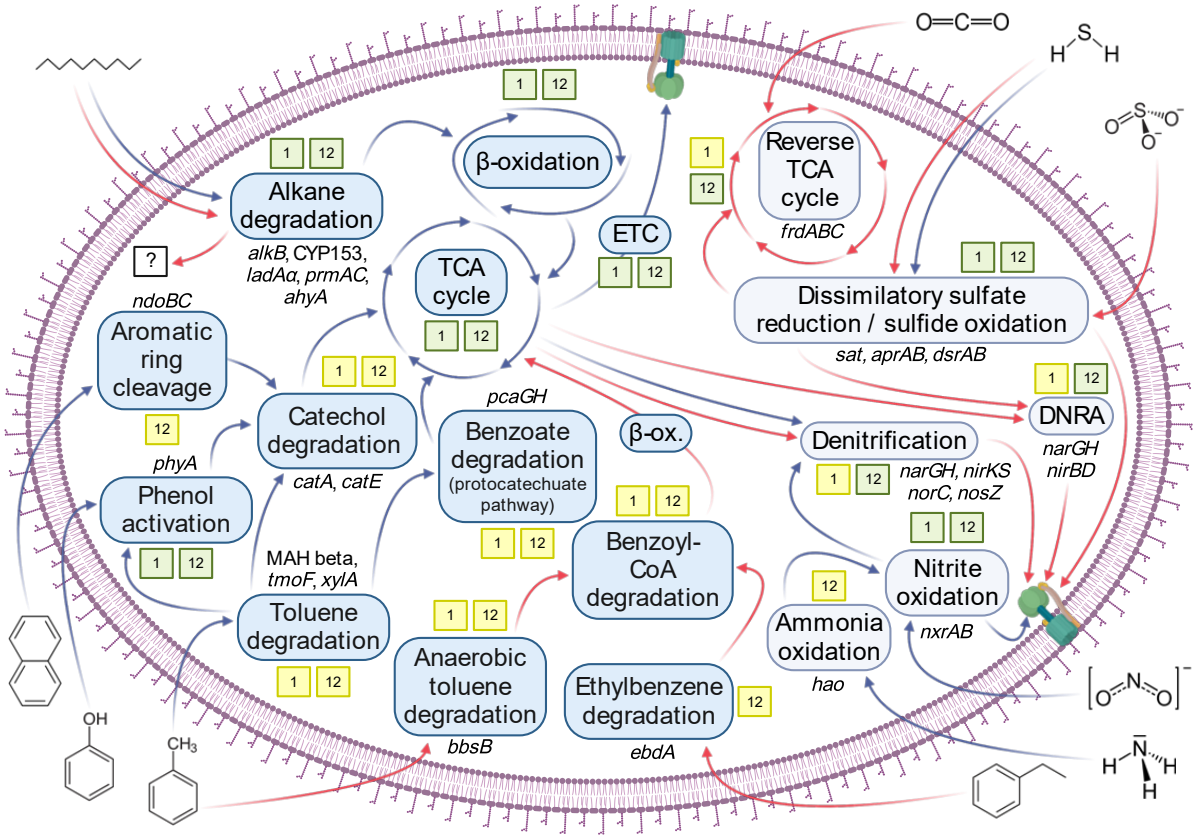

Fig. 2.6 Cell schematic illustrating the diverse metabolisms of MAG1 and MAG12. MAG1 was classified as *Rhodococcus fascians* and was recovered from the 2018 Dump beach metagenome. MAG12 was classified as inside the putatively novel genus QNFC01 in the family *Immundisolibacteraceae* and was recovered from the 2018 Alert metagenome. Genes next to their pathways were detected using CANT-HYD and KEGG annotations. Green and yellow boxes represent complete and incomplete metabolic pathways for each MAG, respectively. Blue and red arrows represent aerobic and anaerobic pathways, respectively.

On the other hand, complete and partial pathways for various anaerobic respiration metabolisms as well as other metabolisms that can be performed in the absence of hydrocarbons were present in MAG1 and MAG12 as well as in other high- and medium-quality MAGs (Fig. 2.6; Tables S8 and S9). Nitrite oxidation and DSR were the most prevalent pathways we observed with 31 (73.8%) and 30 (71.4%) MAGs having complete pathways for these processes, respectively. Anaplerotic pathways were also prevalent with 18 (42.9%) MAGs possessing the complete set of genes for these pathways and 24 (57.1%) having partial pathways. DNRA (39 MAGs, 92.9%), denitrification (41 MAGs, 97.6%), and the rTCA cycle (34 MAGs, 81.0%) were partially present in most MAGs. Ammonia oxidation was the least prevalent metabolism with 2 (4.8%) and 18 (42.9%) of MAGs possessing a complete or partial pathway, respectively.

2.4.5. The pangenome of *Rhodococcus* reveals the ubiquity of alkane degradation in the Arctic

Given the high prevalence of *Rhodococcus* MAGs in our dataset, we conducted a pangenomic analysis for NWP *Rhodococcus* (Fig. 2.7) by comparing our 10 *Rhodococcus* MAGs with the genomes of 7 *Rhodococcus* strains isolated from Tupirvik beach sediments capable of growing on ultra-low sulfur fuel oil (ULSFO) as the sole carbon source [53]. Aerobic alkane degradation appears to be part of the core pangenome of Arctic *Rhodococcus* as *alkB* was present in all the MAGs and isolates with more than one copy detected in all but two MAGs (MAG10 and MAG41). In addition, *ladAα* was present in 14 (82.4%) of the studied genomes (Table S2.6). Three other alkane degradation genes (*cyp153* and *prmA/C*) were also detected in 52.9% of the genomes.

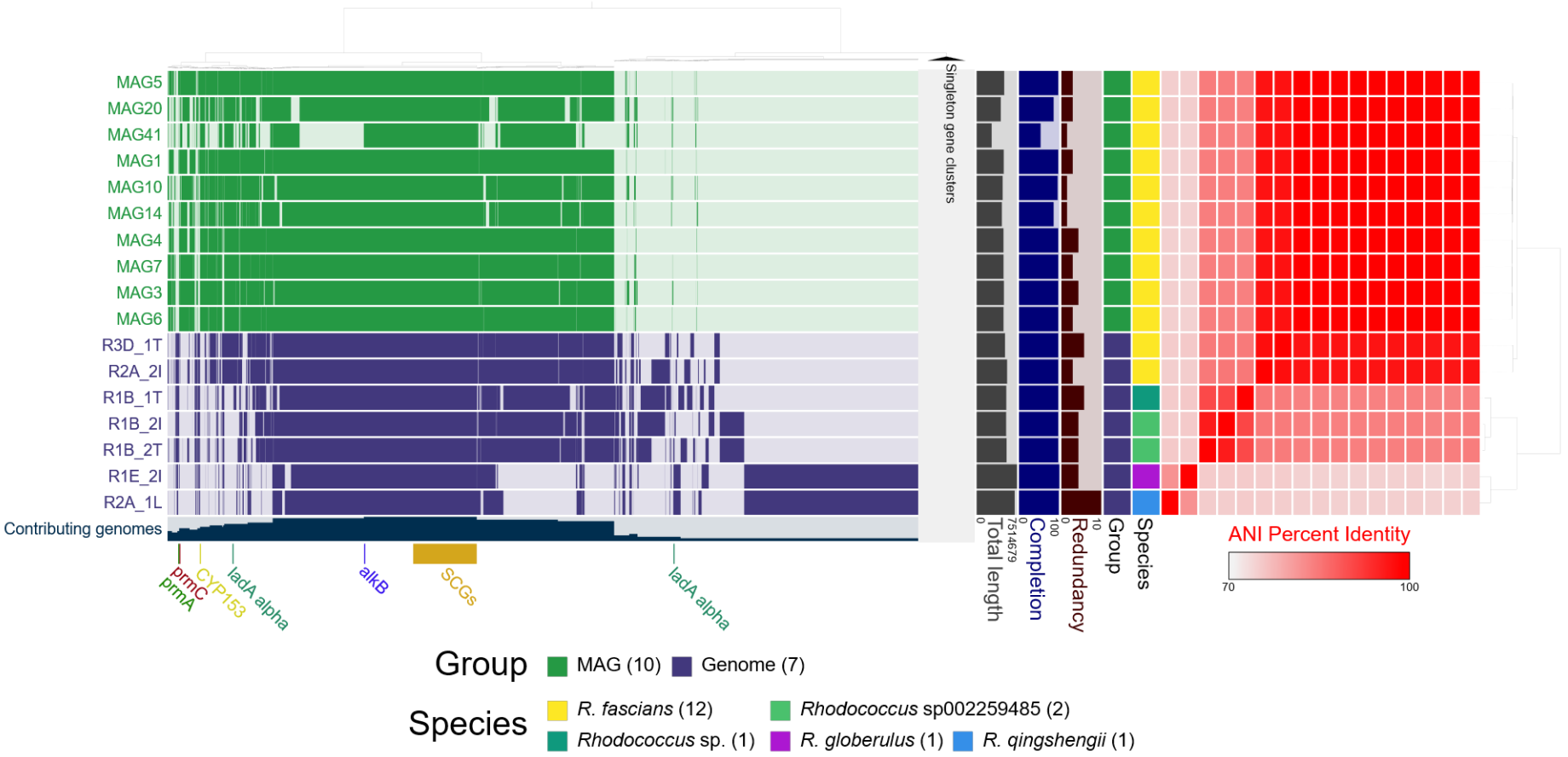

Fig. 2.7 Pangenome of Arctic *Rhodococcus*. MAGs recovered in this study were compared to the genomes of *Rhodococcus* strains isolated from Tupirvik beach sediment and grown using ultra low sulfur fuel oil (ULSFO) as the sole carbon source. The pangenome shows that the alkane monooxygenase (*alkB*) is part of the core pangenome and that other alkane degradation pathways part of the accessory pangenome are prevalent in many of these genomes. SCGs: single-copy core genes; ANI: average nucleotide identity.

2.5. Discussion

To our knowledge this is the first metagenomic survey of Canadian high Arctic beaches describing their community composition along with their functional potential, particularly with regards to hydrocarbon biodegradation. Our statistical comparison of the metagenomes of beaches sampled in two different years suggests that the microbial communities of these beaches might remain relatively constant throughout the years. This could indicate that the results in this study, as well as future metagenomic surveys on other Arctic beaches along the NWP, could be valid longitudinally over moderate (several years) timescales. The creation of a genomic database of NWP beach microbial communities, including those studied here, would be of value for the Canadian government and other stakeholders to define baseline microbiome profiles, high-risk shorelines, and safer travel routes, as well as improving preparedness and contingency plans in case of an oil spill [80–82]. With regards to the comparison of the microbial community among the four sampled regions, we observed no statistical differences either. However, it should be noted that the limited number of samples for certain regions drastically reduces the statistical power of this analysis. This can also cause non-homogeneous multivariate dispersions among groups (PERMDISP: $F = 25.53$, $P = 0.001$) that could lead to overly conservative PERMANOVA results [83]. Differences in the taxonomic composition of the supratidal and intertidal zone sediments from Assistance Bay were observed, but it was not possible to produce statistical evidence of these differences given that we only had one sample per zone. However, we did observe similar abundances of alkane degradation genes for both zones which suggests that bioinformatic analyses based solely on taxonomy are not sufficient to determine hydrocarbon degradation potential. Obtaining a larger number of samples from sites in these regions could help to determine more clearly the similarities and differences in the marine beach microbiomes of the various areas of the NWP. Future studies should also

include robust environmental and hydrocarbon concentration data to be able to associate patterns in the microbial community to these variables.

The microbial communities of these NWP beaches were dominated by *Pseudomonadota*, *Actinomycetota*, and *Bacteroidota* which is consistent with a previous study which described the community composition of the same beaches using 16S rRNA gene sequencing [48] and with a metagenomic study of the same area in Alert where our sample originated [38]. We observed a larger proportion of *Actinomycetota* in our metagenomes, mostly due to the high abundance of *Rhodococcus* sequences, compared to the composition of a clone library obtained from a beach in Spitsbergen, Norway [16] as well as for sea ice and seawater 16S rRNA gene and metagenomic libraries of samples taken around Cornwallis Island [84, 85] and for 16S rRNA gene sequencing of Labrador Sea seawater [42]. The higher proportion of this phylum has been associated with low (<10%) organic matter in Arctic and sub-Arctic soils [39, 41]. This is consistent with the oligotrophic nature of the beaches in this study which had organic matter contents ranging from 0.26% – 0.95% [48]. Our MAG novelty at the genus level is consistent with a compendium of marine environments [86], but lower than previous metagenomic studies conducted using water from the Baltic Sea (90.9%) [87] and from various sites across the Arctic Ocean (83.2%) [88].

The taxonomic classification obtained from our metagenomic survey suggests that the microbial communities of the studied NWP shorelines have the genomic capacity to bioremediate a hydrocarbon spill. Among the 20 most abundant genera, bacteria belonging to 13 of those genera are known to be capable of degrading various types of hydrocarbons and the abundance of another 4 genera has been positively correlated with the presence of hydrocarbons (Table S2.3). Previous studies have also observed the increase in abundance of the genera *Rhodococcus*, *Flavobacterium*, and *Psychrobacter* in microcosms grown using Tank farm beach sediment and ULSFO as the sole source of carbon as well as Tupirvik beach

sediment amended with marine diesel [48, 49]. We recovered MAGs belonging to known hydrocarbon-degrading taxa such as the families *Alcanivoracaceae* [89] and *Immundisolibacteraceae* [90] and a bin from the family *Cycloclasticaceae* [91], as well as MAGs belonging to 8 genera associated with hydrocarbon degradation (Table S2.4). Also among our MAGs are those classified to *Ilumatobacter*, *Rhodococcus*, *Sulfitobacter*, *Cycloclasticus*, *Loktanella*, and *Granulosicoccus*; genera that are present in the Tupirvik and Tank farm microcosm studies using fuels as a carbon source [48, 49]. This was further corroborated with our CANT-HYD and KEGG annotation results which showed the presence of one or more key hydrocarbon-degrading genes as well as complete degradation pathways in our metagenomes and MAGs. We obtained similar abundances of CANT-HYD biomarker genes compared to seawater samples taken by the TARA Oceans survey from marine environments around the world, including various polar sites [73].

Hydrocarbon analyses showed that PHCs, SVOCs, and VOCs were below the detection limit for most beaches, with the exception of Dump beach, Tank farm, and Nanisivik – East (Table S2.7). However, hydrocarbon concentrations detected for those three beach sediments were still below the Canada-wide Standards for petroleum hydrocarbons in soil for industrial use [92]. Based on this, our metagenomic results are consistent with previous studies that have shown the ubiquity of hydrocarbon degradation pathways in marine environments in the absence of a hydrocarbon spill [93, 94]. The presence of these organisms and their pathways suggest that there is a natural hydrocarbon cycle occurring in marine environments that is sustaining hydrocarbon degrading populations in pristine environments. The first explanation for this phenomenon is that some “obligate” hydrocarbon degraders can grow using non-hydrocarbon organic compounds such as dissolved organic carbon and cellular components of lysed marine cells [95, 96]. The second explanation is presence of hydrocarbon seeps that release short-chain gaseous alkanes and liquid alkanes and aromatic hydrocarbons [52, 97]. A

hydrocarbon seep has been found in the Canadian Arctic near Scott Inlet (~900 km from Cornwallis Island), but it was observed that hydrocarbon concentrations decrease with distance from the seep and background methane levels are observed in the upper regions of the water column [97]. The third explanation is the existence of a cryptic marine alkane cycle in which cyanobacteria and eukaryotic phytoplankton produce long-chain alkanes and alkenes which are then quickly metabolized by hydrocarbon degraders that are closely associated with these photosynthetic organisms [94, 98, 99]. Hydrocarbon biosynthesis is suggested to be a universal process in cyanobacteria [100] and alkanes and alkenes appear to be required to maintain their membrane flexibility, which is required for cell division and growth [101]. Alkane-synthesizing cyanobacteria have been isolated and detected in metagenomic studies of Arctic ponds [98, 102]. However, we did not find any 16S rRNA gene sequences or MAGs assigned to cyanobacteria in our dataset. We did observe cyanobacterial sequences in the MetaErg classification, but they only accounted for 0.2% ± 0.11 of the overall metagenomic reads.

Mono- or polycyclic aromatic hydrocarbon degradation pathways were less prevalent compared to the highly prevalent alkane metabolism detected in the shoreline metagenomes. MAHs and PAHs are generally more recalcitrant to biodegradation due to their greater size or complexity, thus requiring multi-operon metabolic pathways [11, 103]. The low prevalence of these complex pathways in the NWP beach microbiomes could explain why PAHs tend to remain in Arctic environments for longer periods of time compared to their aliphatic counterparts [27, 28, 46, 50]. This is in line with the results of the microcosm experiment carried out previously using beach sediment from Tank farm which showed relatively higher rates of alkane biodegradation compared to the PAH degradation rates [48]. This study also performed radiorespiration assays using Tank farm, Nanisivik, and Cambridge Bay sediments supplemented with ^{14}C-labelled hexadecane and naphthalene and confirmed that respiration rates were higher in the hexadecane microcosms [48], which could be explained by the lower

prevalence of genes in these pathways that we observed in the metagenomes of these beaches. Anaerobic hydrocarbon degradation genes were only observed in the Alert beach sediment. While we did not quantify dissolved oxygen for the Alert sample, we did observe high oxygen concentrations in the Resolute samples. It has been observed that oxygen diffusion decreases in soils as water freezes [104] which, combined with the close to freezing temperatures of NWP shorelines, could result in microscopic particles of frozen beach sediment where anaerobic conditions could be occurring. Nonetheless, we observed the presence of genes encoding for other anaerobic metabolic pathways in multiple MAGs from the studied beaches (Table S2.8, S9). The reduced number of reference sequences that were used to create the HMMs for the anaerobic pathways in CANT-HYD could cause divergent sequences to not be detected [73], which could explain why we only observed anaerobic hydrocarbon degradation genes at Alert. Additionally, increasing the sequencing depth could help detect the presence of low abundance genes, such as those for an anaerobic metabolism in well oxygenated beaches.

It is worth noting that we found genes related to MAH and PAH biodegradation for the beaches that appear to have the highest baseline levels of hydrocarbon contamination. Similar to Alert [38, 40] there is a known history of hydrocarbon contamination at Nanisivik, but we did not detect elevated levels of hydrocarbon contamination in the sampled sediments (Table S2.7). There are other sites where past and current human activity could be causing smaller undocumented releases of hydrocarbons. For example, there is a relatively high volume of shipping activity at the main dock where the sample from Cambridge Bay was taken and the Tank farm is an active fuelling station. This suggests that hydrocarbon-degrading microorganisms inhabiting NWP shorelines could thrive when hydrocarbons are released into their environment. This was demonstrated in the microcosm experiments using Tank farm and Tupirvik beach sediment in which a higher abundance of hydrocarbon-degrading bacteria and

genes were observed for samples incubated with fuels compared to the unoiled controls [48, 49].

The 16S rRNA gene and MAG taxonomies indicated a high prevalence of *Rhodococcus* in NWP shorelines. This is consistent with previous studies showing that *Rhodococcus* appears to be an abundant genus in Arctic and Antarctic marine and terrestrial environments with the genus comprising up to 34% of the community [105–108]. While we detected sequences belonging to other *Rhodococcus* species in our metagenomes, we were only able to obtain MAGs classified as *R. fascians*. Previous studies have shown that *R. fascians* is often present in large proportions in Arctic marine environments [105, 108] and seasonal dominance of this phytopathogenic species has been observed in a Norwegian fjord following the collapse of phytoplankton blooms [107].

Our hybrid annotation approach in which we complemented CANT-HYD hits with KEGG orthologs allowed us to detect the presence of multiple marker genes for alkane degradation along with the complete downstream pathways required to fully metabolize these hydrocarbons. This suggests that MAG1 is capable of degrading short-, medium-, and long-chain (C_3, C_5 – C_{13}, and C_{15} – C_{36}) alkanes [74, 75, 109, 110] and MAG12 has the genetic potential to degrade short- and medium (C_5 – C_{13}) alkanes aerobically [75] and anaerobically [77]. We observed the presence of the first step of the degradation of phenols (*phyA*; K03380) in both MAGs and the catalytic subunits of the naphthalene 1,2-dioxygenase (*ndoBC*) in MAG12. However, given that we only detected a subset of the genes required for complete degradation of aromatic compounds with this hybrid approach, we cannot conclusively state that these two MAGs can perform these metabolisms. *R. fascians* has been grown using various types of MAHs and PAHs [111]. *R. fascians* can also produce biosurfactants capable of solubilizing anthracene [112] and emulsifying kerosene [113]. Multiple *Rhodococcus* isolates, including *R. fascians*, obtained from Tupirvik beach sediment have been grown using ULSFO

as the carbon source at 5 °C [53]. The genetic similarity of these isolates with our MAGs (Fig. 2.7) supports the hydrocarbon degradative potential of the *R. fascians* MAGs of NWP beaches even at cold temperatures. *Immundisolibacter cernigliae*, the only described species from *Immundisolibacteraceae*, is capable of growing on a wide range of PAHs at mesophilic temperatures [90], but its ability to grow under cold conditions has not been reported. The genus QNFC01 was first detected from deep oceanic sediments close to a hydrothermal vent [114], which is in accordance with the cold tolerance and anaerobic metabolism we see for MAG12. This is important as the low temperatures encountered in the Arctic can limit hydrocarbon biodegradation rates [25].

Similar to the aerobic aromatic degradation pathways, we detected a limited number of genes for their anaerobic counterparts. While not direct evidence of anaerobic hydrocarbon biodegradation potential, we did detect pathways that could be coupled with these metabolisms. For example, denitrification, DNRA, and DSR were all present in the two studied MAGs and these processes often occur as anaerobic alternatives to aerobic respiration of hydrocarbons when oxygen conditions are limited, using nitrate or sulfate as the terminal electron acceptors [77, 115]. We observed high levels of oxygen saturation in pore water from the beach sediments in the Resolute region (Table S2.1), which suggests that anaerobic processes are not occurring at high rates during the Arctic summer.

Our metabolic reconstruction of MAG1 and MAG12 also revealed that these strains not only have potential as hydrocarbon degraders, but also have pathways for other aerobic and anaerobic metabolic processes. These metabolisms include nitrite oxidation, denitrification, DNRA, DSR, sulfide oxidation, and carbon fixation through the rTCA cycle (Fig. 2.6). *Rhodococcus* strains are capable of performing simultaneous heterotrophic nitrification and aerobic denitrification [116] and we observed the genomic potential for both processes in our MAGs. Autotrophic denitrification and DNRA can be coupled with sulfide [117] and sulfite

[118, 119] oxidation, but these processes have not yet been shown to occur in *Rhodococcus*. For *I. cernigliae*, no growth under anaerobic conditions has been observed [90], but there is the potential for anaerobic metabolism in QNFC01 MAGs that were recovered from deep ocean sediments [114].

Finally, both sulfide and nitrite oxidation can be coupled to CO_2 fixation in chemolithoautotrophic microbes [120]. We observed the presence of genes encoding fumarate reductase (*frdABC*), 2-oxoglutarate synthase (*korAB*), and ATP-citrate lyase (*ACLY*) in MAG12 and only *frdABC* in MAG1. These genes encode the key non-reversible enzymes involved in the reverse TCA cycle [121] and further corroborate the genomic capacity of MAG12 to perform various anaerobic metabolisms. Citrate synthase, which performs the opposite reaction to the ATP-citrate lyase in the TCA cycle, is also able to operate reversibly in a process that is not easily detectable bioinformatically but still present in many organisms not thought to be capable of CO_2 fixation [122], which could be the case for MAG1. A related species, *Rhodococcus erythropolis* N9T-4, is capable of growth using trace CO_2 as a carbon source with a novel CO_2 fixation pathway which has not yet been fully described [123]. Various *Rhodococcus* strains can perform heterotrophic CO_2 fixation as part of the propane and propylene degradation pathways [123–125] and to replenish TCA metabolites as part of anaplerotic pathways [126, 127]. MAG1 does possess a propane monooxygenase (*prmAC*) and the carboxylases involved in the anaplerotic pathways (pyruvate carboxylase, *pycAB*; phosphoenolpyruvate carboxylase, *ppc*; and malate dehydrogenase, *maeB*) and we detected *pycAB* and *maeB* in MAG12. Anaplerotic pathways appear to be ubiquitous in Arctic soils, particularly in permafrost, which tends to be the most carbon-poor soil horizon [128].

These carbon and nitrogen metabolisms are also present in other medium- and high-quality MAGs (Tables S7 and S8). We quantified nitrate, nitrite, and ammonia concentrations in the pore water from beaches of the Resolute region with ammonia being the most abundant

form present in these sediments (Table S2.1). This could suggest that ammonia has been produced which could indicate that processes such as ammonification or DNRA could be occurring at high rates in these environments. We have also observed the presence of sulfide at Assistance Bay in 2022 (unpublished data) which supports the findings in the present study of a high prevalence of DSR genes in our metagenomes. Future research should use expression-based molecular techniques to determine whether these mechanisms are indeed occurring in the studied Arctic beaches.

The capabilities of these MAGs to perform such a wide variety of metabolic processes that tend to be associated with alternative sources of energy and nutrients are consistent with the oligotrophic conditions encountered across NWP beaches (Table S2.1). It is then very likely that bioremediation efforts on NWP shorelines will probably require the addition of N and P fertilizers in order to stimulate the microbial communities enough so that they can overcome their nutrient limitations, especially of decreased N and P concentrations expected after a hydrocarbon spill due to the increased metabolic activity from the growing hydrocarbon-degrading microbial populations colonizing the area of the spill [36]. The side effects of fertilizer use on the microbial communities besides stimulation of hydrocarbon degraders, for example eutrophication or anoxia [129], should also be evaluated before applying these products during a hydrocarbon spill. Further studies using the isolates we have obtained from these environments [53] will evaluate the physiological capabilities of Arctic *R. fascians* and other microorganisms to perform the multiple metabolisms that we described in MAG1 and MAG12. This will improve our understanding of the microbial ecology of these sites and help guide the optimization of bioremediation strategies aimed at enriching these organisms in contaminated shorelines so they can be used to clean up impacted beaches.

2.6. Conclusions

In this study, we described the microbial communities of marine beaches across the Canadian high Arctic based on a metagenomic survey focusing on the genomic potential for hydrocarbon biodegradation in these microbiomes. Our results showed that the microbial communities on these beaches harbour various hydrocarbon degradation pathways, mostly for the degradation of alkanes. This suggests that the microbial communities of Arctic beaches may be able to adapt and respond in the case of a hydrocarbon spill, making bioremediation a potential clean up strategy. We also described the presence of other nitrogen and sulfur metabolisms, such as nitrite oxidation, DNRA, and DSR, which these microbes might be performing in their environment using other sources of carbon. Future studies should focus on *in situ* and laboratory studies that confirm whether the microorganisms from these beaches are in fact capable of carrying out the metabolic processes described here under the cold and oligotrophic conditions that are observed across NWP shorelines.

2.7. Availability of data and material

The metagenome and MAG datasets supporting the conclusions of this article are available in the NCBI Sequence Read Archive (SRA) repository under the BioProject accession number PRJNA1046404. The scripts used for the bioinformatics analyses were deposited on GitHub (https://github.com/estebangongora/NWP_beach_metagenomes).

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2.9. Author's contributions

EG and LGW conceived the study. EG designed the study and methodology. EG, LGW, and CWG collected the sediment samples. EG performed and interpreted the data analysis and wrote the manuscript. All the authors reviewed, revised, and approved the manuscript.

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2.11. Competing interests

The authors declare that they have no competing interests.

2.12. Ethics approval, consent to participate, and consent for publication

Not applicable

2.12. References

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Connecting text

In the previous chapter, I showed that hydrocarbon degradation genes are quite ubiquitous in the baseline microbial communities of beaches along the NWP that have not experienced any recorded hydrocarbon contamination. While these results are encouraging as they show that the microbes inhabiting these beaches do have the genomic potential to degrade hydrocarbons, this does not confirm that they will be capable of completely degrading a hydrocarbon spill that washes into one of these Arctic beaches. In order to obtain stronger evidence of the capabilities of these microorganisms, I deployed mesocosms that simulate a fuel spill on a NWP beach with the goal of describing how the hydrocarbon biodegradation process occurs *in situ* using metagenomic and metatranscriptomic tools. I included mesocosms with three different kinds of fuels that represent legacy, current, and future fuels used by the shipping industry. This is the first description of the biodegradation of the new generation of LSFOs that have recently been introduced for use in shipping vessels across the world.

This manuscript will be submitted to Science of the Total Environment.

Supplementary material can be found in the following appendices:

Appendix 3.1. Supplementary figures (Figures S3.1-S3.6), supplementary tables (Table S3.3), and legends for supplementary tables in Appendix 3.2 (Tables S3.1, S3.2, S3.4-S3.10).

Appendix 3.2. Supplementary tables (Tables S3.1, S3.2, S3.4-S3.10).

Chapter 3. *In situ* mesocosm experiment shows the capability of the microbial community of a Canadian high Arctic shoreline to degrade the new generation of ship fuels

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3.1. Abstract

The warming effects of climate change are leading to a reduction in sea ice which will allow the opening of various shipping routes across the Arctic in the upcoming decades. The increase of ships transiting this region also brings the possibility of a hydrocarbon spills. The behaviour, particularly the biodegradability, of the new generation of low sulfur fuels which are currently being used by vessels around the world has not been assessed on Arctic beaches, where it is the likely destination following a spill. In this study, we deployed mesocosm experiments on a remote beach in the Canadian high Arctic for 33 days using two different types of low sulfur fuels: marine diesel and the new ultra-low sulfur fuel oil (ULSFO). We also compared these fuels to the now phased out Bunker C fuel oil. Our results showed that the low sulfur fuels were more easily biodegraded (Marine diesel: 72.8% biodegradation, 2.9% non-biological removal; ULSFO: 37.6% biodegradation, 10.0% non-biological removal) compared to Bunker C which is mostly removed from beach sediments by other natural processes such as dissolution into the water (14.6% biodegradation, 62.8% non-biological removal). The microbial communities of the shoreline sediments adapted to the presence of the fuels with an increase in the proportion of *Pseudomonadota* bacteria, mostly from the family *Moraxellaceae* (11.7% of the 16S rRNA gene amplicon-based community composition and 38.1% of the recovered metagenome-assembled genomes). These microorganisms expressed multiple genes associated with the biodegradation of aliphatic hydrocarbons. However, we did not find many genes associated with the degradation of aromatic hydrocarbons. This study provides the first evidence of the biodegradability of one of the new low sulfur fuel oils under *in situ* Arctic environmental conditions. Nonetheless, our results also suggest that a combination of a limited number of available hydrocarbon biodegradation pathways, combined with the cold and nutrient-poor environmental conditions encountered in the Arctic could negatively affect the efficacy of

bioremediation as a cleanup strategy. Additional techniques such as the addition of N and P fertilizers to stimulate microbial metabolism should be explored.

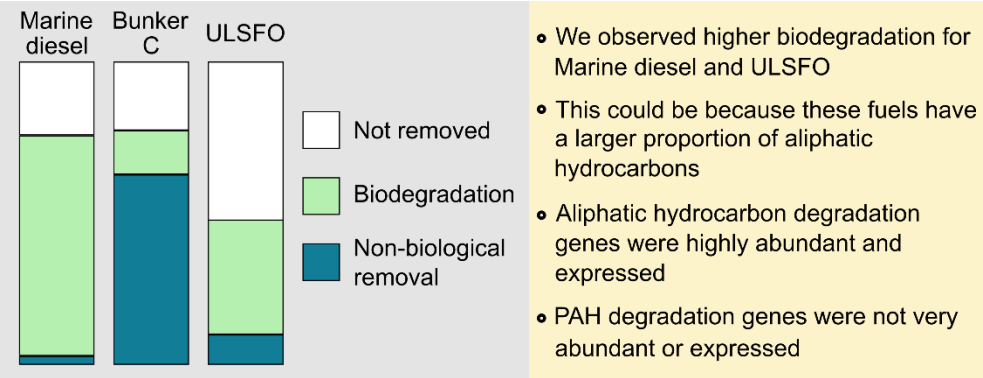
3.1.1. Highlights

- The natural attenuation of low sulfur fuels under Arctic conditions was characterized
- Low sulfur fuels were more easily biodegraded compared to Bunker C fuel oil
- Arctic beach bacteria can adapt to the presence of oils on the shorelines
- The presence of alkane and few aromatic hydrocarbon degradation genes was confirmed

3.1.2. Keywords

Ultra-low sulfur fuel oil, biodegradation, microbiome, beach sediment, Northwest Passage.

3.1.3. Graphical abstract



3.2. Introduction

A reduction of sea ice in the Arctic caused by the warming effects of climate change is expected to result in ice-free waters during the summer months by the middle of the century (Shen et al., 2023). Open water conditions will result in the opening of several routes that could reduce shipping transit times significantly (Smith and Stephenson, 2013). One of these routes is the Northwest Passage (NWP) in the Canadian Arctic Archipelago which connects the Atlantic and Pacific Oceans. Several factors, including the lack of international consensus on the legislation that will regulate traffic through the NWP (Bartenstein, 2019), the movement of drifting sea ice from the northernmost parts of the Arctic into the channels of the NWP (Mudryk et al., 2021), and the increase of wave heights and storm frequency (Liu et al., 2016), will pose environmental risks for the region. One of these risks is a hydrocarbon spill from transiting ships. Another potential risk of an accidental spill could occur during fuel transfer as most communities in the Canadian high Arctic rely on fuel oil for their energetic needs which is brought up North via bunker ships. Any cleanup response in case of such a spill is expected to be slow and limited due to the geographical isolation of the high Arctic (Emmerson and Lahn, 2012) with a considerable proportion of the fuel possibly washing up onto a shoreline.

The reduced accessibility of the NWP as well as the high costs of accessing such remote locations also means that there will be less equipment available to deploy specialized remediation plans (Emmerson and Lahn, 2012). For these reasons, the implementation of simpler cleanup options, such as natural attenuation and bioremediation, should be considered for a hydrocarbon spill in the NWP (Góngora et al., 2022; Péquin et al., 2022). Microorganisms capable of biodegrading hydrocarbons have been observed on Arctic and sub-Arctic shorelines during experimental spills (Garrett et al., 2003; Grossman et al., 2000; Sergy and Blackall, 1987), as well as for the cleanup of the *Exxon Valdez* oil spill (Bragg et al., 1994; Pritchard et al., 1992). It has also been observed that these microbes are present in various beaches

throughout the NWP (Freyria et al., 2024; Góngora et al., 2024) and are capable of degrading ship fuel (Chen et al., 2024; Ellis et al., 2022; Lirette et al., 2024). However, the cold and oligotrophic conditions encountered in the Arctic could limit the metabolic capabilities of potential hydrocarbon-degrading bacteria (Durand et al., 2023; Ellis et al., 2022; Gomes et al., 2022; Hunnie et al., 2023; Schreiber et al., 2023).

The Deepwater Horizon spill in the Gulf of Mexico brought a new lens to the field of bioremediation as it allowed for the use of state-of-the-art omics techniques to help understand how the microbial communities are responding to the large inputs of oil in their environment (Bacosa et al., 2018; Kleindienst et al., 2015; Lamendella et al., 2014; Mason et al., 2012; Yergeau et al., 2015). Recent studies have started to characterize the changes in microbial community composition and metabolism that follow the addition of hydrocarbons in Arctic waters (Cao et al., 2022; Garneau et al., 2016; Gofstein and Leigh, 2023; Lofthus et al., 2021; Pyke et al., 2023; Vergeynst et al., 2019a) and on tidal sediments (Durand et al., 2023; Ellis et al., 2022; Lirette et al., 2024). However, while these laboratory experiments have attempted to simulate Arctic weather, they cannot fully account for the unpredictable environmental conditions that would be encountered in the field in the case of a spill on a NWP beach.

In this study, we deployed mesocosms on a NWP tidal beach to determine the *in situ* hydrocarbon biodegradation metabolic capacity of the microorganisms inhabiting this beach using three types of fuels used by the shipping industry: Marine diesel, Bunker C, and ultra-low sulfur fuel oil (ULSFO). We hypothesized that the lighter, aliphatic-rich fuels (Marine diesel and ULSFO) would be more biodegradable than the heavier, aromatic-rich Bunker C fuel oil. We also hypothesized that there will be limited aromatic hydrocarbon degradation due to the reduced presence of genes associated with the degradation of these compounds in NWP beaches (Góngora et al., 2024). We evaluated the biodegradation of these fuels over a period of one month, representative to the duration of the warmest part of the Arctic summer. We

observed that Marine diesel and ULSFO were mostly removed from the mesocosms by biodegradation while Bunker C was mostly removed by other natural processes such as physical removal by tidal action. Metagenomic and metatranscriptomic analyses of the beach microbiome showed that the reduced presence and expression of key aromatic hydrocarbon degradation genes resulted in the lower biodegradation activity for Bunker C. These results allowed us to understand the genetic limitations of NWP beach microbes and provide insights on how response teams could stimulate these organisms to improve their hydrocarbon-degrading metabolic activity.

3.3. Materials and methods

3.3.1. Experimental setup and sample collection

Assistance Bay (74.6509º N, 94.2983º W) is located approximately 17 km south-east from the hamlet of Resolute on Cornwallis Island, Nunavut, Canada (Fig. 3.1). This uninhabited beach was chosen for this experiment as it directly faces the NWP and it is expected that Resolute, the second northernmost town in Canada, will be a central stopover hub once the NWP becomes ice free. The mesocosms consisted of a Fluortex hydrophobic fluorocarbon-based netting (product reference 09-250/39, Sefar) which was coated with one of three fuels commonly used by the shipping industry: Marine diesel (Glencore Limited), Bunker C (provided by the Canadian Association of Petroleum Producers), and ULSFO (Shell Trading Rotterdam B.V.). The marine diesel used is a light refined ultra-low sulfur diesel that was obtained from the CCGS Amundsen, a research vessel that navigates the NWP during the summer. Bunker C is a residual heavy fuel oil previously used on ships that has been phased out due to stricter sulfur emission regulations imposed by the International Convention for the Prevention of Pollution from Ships (MARPOL; Vedachalam et al., 2022). ULSFO is a refined fuel oil designed to comply with the MARPOL emission regulations.

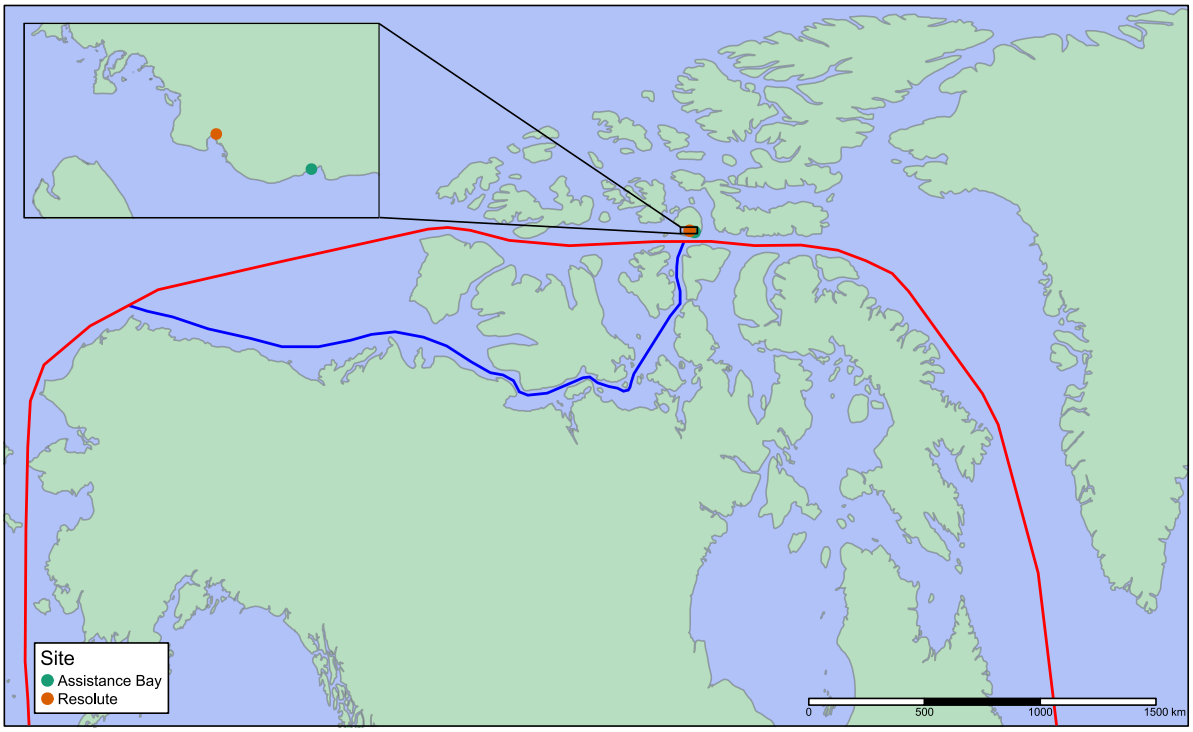


Fig. 3.1 Map of the Canadian high Arctic with the location of the study site in Assistance Bay. Lines show approximate current (blue) and future (red) routes that could be used by the shipping industry to transit the NWP.

The mesocosms were assembled using 30 × 15 cm pieces of netting that were washed with HPLC grade dichloromethane (DCM; Applied Biosystems) to remove any residual contaminants that might have been deposited on the netting during handling and cutting. The netting was then wrapped around 15 × 15 × 0.5 cm stainless steel plates and secured using nylon fishing line to anchor the netting and prevent it from being washed away by tidal action. The netting-wrapped plates were coated with fuel with the aid of a nylon brush until the netting was saturated. An environmental control consisting of netting-wrapped plates with no fuel added was included with the other treatments. The mesocosms were deployed in duplicates on Assistance Bay in July 9, 2019 in the intertidal and supratidal beach zones. The intertidal zone represents the area of the shoreline between the low and high tide and mesocosms in this zone received action from the regular tide cycle. The supratidal zone is located above the high tide

line and was chosen to represent a scenario in which a storm temporarily raises the tide line and washes the spilled fuel to an area that regular tide action would not normally reach. The mesocosms were placed at a depth of 5 cm and the netting-wrapped plates were separated from each other by approximately 25 cm. Replicate sets were separated from each other by approximately 1 m.

Samples were recovered in August 11, 2019 after 33 days. The mesocosms were individually packed into sterile Whirl-Pak bags and taken immediately to the Polar Continental Shelf Project laboratory in Resolute for processing. The netting was aseptically removed from the plates and cut into six 15 × 5 cm fragments. Three of the fragments were individually rolled, inserted into sterile 15 ml Falcon tubes and DNA/RNA Shield (Zymo Research) was added into the tubes until the netting was fully submerged, followed by storage at -80 °C. Samples were transported in coolers to McGill University (Montréal, Canada) where they were stored at -80 °C until they were processed for nucleic acid extraction. The other three fragments were individually rolled, inserted into 20 ml amber glass vials with closed caps with silicone liners (Thermo Scientific) and 10 ml of DCM were added to the vials, followed by storage at 4 °C. Samples were transported in coolers to McGill University where they were stored at 4 °C until they were processed for hydrocarbon analysis.

3.3.2. Hydrocarbon analyses

Samples were analyzed by the Bigelow Laboratory for Ocean Sciences to quantify hydrocarbons on the netting by GC/MS using a modified EPA method 8270D as described elsewhere (Aeppli et al., 2018). A time zero (T_0) control for all fuels prepared the same way as the treatment mesocosms (as described in section 2.1) was also sent for analysis.

3.3.3. Nucleic acids extraction

The DNA/RNA Shield-preserved netting was allowed to thaw on ice. The tubes were vortexed for 90 s to allow any bound cells and particles to be detached from the netting and resuspended into the supernatant. The solutions were allowed to settle on ice until the foam produced by the vortex dissipated. DNA and RNA were then extracted into separate fractions using the ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research) following the manufacturer's instructions with minor modifications. One ml of the supernatant was directly added to the ZR BashingBead Lysis Tube without adding more DNA/RNA Shield and the lysis tubes were shaken for 45 s on a Mini-Beadbeater-16 (BioSpec Products). All centrifugation steps were carried out at 16,000 g unless stated otherwise by the original protocol. DNA/RNA was eluted in 50 µl of ZymoBIOMICS DNase/RNase-Free Water in the final elution step. An extraction control consisting of 500 µl of ZymoBIOMICS DNase/RNase-Free Water and 500 µl of DNA/RNA Shield was processed with the samples, as described above. Extracted DNA was stored at -20 °C until processed. Extracted RNA was treated with TURBO DNase (Invitrogen) following the manufacturer's instructions as we detected DNA contamination in the RNA samples after performing the DNAse treatment included in the ZymoBIOMICS kit. RNA was then purified using the Monarch RNA Cleanup Kit (New England Biolabs, NEB) following the manufacturer's instructions with the following modifications: ethanol (300 µl) was added in step 2 to include small RNAs and the final elution volume was 20 µl. Clean RNA was stored at -80 °C until processed.

3.3.4. Library preparation and sequencing

The 16S rRNA gene was amplified using primers 515F-Y (5′-GTGYCAGCMGCCGCGGTAA) and 926R (5′-CCGYCAATTYMTTTRAGTTT) containing Illumina overhang adapter sequences (Parada et al., 2016). PCR reactions (25 µl) containing 0.5 U of KAPA HiFi DNA Polymerase (Roche), 0.6 µM of each primer, 0.3 mM of KAPA

dNTP Mix (Roche), 1X KAPA HiFi Fidelity Buffer (Roche), and 1 µl of DNA were performed under the following conditions: initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 45 s, and a final extension step at 72°C for 10 min. Reactions were purified using Sera-Mag Select magnetic beads (Cytiva) with a 0.8 bead-to-PCR volume ratio. Indexing was performed using the Nextera XT Index Kit v2 (Illumina) following manufacturer's instructions. Indexed samples were purified with Sera-Mag Select magnetic beads (1.12 bead-to-PCR volume ratio) and quantified using the Qubit fluorometer (Invitrogen). Samples were pooled in equimolar ratios of 4 nM and sequenced with a 2 × 300 bp v3 flow cell with an Illumina MiSeq platform. Adapters and indices were removed with the Illumina FASTQ file generation pipeline.

Metagenomic libraries were prepared using the Nextera XT DNA Library Prep Kit (Illumina) and indexed using the Nextera XT Index Kit v2 following the manufacturer's instructions. The rRNA was depleted from the RNA samples using the NEBNext rRNA Depletion Kit Bacteria (NEB) and the rRNA-depleted RNA was used to prepare metatranscriptomic libraries with the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB) and indexed using the NEBNext Dual Index Multiplex Oligos for Illumina (NEB). The metagenomic and metatranscriptomic indexed libraries were sequenced by Genome Québec with an Illumina NovaSeq 6000 platform using PE100 and PE150 flow cells, respectively.

3.3.5. Bioinformatics and statistical analyses

3.3.5.1. 16S rRNA gene amplicon analyses

Amplicon sequence variants (ASVs) were obtained from the 16S rRNA gene reads using DADA2 v1.26.0 (Callahan et al., 2016a). ASVs were classified using the Silva 138.1 database (Quast et al., 2013). The negative control reads were used to remove potential contaminant ASVs with decontam v1.22.0 (Davis et al., 2018) using the frequency method. Mitochondrial,

chloroplast, and ASVs unclassified at the phylum level were also removed. We averaged ASV abundances across 1000 rarefactions to the minimum library size of 5727 as described previously (Sugden et al., 2022). A phylogenetic tree was inferred using phangorn v2.11.1 (Schliep, 2011) using the method described elsewhere (Callahan et al., 2016b). The resulting ASV table was imported to phyloseq v1.46.0 (McMurdie and Holmes, 2013) for statistical analyses.

All statistical analyses were performed in R 4.3.2 (R Core Team, 2024). We tested differences in hydrocarbon removal between removal type (natural attenuation vs biodegradation), location (intertidal vs supratidal), and among fuel treatments and type of hydrocarbons (aliphatics vs PAHs) using a four-way ANOVA with a Tukey's honest significant differences as a post-hoc test. Shannon and Faith's phylogenetic diversities were calculated for all samples. Faith's phylogenetic diversity (Faith's PD) was estimated using picante v1.8.2 (Kembel et al., 2010) and differences among treatments and between locations (intertidal vs. supratidal zones) were calculated with an ANOVA followed by Tukey's honest significant differences *post-hoc* test. Bray-Curtis dissimilarities and weighted UniFrac distances were calculated to test for differences in community composition among treatments and locations with a PERMANOVA (Anderson, 2001) after using PERMDISP (Anderson and Walsh, 2013) to test for homogeneity in multivariate dispersions with vegan v2.6-4 (Oksanen et al., 2022). We then tested for pairwise differences using a pairwise PERMANOVA (Martinez Arbizu, 2020). We determined differentially abundant ASVs between treatments and locations with DESeq2 (Love et al., 2014). We evaluated the influence of aliphatic and polycyclic aromatic hydrocarbons on the microbial community with a distance-based redundancy analysis (dbRDA) in vegan.

3.3.5.2. Metagenomic analyses

Low quality bases and sequencing adapters were trimmed with Trimmomatic v0.39.1 (Bolger et al., 2014). Metagenomic reads were individually assembled for each sample using metaSPAdes v3.14.1 (Nurk et al., 2017). Reads were mapped to the assembled metagenomes with BBMap v38.91 (Bushnell, n.d.). We annotated and classified the metagenomes using MetaErg v1.2.0 (Dong and Strous, 2019). We also classified the assembled metagenomic reads with Kaiju v1.10.1 (Menzel et al., 2016).

We performed genome binning with MetaBAT2 v2.15-6 (Kang et al., 2019), MaxBin2 v2.2.7 (Wu et al., 2016), and SemiBin2 v1.5.1 (Pan et al., 2023) after which the resulting bins from the three evaluated algorithms were dereplicated and aggregated using DAS Tool v1.1.6 (Sieber et al., 2018). Completeness and contamination of the dereplicated bins were estimated with CheckM2 v0.1.3 (Chklovski et al., 2022) and overall bin statistics were determined with CheckM v1.2.2 (Parks et al., 2017). Medium- (>50% completeness and <10% contamination) and high-quality (>90% completeness and <5% contamination) metagenome-assembled genomes (MAGs) were classified with GTDB-Tk v2.1.0 (Chaumeil et al., 2022) using the Genome Taxonomy Database (GTDB) r214 (Parks et al., 2022) and individually annotated with MetaErg.

Given that the majority of the bins we obtained could only be assigned to the family *Moraxellaceae*, we inferred a maximum likelihood (ML) phylogenomic tree of our *Moraxellaceae* MAGs along genome sequences of isolates from the *Moraxellaceae* and other families in the order *Pseudomonadales* (Table S3.1) to determine the phylogenetic position of these MAGs within this clade. For this, we first extracted and concatenated the amino acid sequences of single-copy core genes from the selected genomes based on the Bacteria_71 collection in anvi'o v7.1 (Eren et al., 2020) and then inferred the ML with IQ-TREE (Nguyen et al., 2015) using the WAG protein substitution model. UFBoot (Minh et al., 2013) was used

to perform bootstrapping and the SH-aLRT test with 1000 replicates to provide support for the resulting ML tree. We also calculated average nucleotide identity values (ANI) with the pyANI module (Pritchard et al., 2016) in anvi'o to further help to infer the phylogenetic relationship of these MAGs.

We used CANT-HYD with an e-value of 10^{-50} (Khot et al., 2022) to further annotate the metagenomes and MAGs for genes associated with hydrocarbon degradation. We used an e-value instead of the recommended noise or trusted cutoff values because we noted that these cutoffs were being too stringent and overlooked many sequences that were being classified as hydrocarbon degradation genes. To account for any false positives, we compared the Swiss-Prot annotation obtained with MetaErg to the CANT-HYD results for any genes that had a score lower than the CANT-HYD noise cutoff score and we only kept those hits for which the annotation from both tools coincided.

3.3.5.3. Metatranscriptomic analyses

Metatranscriptomic reads were also trimmed with Trimmomatic. Trimmed reads were then filtered using SortMeRNA v4.3.6 with database v4.3.4 (Kopylova et al., 2012) to remove rRNA reads, with BBMap using the RemoveHuman masked reference genome to remove human reads, and with DeconSeq v0.4.3 (Schmieder and Edwards, 2011) to remove reads detected in the samples from the negative control. The remaining reads were aligned against the assembled metagenomes using Bowtie 2 v2.5.1 (Langmead and Salzberg, 2012) and the aligned reads were counted using HTSeq v 2.0.2 (Putri et al., 2022).

3.4. Results

3.4.1. Hydrocarbon natural attenuation and biodegradation potential

Hydrocarbon analysis of the residual fuel after one month of exposure to the Arctic shoreline showed that Marine diesel (75.7 ± 6.49%) and Bunker C (77.3 ± 4.30%) had similar

percentages of fuel removed by natural attenuation in both the intertidal and supratidal zones while the ULSFO (47.6 ± 4.61%) had a statistically lower percentage of fuel removed in both zones (Fig. 3.2, Table S3.2). To account for the proportion of the fuel that was removed by biodegradation, we normalized the measured hydrocarbon mass against the conserved internal marker 17α(H), 21β(H)-hopane (Prince et al., 1994). Given the low concentrations of hopane in diesel fuels, we normalized the Marine diesel values using the nC17/pristane ratio (Prince et al., 1994). Marine diesel had the highest proportion of biodegradation (72.8 ± 7.70%), followed by ULSFO (37.6 ± 4.21%), and Bunker C was the fuel with the lowest estimated biodegradation (14.6 ± 4.87%). There were statistical differences between the natural attenuation and biodegradation removal percentages for Bunker C and ULSFO, but not for Marine diesel. Marine diesel was the only fuel for which we detected a higher removal (9.8%) in the supratidal zone compared to the intertidal zone. We did not observe statistical differences between the locations in the tidal zones for the other fuels. The removal of aliphatic hydrocarbons in Marine diesel and ULSFO was higher compared to the polycyclic aromatic hydrocarbon (PAH) removal (24.2 and 11.8%, respectively), but there were no differences in the removal of the two groups of hydrocarbons for Bunker C.

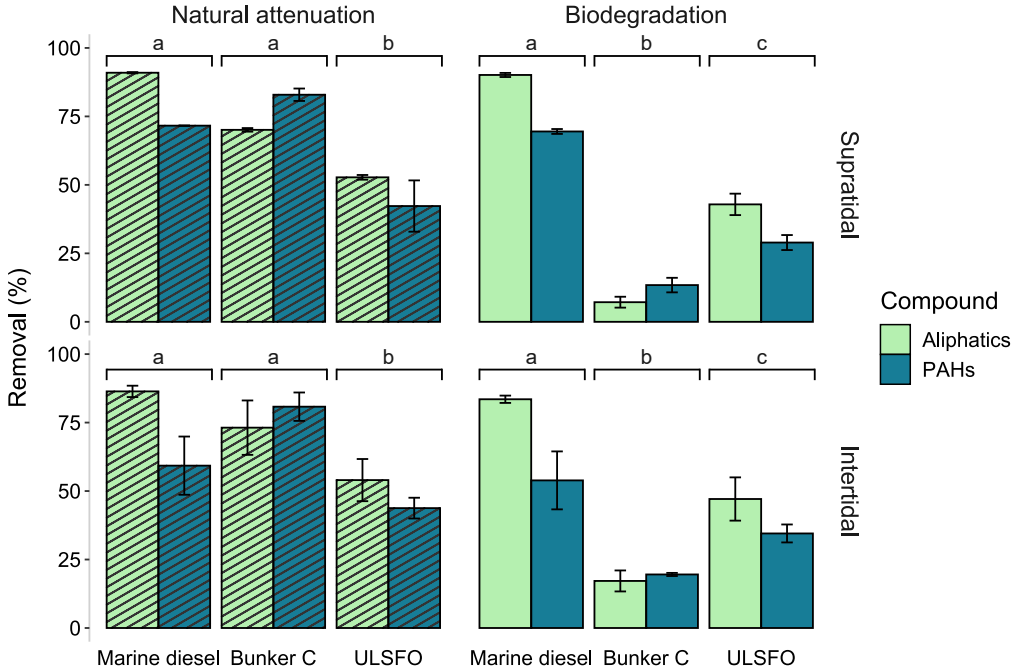


Fig. 3.2 Removal percentages of the studied fuels by natural attenuation and biodegradation and by compound group. Letters represent statistical differences among the fuels for each of the panels. No statistical differences between aliphatics and PAHs were observed. Error bars represent standard deviation (n = 2 for each combination of fuel, location, and removal type).

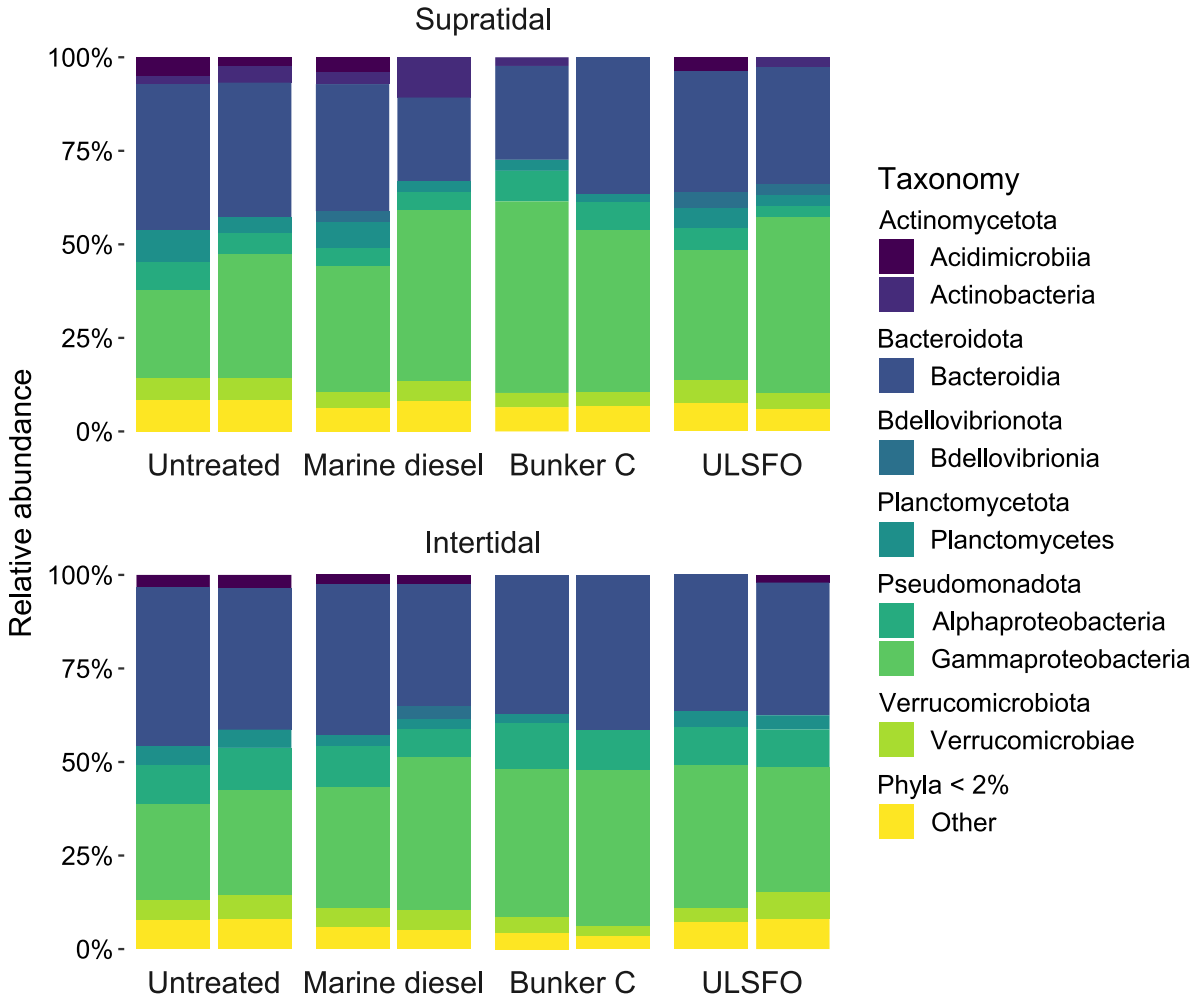


Fig. 3.3 Taxonomy of the microbial communities of the *in situ* mesocosms after 33 days based on 16S rRNA gene sequencing. Taxa with a relative abundance lower than 2% were pooled into "Other".

3.4.2. The beach sediment microbiome responds to fuel presence

We first determined the composition of the microbial community of the Assistance Bay beach sediments in the mesocosms using 16S rRNA gene amplicon sequencing. The Assistance Bay microbiome was dominated by the phyla *Bacteroidota* and *Pseudomonadota*, based on the taxonomic composition of the 1777 obtained ASVs (Fig. 3.3 and S1; Table S3.3). With the exception of the untreated control having a higher Shannon diversity index compared to the Bunker C samples, there were no differences in the alpha diversity indices between the treatments (Fig. S4.2; Table S3.2b). We did observe differences among the treatments (PERMANOVA, pseudo-F = 4.40, R^2 = 0.38, P = 0.001) and between locations (PERMANOVA, pseudo-F = 10.48, R^2 = 0.30, P = 0.001) based on weighted UniFrac distances (Fig. 3.4a) and Bray-Curtis dissimilarities (treatment PERMANOVA, pseudo-F = 2.46, R^2 = 0.27, P = 0.008; location PERMANOVA, pseudo-F = 8.68, R^2 = 0.32, P = 0.001; Fig. 3.4b). Pairwise PERMANOVA comparisons detected differences between the untreated controls and the fuel treatments as well between the community composition of the Bunker C and ULSFO samples (Table S3.4).

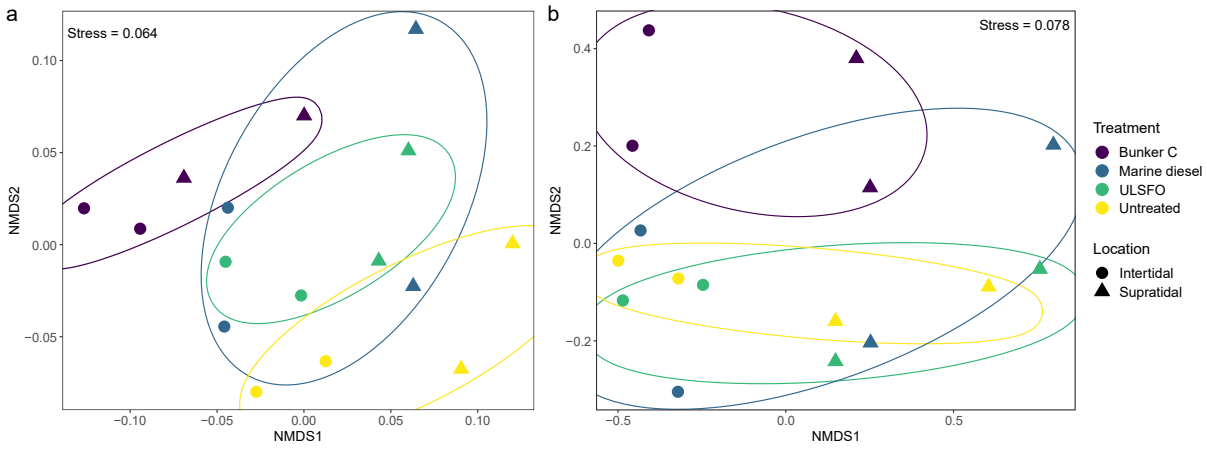


Fig. 3.4 Non-metric multidimensional scaling (NMDS) ordination of (**a**) weighted UniFrac distances and (**b**) Bray-Curtis dissimilarities for the 16S rRNA gene community composition showing how communities adapted in response to the presence of the added fuels.

ASVs belonging to the phylum *Pseudomonadota*, and especially those belonging to the class *Gammaproteobacteria*, were differentially more abundant in the sediments exposed to fuel compared to the untreated control (Fig. 3.5; Table S3.5). These observed differences were mainly caused by a higher abundance of ASVs classified in the genus *Alkanindiges* (Fig. S4.3a), which also included some of the most abundant ASVs observed in the experiment (Fig. S4.1). The DESeq2 results agreed with the PERMANOVA results between locations (Fig. S4.3; Table S3.6). We observed the largest number of differentially abundant ASVs between the intertidal and supratidal zones for the untreated control (51 ASVs) and the samples with Marine diesel (53 ASVs). ULSFO samples had 17 differentially abundant ASVs between zones and the Bunker C samples only had one.

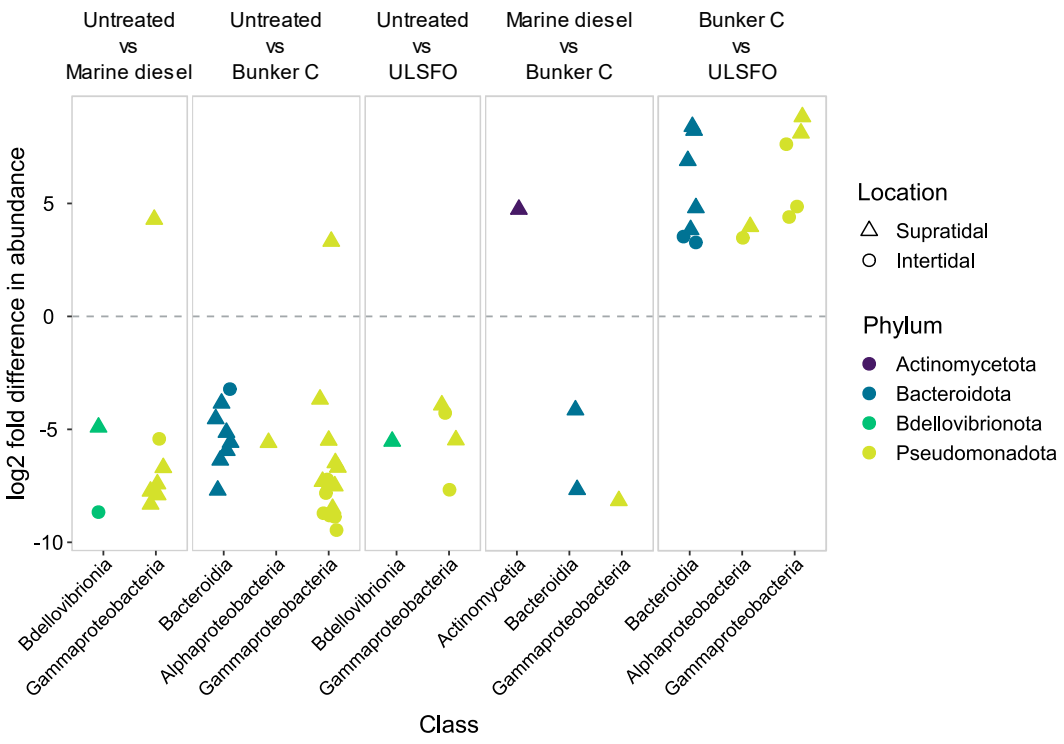


Fig. 3.5 DESeq2 results showing the differentially abundant ASVs detected among treatments by class. Positive values represent ASVs that were more abundant in the first treatment in the contrast and negative values represent ASVs that were more abundant in the second treatment of the contrast.

The dbRDA showed that the community composition of the ULSFO mesocosms was correlated to the amount of aliphatics in the sample and the composition of the Bunker C mesocosms was more correlated by the amount of PAHs, while the untreated control and Marine diesel samples were negatively correlated with the measured hydrocarbons (Fig. 3.6 and S6). The dbRDA also showed that the amount of aliphatics and PAHs explained 21.5% (dbRDA permutation test, F = 2.78, p = 0.001) and 28.7% (dbRDA permutation test, F = 4.19, p = 0.002) of the variability of the microbial community composition of the mesocosms based on Bray-Curtis dissimilarities and weighted UniFrac distances, respectively.

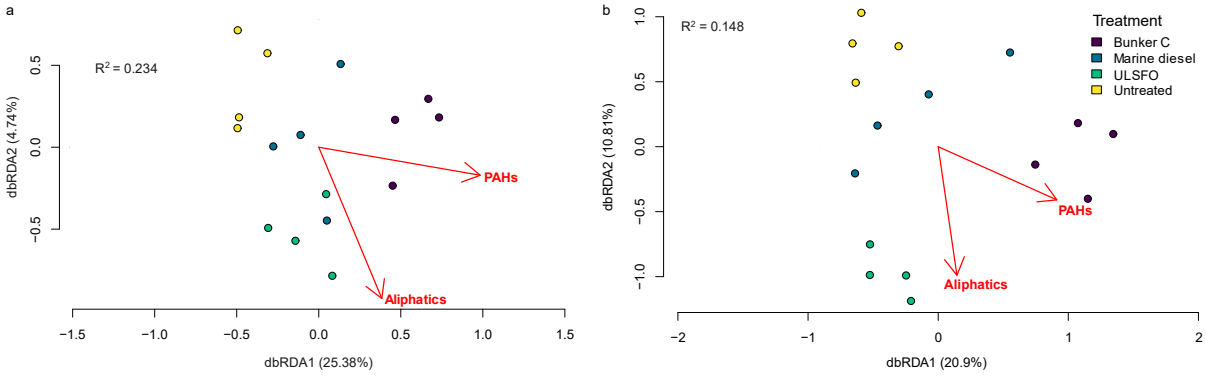


Fig. 3.6 Distance-based redundancy analysis (db-RDA) illustrating the influence of the type of hydrocarbon on the 16S rRNA gene (**a**) weighted UniFrac and (**b**) Bray-Curtis dissimilarities community composition.

3.4.3. Hydrocarbon degradation pathways are present in the Arctic shoreline microbiome

We detected a larger number of different genes associated with the aerobic degradation of alkanes in the metagenomes compared to other hydrocarbon metabolic pathways (Fig. 3.7). There were 5 alkane degradation genes present, among which *alkB* and CYP153 were the most expressed in the metatranscriptomes. We observed 4 genes related to monocyclic aromatic hydrocarbons (MAH) aerobic degradation (MAH alpha, *tomA1*, *tomA3*, and *tomA4*) and 3

related to aerobic PAH degradation (*ndoB*, *ndoC*, and non-*ndoB* PAH dioxygenase). Anaerobic degradation genes were not detected in the metagenomes or expressed in the metatranscriptomes. While we observed the expression of hydrocarbon degradation genes in the untreated controls, the expression levels were lower compared to the fuel treatments.

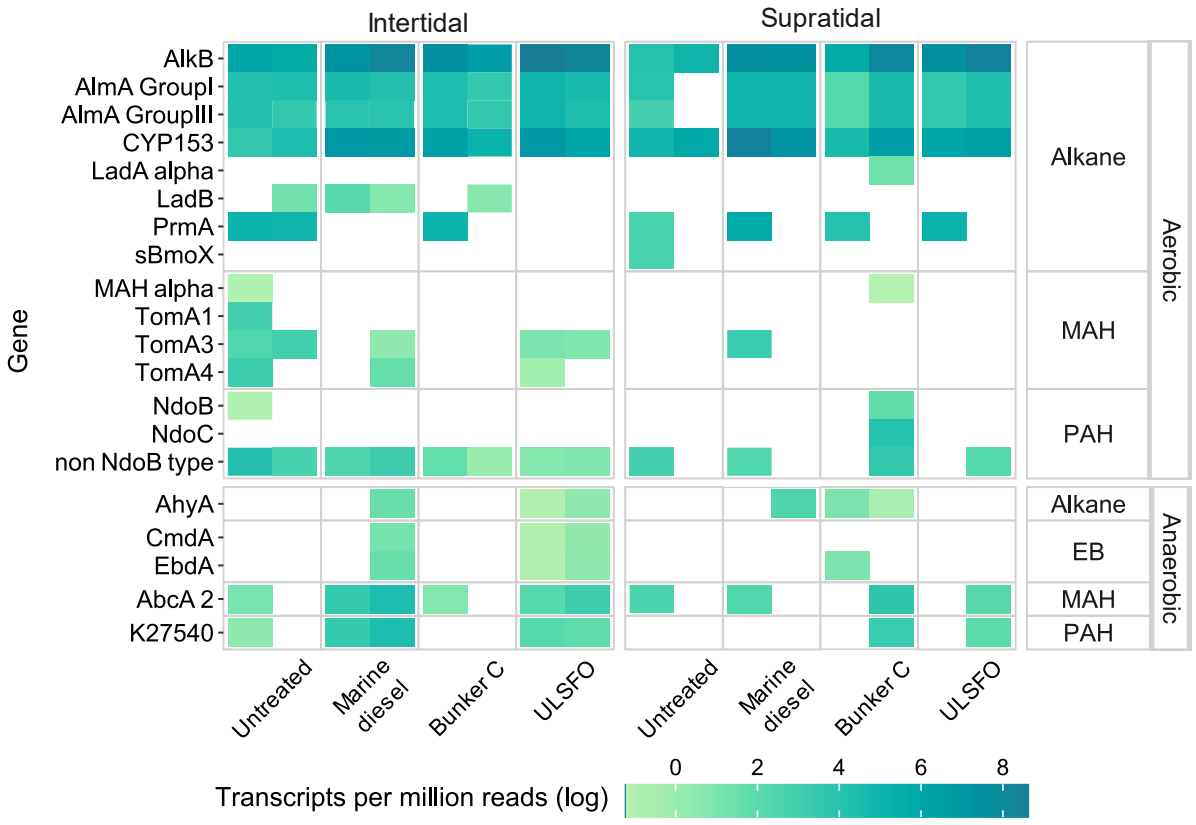


Fig. 3.7 Expression of CANT-HYD marker hydrocarbon degradation genes present in metagenomes. Metatranscriptomic counts were normalized to log transcripts per million reads. MAH: monocyclic aromatic hydrocarbon; PAH: polycyclic aromatic hydrocarbon.

We recovered the largest number of MAGs that contained hydrocarbon degradation genes in the Marine diesel metagenomes (14 MAGs), followed by the ULSFO (9 MAGs), Bunker C (6 MAGs), and untreated control (5 MAGs) samples. The medium- and high-quality MAGs mostly expressed aerobic alkane degradation genes (Fig. 3.8). The majority of the MAGs in which we detected the expression of these genes belonged to the family

Moraxellaceae with many being classified into the putatively novel genus JAGLBJ01 (Table S3.7). Multiple genes belonging to aerobic aromatic hydrocarbon pathways (*tomA1*, *tomA3*, *tomA4*, and non-*ndoB*) were expressed in MAGs from the families *Pseudomonadaceae* and *Commamonadaceae* (Fig. 3.8).

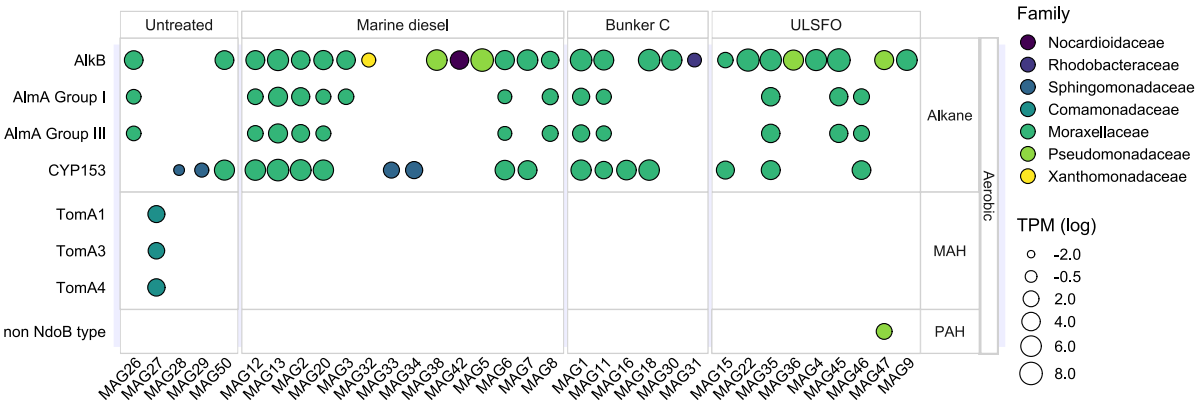


Fig. 3.8 Expression of CANT-HYD marker hydrocarbon degradation genes in the recovered MAGs. Metatranscriptomic counts were normalized to log transcripts per million reads. MAH: monocyclic aromatic hydrocarbon; PAH: polycyclic aromatic hydrocarbon.

3.5. Discussion

To our knowledge, this is the first study to use state-of-the-art microbial ecology techniques to understand the impact of fuels used by the shipping industry on the microbiomes of Arctic beaches and the role that the microbes play in the removal of these hydrocarbons from beach sediments. It is also the first study that evaluated the natural attenuation potential of ULSFO under the *in situ* conditions that the fuel would experience if there was a spill that washes onto an Arctic shoreline.

After one month of *in situ* incubation, we observed varying levels of fuel depletion among the studied fuels. Both Marine diesel and Bunker C showed similar levels of hydrocarbon loss by natural attenuation with around 75.7% and 77.3% of the fuel being

removed, respectively, while ULSFO appeared to be more persistent in the mesocosms with 47.6% of the fuel removed by natural processes. Lighter fuels have high biodegradation rates compared to heavier fuel and crude oils, mostly due to the higher biodegradability of alkanes (Murphy et al., 2021; Vergeynst et al., 2019b). The Marine diesel used in this study was comprised mostly of aliphatic hydrocarbons (Fig. S4.4) and was mainly depleted by biodegradation (72.8 ± 7.70%). The Marine diesel biodegradation capabilities of the Assistance Bay microbiota under natural environmental conditions appeared to be much higher than what was reported by previous studies in other cold environments. Pure cultures of bacterial strains isolated from Antarctic seawater achieved a maximum biodegradation of 57.66% of the added diesel oil at 4 °C after 60 days (Michaud et al., 2004). Microcosms evaluating biodegradation of diesel using seawater from the Labrador Sea at 4 °C saw 43% and 46% losses for alkanes and PAHs, respectively, after 71 days (Murphy et al., 2021). *In situ* experiments in a Greenland fjord using the same type of Fluortex netting used for this study showed 18 – 29% removal of nC_{13-26}-alkanes in the marine gas oil samples after 24 days (Vergeynst et al., 2019b).

Even though hydrocarbon removal for Bunker C was higher compared to ULSFO, if we consider the overall hydrocarbon mass loss (Fig. S4.4), the total masses for both fuels reached similar levels at the end of the experiment (Table S3.2c). Additionally, 62.8 ± 5.40% of the Bunker C was removed from the mesocosms by the non-biological processes, but only 10.0 ± 3.94% of ULSFO was removed by these processes (Table S3.2). We also observed that PAHs with fewer aromatic rings and alkyl substitutions were more depleted compared to higher complexity PAHs (Table S3.8). This is a depletion pattern that has been associated with removal by dissolution due to the higher water solubility of the less complex aromatic compounds (Vergeynst et al., 2019b). This means that a larger proportion of ULSFO is being removed by biodegradation from the Assistance Bay coastal environment, while most of the Bunker C is only being relocated from the shoreline into the water column, where it is still of environmental

concern. Our results thus suggest that ULSFO is not only a more environmentally friendly alternative to Bunker C due to its lower sulfur oxide emissions, but it also could be more easily cleaned up from the coastal zone due to its higher biodegradative potential. It must be noted, however, that this does not take into consideration other non-biological degradation mechanisms, such as photo-oxidation (Vergeynst et al., 2019b, 2019a). While photo-oxidation may not have influenced the hydrocarbon removal rates in our experiments given the fact that the mesocosms were buried and thus negligible amounts of light should have reached them, photo-oxidation should be tested in future studies to provide a more complete picture of the fate of these fuels in Arctic shorelines.

Our results showed that microorganisms in the studied beach sediments have a higher ULSFO biodegradative potential compared to previous studies. A microcosm study using sediment from a beach in Resolute and ULSFO found a non-significant decrease in alkane and PAH concentrations after 55 days of incubation (Ellis et al., 2022). Similarly, a study evaluating the ULSFO biodegradation capabilities of strains from Resolute beach sediment detected a non-significant reduction of the aliphatic and PAH fractions after incubation for 3 months at 5 °C (Lirette et al., 2024). The *in situ* mesocosm experiment in Greenland seawater observed a 3 – 11% removal of $nC_{13–26}$-alkanes for troll blend crude oil (a low sulfur crude oil) after 24 days and substantial biodegradation was only detected after 112 days (Vergeynst et al., 2019b).

We also obtained clear evidence of how the microbial communities changed in response to the addition of fuel into their environment. The first and third most abundant ASVs detected in our samples were classified to the genus *Alkanindiges* (Table S3.3). We also detected multiple *Alkanindiges* ASVs that were differentially more abundant in the fuel treatments compared to the untreated controls (Table S3.5). Bacteria from this genus have been found as key members of the microbial communities from hydrocarbon biodegradation experiments in Arctic seawater and sub-Arctic soils (Chang et al., 2011; Vergeynst et al., 2019b). *Alkanindiges*

are associated with the degradation of complex aliphatic compounds such as long-chain linear and branched alkanes (Bogan et al., 2003) and appear in the biodegradative microbial succession once short-chain alkanes have been depleted (Vergeynst et al., 2019b). The composition of the residual fuel further supports this as a larger hopane-normalized proportion of long-chain (> C_{20}) alkanes remain compared to shorter chain (< C_{20}) alkanes (Table S3.9). This suggests that the biodegradation occurring on our mesocosms is at an advanced stage.

While we did not find any MAGs identified as *Alkanindiges* in our metagenomes, we did recover various MAGs belonging to the family *Moraxellaceae*, to which the genus *Alkanindiges* also belongs. To investigate whether the *Moraxellaceae* MAGs belong to a previously undescribed clade within *Alkanindiges*, we created a phylogenomic tree of these MAGs along publicly available genomes from the other genera within this family and the order *Pseudomonadales*. Their placement in a phylogenomic tree of *Moraxellaceae* shows that the closest relatives of the majority of our MAGs are *Aquirhabdus parva* and JAGLBJ01 (Fig. S4.5). *A. parva* was isolated from a pristine freshwater lake (Kim et al., 2020) and the JAGLBJ01 MAG was recovered from the metagenome of ringed seal lice (Doña et al., 2021). While there is no evidence that *A. parva* and JAGLBJ01 are capable of hydrocarbon degradation, CANT-HYD revealed the presence of various medium- and long-chain alkane degradation genes in these MAGs (*alkB*, *almA* groups I and III, the alpha and beta subunits of *ladA*, and *ladB* in *A. parva* and *alkB* and *almA* groups I and III in JAGLBJ01). This is also consistent with the present study given that our *Moraxellaceae* MAGs are expressing at least one aerobic alkane degradation gene among which *alkB* and CYP153 were the most prevalent followed by *almA* groups I and III (Fig. 3.8). Only the genome of *A. parvus* contained a gene associated with aromatic hydrocarbon degradation (*dszC*), while none of our *Moraxellaceae* MAGs possessed genes associated with aromatic hydrocarbon degradation pathways.

The majority of our *Moraxellaceae* MAGs form a single clade with two branches that represent two potential new species (Fig. S4.5). Additionally, these MAGs share an ANI of 72.7 ± 0.3% and 72.4 ± 0.3% with the genomes of *A. parva* and JAGLBJ01, respectively (Table S3.10). There is currently only one known species in the genus *Aquirhabdus* which prevents a robust comparison of these ANI values to determine if our MAGs belong to this genus (Barco et al., 2020). However, given that we observed similar ANI values among other genera inside *Moraxellaceae* (Table S3.10), this suggests that this clade could represent a novel genus.

In addition to this potentially new clade, we also found two *Moraxellaceae* MAGs (MAG6 and MAG18) which could represent a new species from the genus *Paraperlucidibaca* based on the higher 78.6 ± 0.3% ANI. This genus was also one of the top 20 most abundant ASVs that we detected (Table S3.3) and we found alkane degradation genes being expressed in the transcriptomes of these MAGs (Fig. 3.8). This is consistent with the analysis of MAGs recovered from a hydrocarbon biodegradation experiment using seawater from the Labrador Sea which showed the alkane degrading capabilities of members of this genus (Murphy et al., 2021).

Bacteroidota was the second most abundant phylum based on the 16S rRNA gene dataset (Fig. 3.3) and comprised 5.4 ± 2.5% of the assembled contigs based on the Kaiju annotation. We also detected multiple differentially abundant *Bacteroidota* ASVs in the Bunker C samples compared to the untreated control and the ULSFO samples (Fig. 3.5; Table S3.5). However, we were not able to recover any medium- or high-quality *Bacteroidota* MAGs (Table S3.7) or hydrocarbon degradation genes associated with this phylum based on the MetaErg annotation. This pattern is consistent with a previous microcosm study using beach sediment from a beach in Resolute with ULSFO and observed a high abundance of *Bacteroidota* ASVs in both the control and fuel-treated samples but did not detect the presence of any hydrocarbon degradation genes that were associated with this phylum (Ellis et al., 2022). The abundance of

Bacteroidota sharply decreases after a spill event (Kwon et al., 2019) but, in the case of the Deepwater Horizon spill, their abundance recovered to baseline levels after three months (Redmond and Valentine, 2012). This appears to be attributed to a higher sensitivity to hydrocarbons from these bacteria in addition to them being potential secondary consumers in the hydrocarbon degradation microbial succession (Kwon et al., 2019; Redmond and Valentine, 2012).

We observed less biodegradation of PAHs in contrast to aliphatic biodegradation for Marine diesel and ULSFO (Fig. 3.2; Table S3.2). This could be explained by the overall lower expression of aromatic hydrocarbon degradation genes in our metatranscriptomes compared to their alkane counterparts (Fig. 3.7 and 3.8). The dbRDA showed that the microbial communities treated with ULSFO and, to a certain extent those treated with Marine diesel, appear to be more correlated by the aliphatics present in these samples than by the PAHs (Fig. 3.6). PAH degradation genes were less prevalent than alkane degradation genes in Arctic beach and benthic sediments as well as in sub-Arctic seawater (Ellis et al., 2022; Ji et al., 2023; Murphy et al., 2021). These results suggest that microbial communities from a wide variety of Arctic marine environments are not properly equipped with the genetic repertoire required to degrade PAHs. Likewise, the microbial communities in the Bunker C samples were more correlated by the amount of PAHs (Fig. 3.6) and were the only treatment with a significantly lower Shannon diversity index compared to the untreated controls (Fig. S4.2; Table S3.2b). Taken together, these results indicate that the higher PAH content of Bunker C negatively impacts the shoreline microbiome, while appearing to be tolerant to these compounds to a certain extent, but is not capable of degrading them.

The results of the present study showed that the shoreline microbiota of a high Arctic beach is capable of biodegrading fuels that are used in ships that will navigate these waters. We also provided the first evidence of the biodegradability of ULSFO under *in situ* conditions.

However, we still did not observe complete removal of the fuels after 33 days of incubation, suggesting that natural attenuation, and more specifically biodegradation, on its own will not be sufficient as the primary clean up solution if a spill washes onto an Arctic shoreline. One of the possible explanations for this is that, even though we have shown alkane degradation genes are present and expressed, the metabolic rate of these microbes is limited due to the environmental conditions. The average daily air temperature in Resolute for 2019 was -13.5 °C and freezing temperatures were recorded for 73.6 % of the year (Environment and Climate Change Canada, n.d.). The mean daily air temperature during the duration of the experiment was 5.7 ± 2.1 °C (Fig. S4.6). While previous studies have observed that hydrocarbon biodegradation by microorganisms from marine environments can still occur at low temperatures (Bacosa et al., 2018; Gofstein and Leigh, 2023; Murphy et al., 2021; Schreiber et al., 2021, 2019; Vergeynst et al., 2019b), these results often occur only after months of incubation or deployment at near, but only rarely below, (Garneau et al., 2016; McFarlin et al., 2014) 0 °C. Our experiment was deployed during the warmest months of the Arctic summer and temperatures started to drop below 0 °C shortly after we recovered the mesocosms (Fig. S4.6) which would further hamper microbial activity. Additionally, we have observed low nutrient concentrations in the pore water collected from the Assistance Bay beach sediment (Góngora et al., 2024). While these values are similar to those obtained for various sampling sites across the Arctic Ocean surveyed by the *Tara* Oceans project (Salazar et al., 2019), it is not clear if the recurrent incoming tide is able to provide sufficient nutrients to replenish those utilized due to the increased metabolic activity of hydrocarbon degraders in this oligotrophic environment. Nutrient limitations along with the short and cold Arctic summer followed by an approximately 9 month-long winter with freezing temperatures, presents a considerable problem for the use of natural attenuation as the main cleanup strategy in case of a fuel spill in the NWP. Future studies should evaluate the impacts of extended deployment times of *in situ*

mesocosms and microcosms under Arctic conditions as well as testing the addition of N and P fertilizers to stimulate hydrocarbon degradation rates. This will help to determine whether bioremediation, more specifically biostimulation, is a feasible treatment option in this type of environment.

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Connecting text

The one-month *in situ* mesocosm provided the first evidence of the biodegradability of ULSFO under Arctic environmental conditions. However, while it was more biodegradable than the legacy Bunker C, the majority of the fuel was still present in the mesocosms by the end of the experiment. I hypothesized that the cold temperatures and oligotrophic conditions of the beach sediment in Assistance Bay could have slowed down the metabolism of these microbes so I tested the use of N and P fertilizers and a prolonged incubation time (one year) as strategies to stimulate the biodegradation activity of the native hydrocarbon-degrading microbes. This is the first study of the long-term biodegradability of the recently introduced ULSFO.

This manuscript will be submitted to Marine Pollution Bulletin.

Supplementary material can be found in the following appendices:

Appendix 4.1. Supplementary figures (Figures S4.1-S4.6) and legends for supplementary tables in Appendix 4.2 (Tables S4.1-S3.8).

Appendix 4.2. Supplementary tables (Tables S3.1-S3.8).

Chapter 4. Long-term patterns of ultra-low sulfur fuel oil bioremediation in Arctic shorelines using *in situ* mesocosms

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4.1. Abstract

New maritime regulations restricting high-sulfur fuels have led to the transition to new low sulfur fuel oils (LFSOs). We do not know how LSFOs will behave in marine environments and how they will respond to available remediation strategies, presenting an environmental risk. The risk will be even higher in the remote high Arctic, especially along the Northwest Passage (NWP), for which an increase in shipping traffic is expected by the end of the century. In this study, we evaluated the long-term (one year) biodegradation potential of the native microbial community of NWP beach sediment using *in situ* mesocosm experiments with two different types of LSFOs: a marine gas oil (Marine diesel) and an ultra-low sulfur fuel oil (ULSFO). We observed that the lighter Marine diesel was biodegraded better (72.0%) than the heavier ULSFO (32.5%). We described composition of the microbial community of the mesocosms using 16S rRNA gene amplicon sequencing and observed a decrease in microbial diversity for the fuel-treated samples compared to the untreated controls. Despite the decrease in overall diversity, we observed significantly higher abundances of known hydrocarbon degrading microbes (e.g., *Oleispira*, *Altererythrobacter*, *Gilvibacter*, *Pseudohongiella*) in the fuel mesocosms. Our study showed the potential to implement biodegradation as a remediation strategy under the cold and oligotrophic environmental conditions present throughout the NWP. However, we also observed that microbes on their own cannot degrade the entirety of the removed fuel and other types of remediation will need to be considered to complement the natural biodegradation observed here.

4.2. Introduction

In 2020, new regulations from the International Maritime Organization (IMO) came into force which set a maximum global cap for sulfur content in ship fuels of 0.5% for open seas and 0.1% within sulfur emission control areas (SECAs) located close to highly populated zones (Vedachalam et al., 2022). These changes have caused the shipping industry to phase out from the use of heavy fuel oils (HFOs; < 3.5% sulfur content) into global cap- and SECA-compliant fuels which include marine diesels and a new generation of low sulfur fuel oils (LSFOs) which can be further categorized as very low sulfur fuel oils (VLSFOs; < 0.5% sulfur content) and ultra low sulfur fuel oils (ULSFOs; < 0.1 % sulfur content). LSFOs comprise a wide variety of products with different hydrocarbon compositions (Daling and Sørheim, 2020; Faksness et al., 2024; Nelson et al., 2022; Yang et al., 2023). These differences are caused by the processes used by refineries to produce LSFOs which include catalytic and thermal cracking, hydrodesulfurization, or blending of heavy fuel oils with low sulfur hydrocarbons (Faksness et al., 2024; Faragher et al., 2024; Kass et al., 2022; Vedachalam et al., 2022; Zou and Yang, 2023).

Climate change is causing a rise in temperatures globally, but especially in the Arctic which leads to a reduction in sea ice coverage and the possibility of summers with ice-free Arctic waters by the middle of the century (Shen et al., 2023). This presents an enticing opportunity for the shipping industry as ice-free conditions will lead to the opening of the Northwest Passage (NWP), a shorter navigation route through the Canadian Arctic Archipelago (CAA) which connects the northern Atlantic and Pacific Oceans (Howell et al., 2023). The ambiguity on the applicability of Canadian and international legislation to regulate shipping through the NWP (Bartenstein, 2019) along with an expected increase of drifting ice coming from the highest regions of the CAA into the NWP channels (Howell et al., 2023; Mudryk et al., 2021) and the rise of storms and waves in the region (Liu et al., 2016) will pose unsafe

conditions for vessels going through the NWP. Additionally, the IMO approved a proposal to create a new SECA in the Canadian Arctic in April 2024 (International Maritime Organization, 2024). This is of importance as ships going through the NWP could be required to use ULSFO which leads to a possibility of these new type of poorly understood fuels to be released into the environment in the case of a spill.

Cleanup strategies such as skimmers, *in situ* burning, dispersants, or sorbent booms may not be effective for LSFOs (Daling and Sørheim, 2020; IMAROS, 2022). Additionally, it has been observed that spilt VLSFO can be neutrally buoyant and moves right under the surface which could reduce its detection capacity (Pålsson et al., 2024) and lead to a significant portion of the fuel reaching the shoreline. The geographical isolation of the highly uninhabited NWP will also slow and limit spill response in Arctic marine environments (Emmerson and Lahn, 2012). Because of these reasons, simpler remediation solutions, such as bioremediation, should be considered. Previous studies have documented the presence and hydrocarbon biodegradative capacity of Arctic microorganisms from marine environments such as seawater (Brakstad et al., 2015; Cao et al., 2022; Gofstein and Leigh, 2023; Gomes et al., 2022; Kampouris et al., 2023; McFarlin et al., 2014; Pyke et al., 2023; Vergeynst et al., 2019b), sea ice (Garneau et al., 2016; Lofthus et al., 2021; Vergeynst et al., 2019a), and beach sediments (Bragg et al., 1994; Y.-J. Chen et al., 2024; Durand et al., 2023; Ellis et al., 2022; Freyria et al., 2024; Garrett et al., 2003; Góngora et al., 2024b; Lirette et al., 2024; Prince et al., 2003). These studies show the feasibility of bioremediation as a primary cleanup strategy.

Nevertheless, the inhospitable, oligotrophic, and cold environment of the high Arctic hinders microbial activity (Durand et al., 2023; Gomes et al., 2022; Hunnie et al., 2023; Schreiber et al., 2023). A previous study from our research group (Góngora et al., 2024a) performed an *in situ* mesocosm study to test the biodegradation capabilities of the microbial community of NWP beach sediment treated with marine diesel, ULSFO, or HFO. After 1

month, higher biodegradation activity was observed in the low sulfur fuels, but particularly for ULSFO, only 47.6% of the fuel was removed from the mesocosms. Here we present a follow-up study to Góngora et al. (2024a) with the objective to test whether prolonged incubation times and the addition of nutrient amendments are able to improve fuel biodegradation. We hypothesized that extending the duration of the experiment to a year would help to account for the slow metabolic rates of microbes inhabiting near 0 °C and often below. We observed a very defined shift in the 16S rRNA gene community composition of the fuel treatments, which indicated that the microbiome was able to adapt to the presence of fuel. This was accompanied by an increase in the percentage of ULSFO removed after a year (62% of the fuel removed), out of which only 32.5% of the fuel was removed by biodegradation. Additionally, we hypothesized that the addition of nutrient amendments in the form of N and P fertilizers would stimulate the microbial community by replenishing the naturally low nutrient concentrations we previously observed in various NWP beaches (Góngora et al., 2024b). However, we did not observe a significant effect of the nutrient amendments on biodegradation or removal effectiveness. These results show that the Arctic environments across the NWP could be under imminent environmental threat if a spill were to occur and that it could take years for a shoreline to recover on its own.

4.3. Materials and methods

4.3.1. Sampling site description and experimental design

Assistance Bay (74.6509º N, 94.2983º W) is an uninhabited beach located approximately 17 km south-east from the hamlet of Resolute on Cornwallis Island, Nunavut, Canada. We chose this location for our experiment because Resolute is expected to be a key stopover hub in the NWP as the route becomes more widely used once sea ice coverage is reduced. Additionally,

Assistance Bay faces the NWP so it represents one of the many remote beaches that could be impacted by a fuel spill from a ship travelling through the NWP.

The mesocosms were prepared as described by (Góngora et al., 2024a) with minor modifications. Fluortex netting (product reference 09-250/39, Sefar) pieces (15 × 15 cm) were washed with HPLC grade dichloromethane (DCM; Applied Biosystems) and attached to 15 × 15 × 0.5 cm stainless steel plates using nylon fishing line. The mounted netting was then saturated with Marine diesel (Glencore Limited) or ultra-low sulfur fuel oil (ULSFO; Shell Trading Rotterdam B.V.). A biostimulation treatment was also added in the form of a combination of an inorganic fertilizer (monoammonium phosphate; MAP; Sigma-Aldrich) and an oleophilic fertilizer (S-200 OilGone; S200 hereafter; International Environmental Products). A combination of these two fertilizers was chosen based on a previous study from our research group which found that both fertilizers led to a higher hydrocarbon removal compared to either of them applied individually (Y.-J. Chen et al., 2024). MAP was added at a concentration of 0.25 g kg^{-1} (Bell et al., 2013) and assuming that approximately 1 kg of sediment surrounds the netting (0.25 g per mesocosm) and S200 was added at a 1:1 ratio to hydrocarbon (following the manufacturer's instruction) and assuming that approximately 3 g of fuel were added to the netting (3 ml per mesocosm). A control only using fertilizers but no added fuel was also included along with an environmental control with no fuel or fertilizer applied. Unlike the one-month experiment where the mesocosms were placed on a sheltered part of the beach, for this study we selected a section of the beach that is fully exposed to tidal action. The treatments and controls were deployed in duplicates on August 7, 2021 on two parts of the tidal zone: the supratidal zone which does not experience frequent tidal activity and the intertidal zone which is regularly submerged during the high tide. Each mesocosm was buried at a depth of 5 cm and with a distance of approximately 25 cm between each plate and replicate sets were separated

by approximately 1 m. A time 0 (T_0) control consisting of a mesocosm with added fuel was buried as described above, then immediately removed, and processed as described below.

Samples were recovered after a year (387 days) on August 28, 2022. Each mesocosm was individually packed in a sterile Whirl-Pak bag and taken to the Polar Continental Shelf Project laboratory in Resolute where they were immediately processed. Under aseptic conditions, the netting was separated from the metal plates and cut into four 7.5 × 7.5 cm pieces. Two of the fragments were individually rolled and placed inside 20 ml amber glass vials with closed caps with silicone liners (Thermo Scientific) and stored at -20 °C. The other two fragments were individually rolled and inserted into sterile 15 ml Falcon tubes. DNA/RNA Shield (Zymo Research) was added into the tubes until the netting was fully submerged and the tubes were stored at -80 °C. The tubes and vials were transported in coolers to McGill University (Montréal, Canada) where the amber vials were stored at -20 °C until they were processed for hydrocarbon analysis and the Falcon tubes were stored at -80 °C until processed for DNA/RNA extractions.

4.3.2. Hydrocarbon analyses

Samples were sent to the Bigelow Laboratory for Ocean Sciences for hydrocarbon quantification of the mesocosms. Samples were analyzed by GC/MS using a modified EPA method 8270D as described elsewhere (Aeppli et al., 2018). A time zero (T_0) control for all fuel and tidal zone combinations prepared the same way as the treatment mesocosms (as described above) was also sent for analysis (four T_0 controls in total). To account for the proportion of the fuel that was biodegraded, we normalized hydrocarbon masses in ULSFO to the conserved internal marker 17α(H), 21β(H)-hopane (Prince et al., 1994). Given the low concentrations of hopane in diesel fuels, we used the nC17/pristane ratio to normalize the Marine diesel masses (Prince et al., 1994).

4.3.3. Nucleic acid extraction

The netting preserved in DNA/RNA Shield was first thawed on ice prior to the nucleic acid extraction. The Falcon tubes were vortexed for 90 s to detach any bound cells and particles from the netting and resuspend them in the supernatant. The solutions were left to settle on ice until the foam produced by vortexing dissipated. DNA was then extracted using the ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research) following the manufacturer's instructions with minor modifications. The supernatant (250 µl) was added to the ZR BashingBead Lysis Tube and mixed with 750 µl DNA/RNA Shield and the lysis tubes were shaken for 5 min on a Mini-Beadbeater-16 (BioSpec Products). All centrifugation steps were carried out at 16,000 g unless stated otherwise by the original protocol and in the final elution step, DNA/RNA was eluted in 50 µl of ZymoBIOMICS DNase/RNase-Free Water. An extraction control consisting of 500 µl of ZymoBIOMICS DNase/RNase-Free Water and 500 µl of DNA/RNA Shield was processed along with the samples as described above. The resulting DNA extractions were stored at -20 °C until processed.

4.3.4. Library preparation and sequencing

The 16S rRNA gene was amplified using primers 515F-Y (5′-GTGYCAGCMGCCGCGGTAA) and 926R (5′-CCGYCAATTYMTTTRAGTTT) containing Illumina overhang adapter sequences (Parada et al., 2016). PCR reactions (25 µL) containing 0.5 U of KAPA HiFi DNA Polymerase (Roche), 0.6 µM of each primer, 0.3 mM of KAPA dNTP Mix (Roche), 1X KAPA HiFi Fidelity Buffer (Roche), and 1 µL of DNA were performed under the following conditions: initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 45 s, and a final extension step at 72°C for 10 min. Reactions were purified using Sera-Mag Select magnetic beads (Cytiva) with a 0.8 bead-to-PCR volume ratio. Indexing was performed using the Nextera XT Index Kit v2 (Illumina) following manufacturer's instructions. Indexed samples were purified with Sera-Mag Select

magnetic beads (1.12 bead-to-PCR volume ratio) and quantified using the Qubit fluorometer (Invitrogen). Samples were pooled in equimolar ratios of 4 nM and sequenced with a 2 × 300 bp v3 flow cell with an Illumina MiSeq platform. Adapters and indices were removed with the Illumina FASTQ file generation pipeline.

4.3.5. Bioinformatics and statistical analyses

All statistical analyses were performed in R 4.4.1(R Core Team, 2024).

4.3.5.1. Hydrocarbon analysis

We tested for differences in hydrocarbon removal between the fertilized and unfertilized samples using a paired t-test or a paired Wilcoxon signed-rank test when the assumption of normality was not met. We could not detect a statistical effect of the use of fertilizer on the amount of removed hydrocarbons (Fig. S4.1; Table S4.1). We also did not find statistical differences in the microbial community (see below). Accordingly, we combined the fertilized and unfertilized samples into their respective categories (e.g., Marine diesel intertidal, etc.) for the remainder of the analyses to increase the statistical power of the tests. We used a two-way ANOVA to test for differences between treatments and locations (intertidal vs. supratidal zones) followed by a Tukey's honest significant differences post-hoc test.

4.3.5.2. 16S rRNA gene amplicons

Amplicon sequence variants (ASVs) were obtained from the 16S rRNA gene reads using DADA2 v1.26.0 (Callahan et al., 2016a). ASVs were classified using the Silva 138.1 database (Quast et al., 2013). The negative control reads were used to remove potential contaminant ASVs with decontam v1.22.0 (Davis et al., 2018) using the frequency method. Mitochondrial, chloroplast, and ASVs unclassified at the phylum level were also removed. We averaged ASV abundances across 1000 rarefactions to the minimum library size of 5727 as described previously (Sugden et al., 2022). A phylogenetic tree was inferred using phangorn v2.11.1

(Schliep, 2011) using the method described elsewhere (Callahan et al., 2016b). The resulting ASV table was imported to phyloseq v1.46.0 (McMurdie and Holmes, 2013) for statistical analyses.

Shannon and Faith's phylogenetic diversities were calculated for all samples. Faith's phylogenetic diversity (Faith's PD) was estimated using picante v1.8.2 (Kembel et al., 2010). We tested for differences in these two diversity metrics between nutrient amendments (fertilized vs. unfertilized) with a paired t-test or a paired Wilcoxon signed-rank test when the assumption of normality was not met. Similar to the hydrocarbon analyses, we did not observe any statistical changes in the microbial communities between the nutrient amendment treatments (Fig. S4.2; Table S4.2) so samples were combined as described above. Differences between treatments and locations were calculated with an ANOVA followed by Tukey's honest significant differences post-hoc test. Bray-Curtis dissimilarities and weighted UniFrac distances were calculated to test for differences in community composition between treatments and locations with a PERMANOVA (Anderson, 2001) after using PERMDISP (Anderson and Walsh, 2013) to test for homogeneity in multivariate dispersions with vegan v2.6-4 (Oksanen et al., 2022). After obtaining significant results for the global PERMANOVA, we tested for pairwise differences among the categories of the significant variables using a pairwise PERMANOVA (Martinez Arbizu, 2020).We determined differentially abundant ASVs between treatments and locations with ANCOM-BC2 (Lin and Peddada, 2024). We evaluated the influence of aliphatic and polycyclic aromatic hydrocarbons on the microbial community with a distance-based redundancy analysis (dbRDA) in vegan.

4.4. Results

4.4.1. Hydrocarbon analysis of the residual fuel

After one year, we observed a significantly higher (ANOVA, $F = 163.26$, $p < 2 \times 10^{-16}$) removal ($79.8 \pm 4.1\%$) of the added Marine diesel compared to the $62.1 \pm 20.3\%$ of the ULSFO removed from the mesocosms (Fig. 4.1). There was a higher removal in the supratidal zone compared to the intertidal zone (ANOVA, $F = 93.29$, $p = 3.13 \times 10^{-12}$) for both Marine diesel and ULSFO. Tukey HSD test results showed differences among individual variable combinations presented below can be found in Table S4.3. After normalizing to account for the proportion of natural attenuation that can be attributed specifically to biodegradation, we determined that $72.0 \pm 15.8\%$ of the Marine diesel was removed by biological processes and there were no statistical differences between the proportion of fuel removed by natural attenuation and by biodegradation for this fuel. On the other hand, only $32.5 \pm 9.6\%$ of the ULSFO was biodegraded and this value was significantly lower than the ULSFO removal by natural attenuation. The removal of Marine diesel was consistently higher than for ULSFO by both natural attenuation and biodegradation for the two tidal zones. The one exception was natural attenuation in the supratidal zone were there were no statistical differences between the fuels. Aliphatic hydrocarbon removal was higher in Marine diesel compared to the PAH removal. For ULSFO, there were no statistical differences between the removal of the two classes of compounds except for the natural attenuation in the intertidal zone for which there was a higher removal of aliphatics compared to PAHs.

4.4.2. Changes in the beach microbiome

The phyla *Pseudomonadota* and *Bacteroidota* comprised the majority of the 2512 ASVs detected in the mesocosms (Fig. 4.2). The top 20 most abundant ASVs also belonged to these two phyla with the exception of ASV18 which belonged to the phylum *Verrucomicrobiota* (Fig.

S4.3; Table S4.6). The ANCOM-BC2 results showed that bacteria from the classes *Bacteroidia* (phylum Bacteroidota), Alphaproteobacteria and Gammaproteobacteria (phylum Pseudomonadota), and Verrucomicrobiia (phylum Verrucomicrobiota) were more abundant in the treated samples compared to the untreated and T0 controls and the majority of these were observed in the supratidal zone (Fig. 4.3). When comparing whether there were any differentially abundant classes between the tidal zones of the same treatment, only the class Alphaproteobacteria was more abundant in the supratidal zone of the ULSFO treatment compared to the samples of this treatment in the intertidal zone (Fig. 4.3b). At the genus level, ANCOM-BC2 showed that 14 genera from the phyla Pseudomonadota and Bacteroidota were more abundant in the fuel treatments compared to the controls and 4 Pseudomonadota genera were more abundant in the controls compared to the treated samples (Fig. S4.4; Table S4.7). We also observed 13 genera from the phyla Bacteroidota and Pseudomonadota with higher abundances in the supratidal zone and 6 genera from the same two phyla that were more abundant in the intertidal zone (Fig. S4.5; Table S4.8).

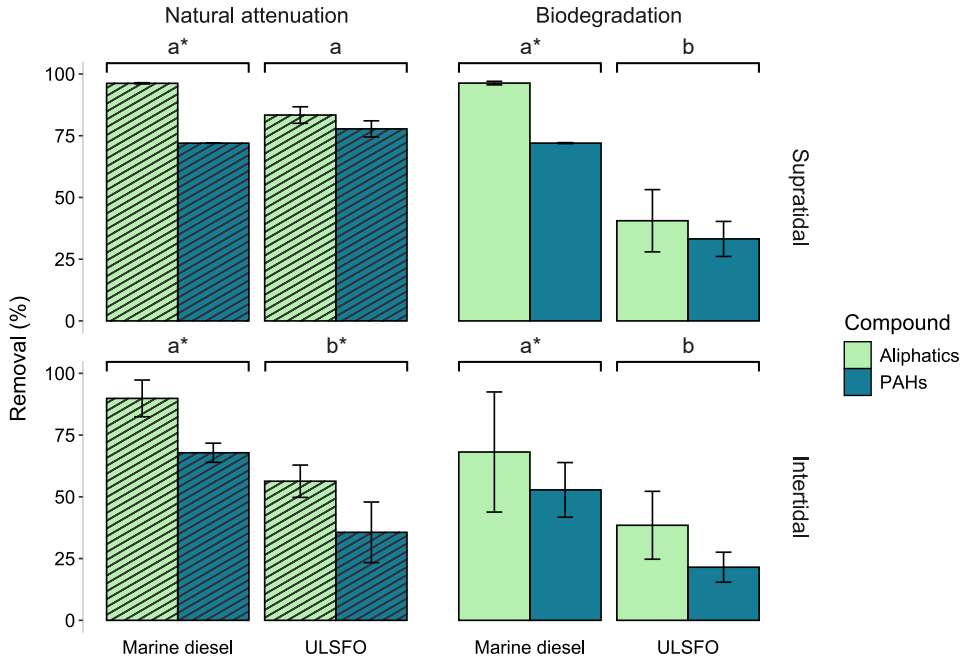

Fig. 4.1. Removal percentages of the studied fuels by natural attenuation and biodegradation and by compound group. Letters represent statistical differences among the fuels for each of the panels. An asterisk (*) represent statistical differences in the removal percentage by compound group inside a given fuel and removal type combination. Error bars represent standard deviation (n = 4 for each combination of fuel, location, and removal type).

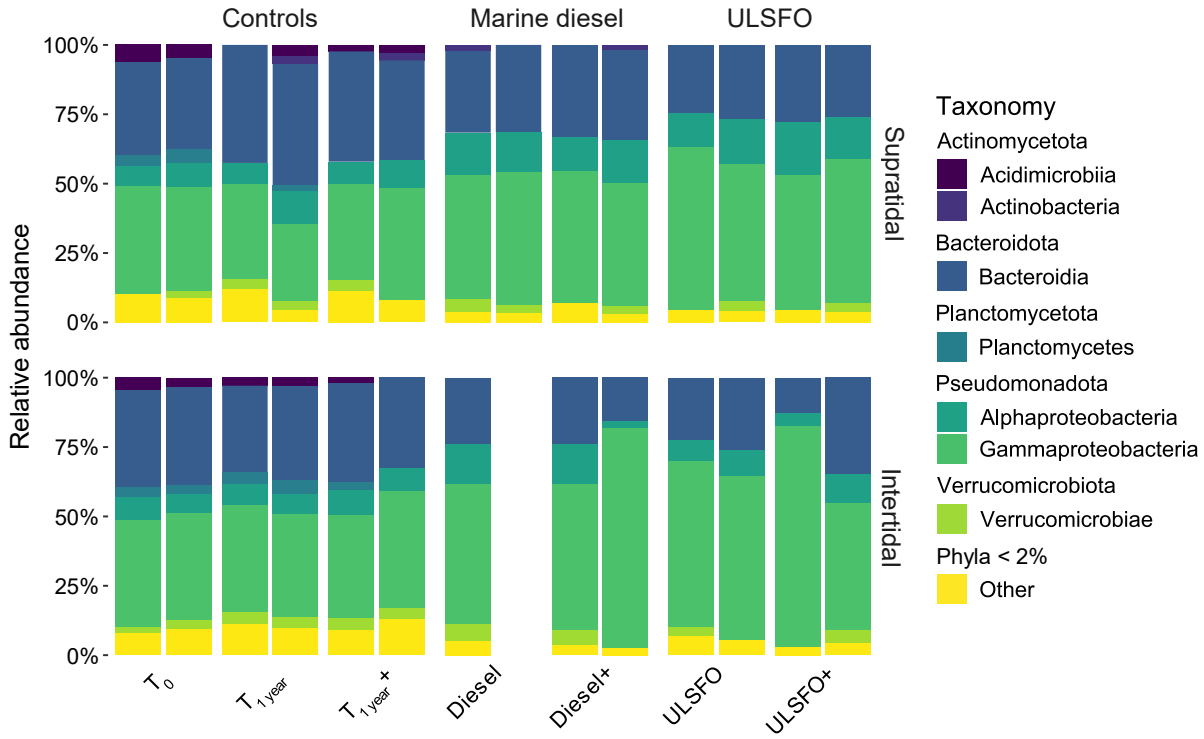

Fig. 4.2. Taxonomy of the microbial communities of the *in situ* mesocosms after a year based on 16S rRNA gene amplicon sequencing. Phyla with a relative abundance lower than 2% were pooled into "Other".

We observed a statistically significant decrease in both Shannon diversity and Faith's PD for the samples treated with fuel compared to the controls for the supratidal zone and a significant decrease in Shannon diversity, but not in Faith's PD for the intertidal zone (Fig. 4.4; Table S4.4). There were no statistical differences between the intertidal and supratidal samples within the same treatment. Similarly, we observed differences in community composition based

on Bray-Curtis dissimilarities and weighted UniFrac distances between the untreated controls and the fuel samples (Fig. 4.5; Table S4.5). However, unlike for the alpha diversity metrics, we did detect differences between the community composition between the intertidal and supratidal samples of the same treatment. The only pairwise comparison for which we did not obtain a statistical difference was for the weighted UniFrac distance between Marine diesel and ULSFO.

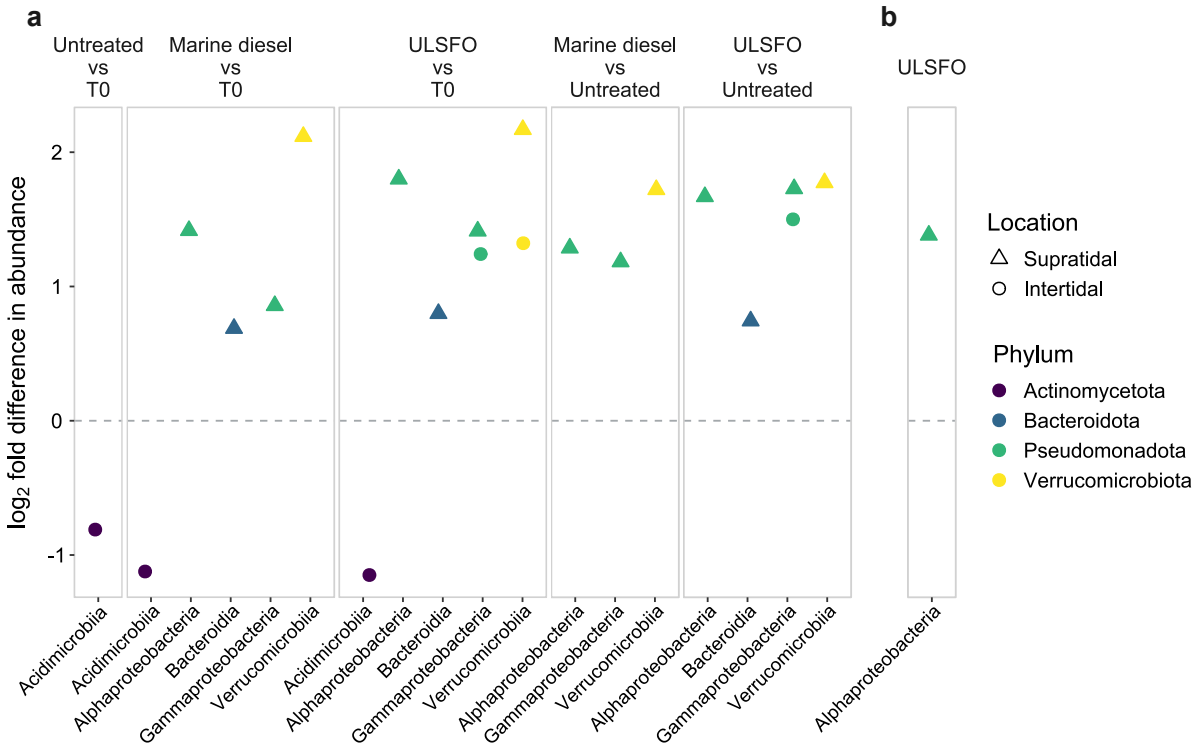

Fig. 4.3. ANCOM-BC2 results showing the differentially abundant ASVs pooled by class. (**a**) Positive values represent ASVs that were more abundant in the first treatment in the contrast and negative values represent ASVs that were more abundant in the second treatment of the contrast. (**b**) Positive values represent ASVs that were more abundant in the supratidal zone and negative values represent ASVs that were more abundant in intertidal zone.

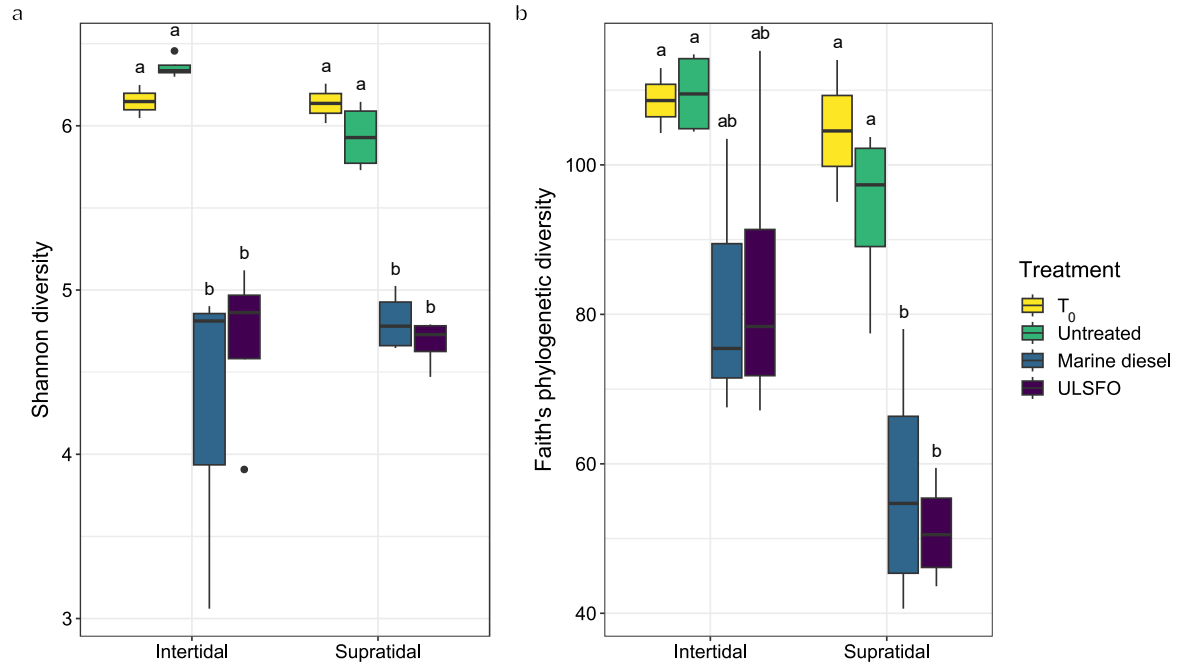

Fig. 4.4. Differences in (**a**) Shannon diversity and (**b**) Faith's phylogenetic diversity among the 16S rRNA gene amplicon community of the treatments.

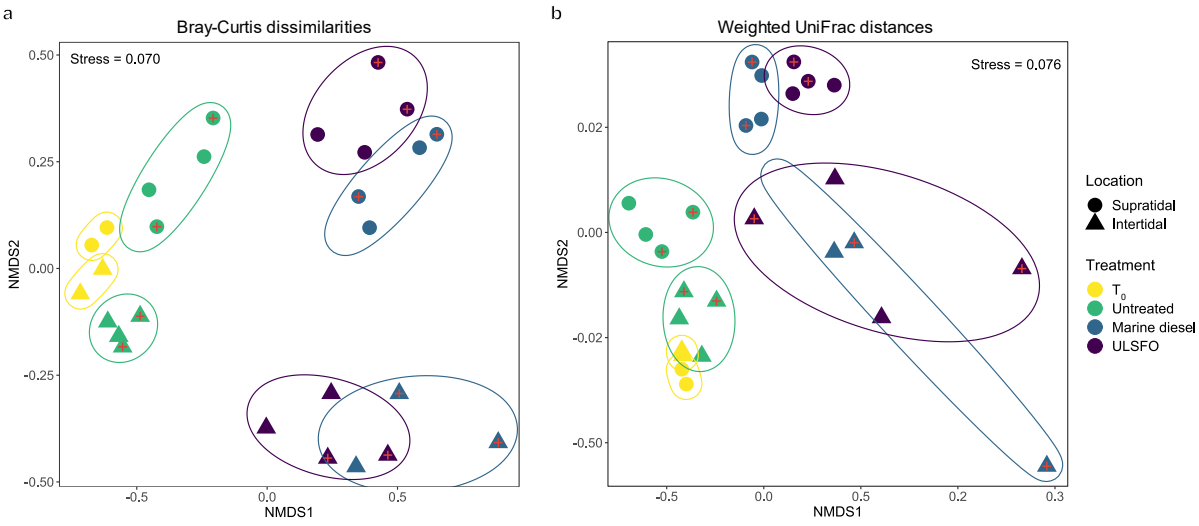

Fig. 4.5. Non-metric multidimensional scaling (NMDS) ordination of Bray-Curtis dissimilarities (**a**) and (**b**) weighted UniFrac distances for the 16S rRNA gene amplicon community composition showing how communities adapted in response to the presence of the added fuels. Symbols with red crosses represent samples for which fertilizers were added at the beginning of the experiment.

The dbRDA further illustrated how the hydrocarbon concentrations explain the differences observed in the microbial communities (Fig. 4.6). For the Bray-Curtis dissimilarities, aliphatic concentrations had a significant effect and explained 20.1% of the variability on the community composition. The ULSFO samples were more strongly driven by aliphatic values, compared to samples with Marine diesel (Fig. 4.6a; dbRDA permutation test, F = 4.99, p = 0.001). Both the aliphatic and PAH concentrations significantly influenced the community composition based on the weighted UniFrac distances (Fig. 4.6b; dbRDA permutation test, F = 4.52, p = 0.004) and explained 26.3% of the community variability. Similar to the Bray-Curtis dbRDA, the ULSFO microbial communities were more strongly driven by the hydrocarbon concentrations compared to the Marine diesel samples.

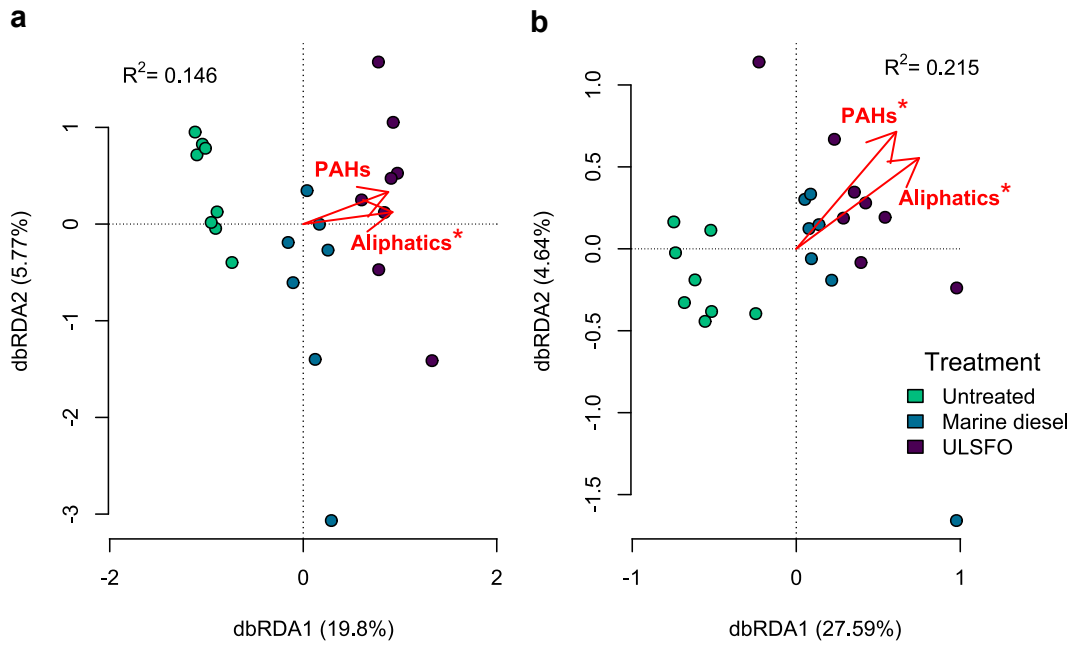

Fig. 4.6. Distance-based redundancy analysis (db-RDA) illustrating the influence of the type of hydrocarbon on the 16S rRNA gene amplicons for (**a**) Bray-Curtis dissimilarities- and (**b**) weighted UniFrac-based community composition.

4.5. Discussion

Previous work at Assistance Bay (Góngora et al., 2024a) showed that the microbial community of this NWP beach was capable of degrading only a portion of the applied fuel after 33 days. In the present study, we followed up the one-month experiment by deploying the *in situ* mesocosms for a full year to determine whether the extended contact time along with the addition of nutrient amendments improved hydrocarbon removal.

We did not observe an effect of the use of fertilizers in hydrocarbon removal (Fig. S4.1), in the alpha diversity metrics (Fig. S4.2) or in the community composition (Fig. 4.5). Previous hydrocarbon biodegradation studies using fertilizer amendments in high Arctic beach sediments obtained mixed results. Ellis et al. (2022) observed an increase in hexadecane radiorespiration rates in nutrient amended microcosms compared to the unamended samples only for one of the three studied beaches and the addition of fertilizers did not improve the naphthalene radiorespiration rates. The authors also saw no differences in ULSFO removal between the fertilized and unfertilized microcosms. In a laboratory experiment simulating tide activity with sediment from a beach in Resolute, Y.-J. Chen et al. (2024) reported an increase in alkane removal after 32 days for the treatment containing ULSFO, MAP, and S200 compared to the fuel only treatment, but did not detect an effect of the nutrient amendments after 92 days. The *In situ* Treatment of Oiled Sediment Shorelines program carried out in a shoreline in Svalbard, Norway (1997–1998) estimated that fertilizer treatments applied during the first two months of the experiment doubled biodegradation rates of the intermediate fuel oil IF-30 after a year (Prince et al., 2003). The Baffin Island Oil Spill (BIOS) project carried out in Baffin Island, Canada (1980-1984) saw an improvement in biodegradative activity of Lago Medio crude oil for the treatment plots supplemented with fertilizers (Eimhjellen and Josefsen, 1984). One explanation for the lack of effect of fertilizers in our study could be the fact that we only added these nutrient amendments once at the beginning of the experiment. It is then possible

that the fertilizer was consumed very quickly by the microbial community in the first days of the experiment and we could not observe the short-term effect with the sampling points selected. This is consistent with the experiment by Y.-J. Chen et al. (2024) which saw a biostimulation effect only in the first time point or with the BIOS project which determined that nutrient amendments increased the biodegradation rates, but not the end-point oil loadings (Owens et al., 2003). Another possibility is that the added fertilizers in our experiment were washed away by tidal action within the first couple of tidal cycles. This is why it has been suggested that constantly monitoring nutrient and adding fertilizers more than once could lead to an optimal biostimulation treatment (Prince et al., 2003).

After a year, we observed a statistically higher hydrocarbon removal of both fuels in the supratidal zone compared to the intertidal zone (Table S4.3). For Marine diesel, the lack of statistical differences between the natural attenuation and biodegradation percentages suggests that most of the removal (72.0%) can be attributed to the metabolic activity of hydrocarbon degraders. On the other hand, we did observe statistical differences between the natural attenuation and biodegradation (32.5%) removal of ULSFO meaning that a substantial proportion (29.6%) of this fuel was removed by non-biological processes. Some of the differences that we observed could be explained by the type of hydrocarbons present in the used fuels (Fig. S4.6). Marine diesel is mostly comprised of aliphatic hydrocarbons (93.3%) while 26.4% of the quantified compounds in ULSFO are PAHs. Our previous studies of the microbial community of Assistance Bay found that there is limited abundance and expression of genes associated with PAH degradation on the microbiome of this beach (Freyria et al., 2024; Góngora et al., 2024a, 2024b) as well as in other beaches across the NWP (Durand et al., 2023; Ellis et al., 2022).

We observed a pronounced difference in the microbial communities of the fuel-treated samples compared to the T_0 and untreated controls (Fig. 4.5; Table S4.5). This separation

between the treated and untreated samples evidences the capacity of the Assistance Bay beach sediment microbial community to adapt to the sudden addition of hydrocarbons as was also observed with the dbRDA which showed a strong correlation of the treated samples with the hydrocarbon concentrations (Fig. 4.6). The ANCOM results also showed that four of the 20 most abundant genera (*Oleispira*, *Altererythrobacter*, *Gilvibacter*, *Pseudohongiella*) were differentially more abundant in the fuel treated samples compared to the untreated controls (Table S4.7). However, the differences also appear to be caused by a reduction in diversity (Fig. 4.4; Table S4.4) showing that the added fuels could have also negatively affected the baseline microbiome while enriching for hydrocarbon degraders. These statistically significant differences in alpha diversity and community composition were not observed in the one-month experiment (Góngora et al., 2024a) which could suggest that the environmental impacts of a fuel spill in the NWP are greater for the microbiota after a longer exposure.

It is also worth noting that there were no differences in Faith's PD between the fuel treatments and the controls for the intertidal zone, but there were differences in weighted UniFrac for these samples. These results could be indicating that the microbial community in the intertidal zone is changing very slowly. The weighted UniFrac NMDS also illustrate how there is a larger dispersion in the fuel-treated intertidal samples compared to their supratidal counterparts (Fig. 4.5b) which further supports that the microbial communities in these samples have not fully stabilized. Additionally, there is a larger number of differentially abundant genera in the supratidal zone compared to the intertidal zone (Fig. S4.5; Table S4.8) which indicates that bacteria from the same genus appear to thrive better in the supratidal zone. We can see that 38.5% of the genera that were most abundant in the supratidal zone are known hydrocarbon degraders, while only 16.7% of the genera that were more abundant in the intertidal zone were hydrocarbon degraders (Table S4.8). On the other hand, 66.7% of the genera more abundant in the intertidal zone (23.1% in the supratidal zone) have been associated

with hydrocarbon degradation, but their biodegradative capabilities have never been confirmed. There have been various documented cases suggesting that the presence of a given taxa inside the microbial community of a hydrocarbon spill does not necessarily mean that said taxa is capable of hydrocarbon biodegradation. For example, *Colwellia* which was differentially more abundant in the supratidal zone of the ULSFO treatments, does not appear to possess or express genes involved in hydrocarbon degradation based on results of microcosm experiments using water from the water plume of the Deepwater Horizon oil spill (Peña-Montenegro et al., 2023). The transcriptomic activity of bacteria from this genus led the authors to hypothesize that *Colwellia* might be opportunistic bacteria, rather than a primary hydrocarbon degrader. Hydrocarbon degradation genes were scarcely distributed across various *Lutibacter* genomes, a genus that was more abundant in the intertidal zone of the T_0, Marine diesel, and ULSFO mesocosms. The authors suggested that this and other genera with few hydrocarbon activation genes could be secondary consumers that use the intermediary metabolic products produced by primary hydrocarbon degraders (S.-C. Chen et al., 2024). A limited colonization of the intertidal mesocosms by hydrocarbon degraders could thus explain why there was less biodegradation compared to the supratidal zone (Fig. 4.1; Table S4.3).

While our results provide some of the first evidence of the *in situ* biodegradation of ULSFO under high Arctic environmental conditions, not much is known about the behaviour and properties of LSFOs in general. Each LSFO has a different chromatographic signature (Daling and Sørheim, 2020; Faksness et al., 2024; Nelson et al., 2022; Yang et al., 2023) which could be beneficial for tracing and identification of a spill from an unknown fuel source, but it could also present issues in determining whether biodegradation could be a feasible remediation solution since the microbial communities could respond differently to each type of LSFO. For example, two different batches of ULSFO produced by Shell contain different proportions of long-chain alkanes (Daling and Sørheim, 2020). LSFOs have been seen to

become very viscous at low temperatures and viscosity further increases when water-oil emulsions are formed (Lee et al., 2023). The Swedish Coast Guard reported that after a VLSFO spill off the coast of Sweden, the fuel tended to cluster into small lumps when it reached the shore (Pålsson et al., 2024). ULSFOs have a high (> 12%) wax content (Daling and Sørheim, 2020; IMAROS, 2022) which further increases the viscosity of these fuels. While the increased viscosity could reduce fuel penetration into the beach sediment, it may also hamper biodegradation as it reduces the surface area where microorganisms come into contact with the hydrocarbons.

4.6. Conclusions

The limited amount of research on LSFOs presents a high risk for governments and response teams across the world as there does not appear to be a reliable cleanup strategy in case a LSFO spill washes onto a shoreline. In this study, we aimed to help to close the knowledge gap by evaluating whether nutrient biostimulation could improve the natural attenuation removal capacity of the microorganisms inhabiting a high Arctic beach in the NWP. We observed that there is indeed a natural biodegradative capacity in the Assistance Bay shoreline microbiome, even if we did not achieve a complete removal of the applied fuel. On the other hand, we observed higher biodegradation for Marine diesel which could be caused by its less complex, alkane-rich nature. We also observed that a single fertilizer application at the beginning of the year-long experiment was not sufficient to provide a long-term improvement in performance to the already present natural attenuation potential of the microbes inhabiting the shoreline. These findings show that the native beach microbiome does not appear to be capable of fully degrading a ULSFO spill if one were to happen on a NWP shoreline. A higher level of monitoring to help determine if more frequent fertilization application events might be needed along with more invasive stimulation techniques such as the application of surface washing agents or mechanical removal are required to achieve a complete cleanup response.

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Chapter 5. Discussion and Conclusion

5.1. General discussion

The Arctic is a fragile ecosystem that is being directly and indirectly impacted by the effects of climate change. Besides the direct warming effect causing the reduction in sea ice coverage, the Arctic is under threat by the increased human presence that has been predicted by the middle of the century and onwards due to the opening of the NWP (Howell et al., 2023; Shen et al., 2023). One of the risks that the increased ship traffic through the NWP will bring is the contamination of Arctic shorelines if a transiting vessel spills the fuel it uses to propel itself. Long before we are able to confirm the presence of hydrocarbons on beach sediments and deploy response teams to contain and clean up the spill, native microorganisms will be the first responders. We already know a great deal about oil spills and how microbes are a crucial part of most, if not all, remediation strategies. However, previous studies on experimental and real life spills under Arctic conditions have mostly focused on whether there is biodegradation occurring, not on which microorganisms are carrying out the biodegradation of these compounds *in situ* and what metabolic pathways they are using. This thesis aimed to bridge this gap of knowledge that might help to explain when the shoreline microbiota can be used to remediate spills and, perhaps more importantly, when the native microbial community is not able to completely degrade hydrocarbons.

5.1.1. The marine hydrocarbon cycle in the NWP

Here, I showed evidence of the presence of hydrocarbon-degrading microorganisms on shorelines representative of those found throughout the NWP. The presence of genes and organisms associated with hydrocarbon biodegradation appears to be quite ubiquitous in marine environments. This appears to be due to the presence of the symbiotic association of

hydrocarbon degraders and producers and the presence of naturally occurring deep hydrocarbon seeps (Dong et al., 2020; Gutierrez, 2018; Lea-Smith et al., 2015; Love et al., 2021; Radwan et al., 2019; Vigneron et al., 2023). Accordingly, the presence of these microorganisms and the genes they utilize was not unexpected in the studied beaches. However, these microbes tend to be found as small proportions of the microbial community (Ellis et al., 2022; Gofstein et al., 2020; Ji et al., 2023; Murphy et al., 2021; Schreiber et al., 2021) so observing their presence as some of the most abundant members of presumably pristine beach sediments is unprecedented. On the other hand, Khot et al. (2022) showed that key hydrocarbon degradation genes were present in the metagenomes of all of the surface seawater from the TARA Oceans project samples they analyzed. These findings support the theory that the ubiquity of hydrocarbon degraders in marine environments is much more widespread than previously thought and limitations in the methodologies used, namely the use of classic culture-dependent microbiology techniques, are underestimating the abundance of hydrocarbon degradation genes.

5.1.2. Hydrocarbon degraders in the baseline shoreline microbiomes

Additionally, a recent study from our research group which characterized the microbial communities of seawater from five of the same beaches studied for the baseline metagenomes in Cornwallis Island (Assistance Bay, Dump beach, Dynamite beach, Tank farm, and Tupirvik) using Nanopore long-read sequencing also observed that bacteria possessing hydrocarbon degradation genes were present in these metagenomes, although at lower frequencies compared to those in sediment samples from the same beaches (Freyria et al., 2024). This result along with the findings in this thesis suggest that beach sediment appears to be more enriched in hydrocarbon degraders compared to surface seawater. For example, known hydrocarbon degraders from the genera *Rhodococcus* and *Nocardia* were the two most abundant genera in

the baseline metagenomes with 26% and 28% average relative abundance across the studied NWP beaches, respectively (Table S2.3). In Freyria et al. (2024), *Granulosicoccus anarcticus* was the most abundant hydrocarbon degrader in all beaches, except for Tank farm, and comprised up to 29% of the hydrocarbon degraders. Metagenomic reads from *Granulosicoccus* were also highly abundant overall in their study. Nonetheless, the very abundant hydrocarbon-degrading taxa from the baseline metagenomes and in Freyria et al. (2024) did not appear as highly abundant after hydrocarbons are added to the beach sediment, as shown in the *in situ* mesocosm experiments.

Interestingly, all three of these genera have been observed in marine environments in association with micro- and macroalgae (El-Gendy et al., 2008; Freyria et al., 2024; Kang et al., 2018; Sinha et al., 2017). *Rhodococcus*, *Nocardia*, and *Granulosicoccus* appear to be generalists and/or opportunists that thrive when algal blooms collapse and consume a plethora of metabolites released when the algae die. For example, the genome of *G. antarcticus* contains a gene (*dmdA*) involved in the metabolism of dimethylsulfoniopropionate (DMSP), an osmolyte produced by phytoplankton and macroalgae as well as other enzymes that could be involved in the metabolism of algal polysaccharides such as alginate lyase (Kang et al., 2018). While the presence of these genes was not detected in the *Rhodococcus fascians* MAGs, Chapter 2 did show that the obtained *R. fascians* MAGs have a wide variety of metabolic pathways. Additionally, *R. fascians* is a known phytopathogenic species (Sinha et al., 2017) and there is evidence that polar *Rhodococcus* strains are able to metabolize DMSP (Qu et al., 2019; Zhang et al., 2022). Not much is known about the association between *Nocardia* and marine algal communities, but *Nocardia* isolates have been obtained from red algae (El-Gendy et al., 2008). This complements the theory of the marine hydrocarbon cycle as one of the main contributors to the presence of microbes capable of hydrocarbon degradation in seemingly hydrocarbon depleted environments (Gutierrez, 2018; Lea-Smith et al., 2015; Love et al., 2021;

McGenity et al., 2021; Radwan et al., 2019; Vigneron et al., 2023) showing that not only specialist bacteria (like "obligate" hydrocarbon degraders) are sustained by algal metabolites, including hydrocarbons, but also generalists and opportunists that also happen to possess hydrocarbon degradation genes benefit from the association with phytoplankton and macroalgae.

However, these three genera are not part of the 20 most abundant ASVs in the one-month (Table S3.3) and one-year mesocosms (Table S4.6). While these opportunist microorganisms contain hydrocarbon degradation genes, it is possible that they could not persist once a large input of hydrocarbons was added during the deployment of the mesocosms. This could be because they still require some of the metabolites produced by other microbes that are not hydrocarbon-tolerant and which did not survive the simulated spill. It could also be that they are outcompeted by the more specialized bacteria that took over the community after a month and after a year.

5.1.3. Shifts in the microbial community after simulated spill scenarios

Some taxa that were abundant, to a lesser extent, in the baseline surveys later became dominant in the *in situ* mesocosm experiments. For example, the genera *Alkanindiges*, *Alcanivorax*, C1-B045, and *Flavobacterium* were all part of the 20 most abundant genera for the one-month mesocosms and *Cycloclasticus* was the second most abundant hydrocarbon degrader in Freyria et al. (2024), after *G. antarcticus*. All these genera were observed as part of the 20 most abundant ASVs in one or both of the mesocosm experiments (Figures S3.1 and S4.3).

The genus with highest average relative abundance in the one month and one year mesocosms was *Alkanindiges* (Figures S3.1 and 4.3 and Tables S3.3 and S4.6). The second most abundant among these genera were *Flavobacterium* in the one month experiment and

Alcanivorax in the one year experiment. In an *in situ* experiment incubated for 74 days using the same type of fluorocarbon netting used in my *in situ* mesocosms to evaluate the biodegradation of marine gas oil in sea ice and seawater from Greenland, the authors did observe that various groups of OTUs did take over the microbial community with *Oleispira antarctica* having the highest maximum relative abundance (46%) in the oiled samples (Vergeynst, Christensen, et al., 2019). Another *in situ* experiment in various sites of the Douglas Channel fjord in western Canada using diluted bitumen-coated clay beads observed that *Pseudohongiella* and C1-B045 were some of the most dominant genera (Schreiber et al., 2021). In this experiment, *Pseudohongiella* was more abundant during the first 3 months of incubation with a maximum average abundance of approximately 29.7% of the microbial community for one of the sites and the proportion then decreasing to a maximum of approximately 11.7% after 12 months. C1-B045 was also more abundant after the first three months (approximately 11.1% average relative abundance for one of the sites) and decreased after 12 months to approximately 3.9% of the average relative abundance of the same site.

The average relative abundances detected the *in situ* mesocosms for the most abundant hydrocarbon genera were not as large as those detected in other *in situ* experiments in cold marine environments using similar methodologies. It thus appears that the native hydrocarbon-degrading genera detected in the baseline metagenomes are not capable of large blooms of bacteria in the mesocosms. This could be occurring because genera such as *Oleispira* (Golyshin et al., 2010), *Pseudohongiella* (L. Xu et al., 2016), *Alcanivorax* (Cappello & Yakimov, 2010), and C1-B045 (Cui et al., 2024) are considered to be marine bacteria while genera such as *Alkanindiges* (Klein et al., 2007; Yadav et al., 2021), *Flavobacterium* (Bernardet & Bowman, 2006), and *Rhodococcus* (Yergeau et al., 2012) have been detected in various marine and non-marine environments. While beach sediments could be expected to harbour both terrestrial and marine microbes, it appears that those associated with more terrestrial environments can grow

more efficiently on the shoreline, as they did not have to colonize a new environment, which could explain why the marine genera are present in very low abundances (or absent altogether) in the one-month mesocosms and only increased in abundance in the one-year mesocosms after they were able to colonize the netting (Figures S3.1 and 4.3 and Tables S3.3 and S4.6). This difference can also be observed in the top 20 most abundant ASVs of the one-year mesocosms; the samples from the intertidal zone contained higher abundances of the marine-associated genera and lower abundances of the sediment-associated genera (i.e., more marine influence as this zone would be more frequently submerged) while the samples from the supratidal zone contained higher abundances of the sediment-associated microbes and lower amounts for the marine taxa (i.e., more terrestrial influence as this zone is submerged for less time during the day).

However, this does not explain why genera such as *Alkanindiges* and *Flavobacterium* had relatively smaller increases in relative abundance compared to taxa from other *in situ* experiments (Schreiber et al., 2021; Vergeynst, Christensen, et al., 2019). This was also the case during the Deepwater Horizon spill where over 80% of the microbial community of the hydrocarbon plume was dominated by a single group of *Oceanospirillales* (Mason et al., 2012). *Alkanindiges* can be capable of such blooms as reported for a microcosm experiment using soil from Chile where bacteria from this genus comprised over 60% of the community after 6 weeks while initially being found at around 0.1% of the initial sample (Fuentes et al., 2016). *Alkanindiges* also comprised up to 75% of the clone libraries of activated sludge foam from a wastewater treatment plant in Illinois. *Flavobacterium* have not been documented to produce such strong bloom events, but they appear to increase in abundance in the late stages of the biodegradation process (Vergeynst, Christensen, et al., 2019).

There are various explanations of why this could be occurring. The first, could be that I only studied initial and final time points in this thesis; it is possible that the selected time

points of one month and one year did not capture key transition events that took part in the microbial succession, such as the blooms of certain taxa (Dubinsky et al., 2013). Another explanation could be that the cold, often below-zero, temperatures experienced in the Resolute region through most of the year (Figure S3.6) hindered microbial colonization and did not allow the bloom of these microbes. Indeed, the netting mesocosms from sea ice and seawater from the coast of Greenland showed that the microbial communities from the open seawater and, to a lesser extent, from the bottom of the sea ice showed blooms of certain taxa, even in the untreated controls (Vergeynst, Christensen, et al., 2019). On the other hand, similar to the top and middle ice samples in Vergeynst, Christensen, et al. (2019), the microbial communities of the untreated controls did not differ statistically from the communities of the T_0 controls in the one-year mesocosms and that the oiled samples did not show a blooming event, even if they did change compared to the T_0 and untreated controls. It is possible that the freezing conditions during that likely occurred during the one-year mesocosms are preventing the appearance of a bloom, even if there still appears to be microbial activity.

5.1.4. Metagenomic and metatranscriptomic evidence of hydrocarbon degradation

Regardless of the taxonomic composition of the microbial community of these beaches, an important aspect of the shoreline microbiome that was observed throughout this thesis was the presence, or absence in some cases, of genes associated with hydrocarbon biodegradation as it is a strong determinant on whether the components of shipping fuel will be degraded or not. Alkane degradation genes were present in the baseline metagenomes and were being expressed after a month of incubation in the mesocosms. However, there were not as many genes involved in the biodegradation of PAHs in the baseline metagenomes and they were almost absent and very poorly expressed in the one-month mesocosms. This is consistent with the results of the hydrocarbon analyses of the two *in situ* mesocosm experiments which showed

that alkanes were preferentially degraded over PAHs and also consistent with previous studies (Chen et al., 2024; Ellis et al., 2022; Garrett et al., 2003; Murphy et al., 2021; Schreiber et al., 2021; Vergeynst, Greer, et al., 2019). Additionally, even for the lightest, most aliphatic-rich fuel tested (Marine diesel), only in the supratidal zone I was able to observe almost complete removal of the added fuel (Figures S3.4 and S4.6).

This is of concern when thinking about the use of bioremediation in the case of a large-scale hydrocarbon spill. The highest amount of oil added in the mesocosms was that for the Bunker C treatments in the one-month mesocosms with approximately 80 mg of total petroleum hydrocarbons (TPH). Assuming that the mesocosms came into contact with one kg of sediment, this would most likely mean that the amount of Bunker C added is already below the allowed levels Canada-wide Standards for petroleum hydrocarbons in soil (30 – 6600 mg/kg), depending on the hydrocarbon fraction, soil type, and land use of the affected area (Canadian Council of Ministers of the Environment, 2008). Even if the fluorocarbon netting we used provides optimal conditions for biodegradation (Vergeynst, Christensen, et al., 2019), the native microbial communities of Assistance Bay do not appear to be capable of removing all of the introduced hydrocarbons after a month or a year. Additionally, it has been observed that increasing the oil concentration further inhibits microbial growth and biodegradation rates (Bacosa et al., 2018; Durand et al., 2023; Murphy et al., 2021) which could mean that we are overestimating the biodegradative capabilities of NWP beaches.

5.1.5. Bioremediation as a potential cleanup strategy for a hydrocarbon spill on the NWP

The results of my thesis bring to light very important questions the Canadian government needs to consider in the upcoming decades: which types of fuels should be allowed to be transported in Canadian Arctic waters? Should we allow the transit of shipping vessels through the NWP altogether? Based on my results, Bunker C presents the worse case

alternative as it is very poorly biodegraded due to its high PAH content. While it appears to be more easily removed from the mesocosms due to physical removal, this oil will remain in either the water column or bound to sediment particles where it is still of environmental concern. Even though Bunker C is technically not allowed based on the 2020 IMO global sulfur cap due to its high sulfur content, this and other heavy fuel oils (HFOs) are still considered as a possibility by the shipping industry because the emission limits can be achieved by the use of scrubber systems than can filter sulfur oxides and other harmful combustion gases so that the released emissions remain below the 0.5/0.1% cap (Fan et al., 2023). Light marine gas oils (MGOs) such as the Marine diesel tested is, in my opinion, the best alternative as it is the fuel that will be biodegraded the fastest and for which the microbial community of NWP shorelines have the appropriate biodegradation genes due to its high aliphatic and low PAH content. However, marine gas oil fuels are very expensive and tend to be used exclusively inside SECAs. ULSFO and similar new generation LSFOs can be considered as a compromise in between HFOs and MGOs due to their higher aliphatic content compared to HFOs but higher PAH content compared to MGOs. This makes them more biodegradable than HFOs, but they are not as biodegradable as MGOs. The high wax content of the new ULSFOs gives them undesirable physicochemical properties such as their elevated pour point (Daling & Sørheim, 2020; Faksness et al., 2024; Faragher et al., 2024; Pålsson et al., 2024) that could make more difficult their ability to be physically or biologically removed from cold environments (Pålsson et al., 2024).

A very important issue that should be noted is that, irrespective of the scientific results provided in this thesis and other past studies, if strict regulations are not imposed at the national and international level, the fuel type choice will ultimately be determined by a financial risk assessment carried out by shipping companies. For example, since both MGOs and new LSFOs tend to be more expensive than HFO, studies have shown that the choice between using

ULSFOs (0.1% sulfur content) inside SECAs and VLSFOs (0.5% sulfur content) in all other areas versus installing a scrubber system using exclusively HFOs will depend on the distance travelled inside the SECA with scrubbers being more cost-efficient if more than 6% of travel occurs inside a SECA (Fan et al., 2020, 2023). The distance that would need to be covered to traverse the NWP is much larger than that of the Chinese SECA evaluated for the Fan et al. (2020) study and is very likely to exceed the estimated 6% of travel distance, which suggests that shipping companies might be more financially incentivized to install scrubbers. There are also other factors that could affect this decision such as the volatility of fuel and scrubber installation prices, the age of the ship, and the size of the company, among others (Abadie & Goicoechea, 2019; Fan et al., 2023; H. Xu & Yang, 2020).

Additionally, ports in some countries such as France, Malaysia, the United States, and China do not accept ships with open-loop scrubbers which discharge the wash water resulting from the scrubber activity from the system into the marine environment (Andersson et al., 2020; Zis & Cullinane, 2020). The contaminated wash water could cause negative environmental impacts if discharged into the ocean without being treated first (Andersson et al., 2020). In 2020, there were around 919 vessels with a scrubber system installed (Fan et al., 2023). However, this number appears to be increasing and as by the end of 2022, there were around 4800 ships that are using scrubbers and, among these, 81% were running open-loop scrubbers (Jönander et al., 2023). This shows that the shipping industry does not appear to be interested in the use of LSFOs over HFOs and are turning towards the use of scrubbers in order to comply with the 2020 IMO regulations. Interestingly, this trend only appears to be part of a transitional period towards ship that run on alternative fuels such as liquefied natural gas (LNG), methanol, ammonia, or hydrogen. In 2022, 61% of the new ship orders were designed to use these alternative fuels with 57% of these using LNG (Zou & Yang, 2023). Another 13.5% of the orders were designed so they could be retrofitted to an alternative fuel in the

future (Zou & Yang, 2023). Nonetheless, the use of alternative fuels will need to come with heavy investment by shipping ports so that they can provide reliable refuelling capacities. Without these, it is expected that alternative fuels such as LNG will not be economically feasible for Arctic shipping routes as was shown in a study looking at the use of LNG on the Northern Sea route (H. Xu & Yang, 2020). However, these new technologies will most likely not be implemented in older ships and thus the risk of a hydrocarbon spill in the Arctic will continue until the shipping industry has fully moved away from traditional fuels such as the ones I studied in this thesis. It is thus necessary for the Canadian government to develop strict regulations that limit what kind of marine fuels will be allowed to be transported along the NWP taking into consideration the poor biodegradability of the fuel oils currently being used and consequently their long-lived environmental impact on the NWP marine system.

5.2. Future work

The results of this thesis provided the first *in situ* evidence of the capabilities of Arctic beach sediment microorganisms to degrade fuels commonly used by the shipping industry. However, there is much that we still do not fully understand about the hydrocarbon biodegradation process in these environments. While I attempted to describe the baseline metagenomes of several beaches which are representative of different regions along the NWP, there are still many other beaches and regions which I was not able to sample. For example, the upper route of the NWP west of Resolute is poorly studied as it is mainly uninhabited which could lead to a spill in this region to remain unnoticed for a longer period of time. On the other hand, the eastern coast of Baffin Island is a much more populated and frequented region were a clean up response could be carried out more quickly and efficiently. Describing the microbial communities in beaches from these regions would complement my results of and provide more reference points to determine baselines that can be used by response teams.

During an actual clean up campaign following a spill, there would be constant monitoring of the spill to assure that the chosen remediation strategy is effective. This is a limitation of my work with the *in situ* mesocosm experiments in which I was only able to take single sample points at the end of the experiments due to logistic and financial reasons. This could be one of the possibilities why the fertilizer treatments showed no effect on the amount of fuel removed after a year. Having a larger number of sampling points would allow us to determine whether fertilizers could have provided some short term (days) beneficial effects on biodegradation rates and whether the inclusion of more fertilizer addition events could have further improved biodegradation in the long term. Additionally, as mentioned earlier in this discussion, the amounts of fuel added to the mesocosms was relatively low in terms of the total volume of fuel that would be present on a beach during a real spill and there is evidence from other studies that a larger amount of fuel could inhibit microbial activity. It will then be

interesting for future studies to determine how the total amount of added fuel affects biodegradation. While a large scale fuel spill field experiment would be an ideal situation to study this, the long term environmental impact that this could cause on the selected beach is an important trade-off that needs to be considered. An alternative could be another mesocosm experiment in which multiple layers of fuel-covered netting are added one on top of the previous one to simulate a thicker fuel slick washing into the beach.

Also based on the results of the mesocosm experiments, I observed that there appeared to be more biodegradation occurring in the supratidal zone. I hypothesized that this could be due to the type of microorganisms that colonize the mesocosms with more terrestrial microbes being able to better adapt to the more contained conditions of the buried netting. In order to test this, future studies could isolate microorganisms from the supratidal and intertidal mesocosms and grow them in both liquid and solid media (or in artificial seawater and sediment) amended with hydrocarbons to simulate the aquatic and terrestrial environments and determine if these isolates perform better in the media that simulates the environment they were isolated from, in the opposite medium, or if they do not have a preference.

While the *in situ* mesocosm experiments allowed us to capture the activity of the microbial community under *in situ* environmental conditions, the opening of the NWP will only occur when temperatures in the Arctic increase. This means that ambient temperatures in these beaches would also be higher than those observed during my experiments. Future studies need to determine if increases of a few degrees could affect the microbial metabolic rates in these environments. Additionally, the duration of the Arctic summer would be longer in the future. These two points could mean that beach microorganisms could be more active for a longer period of time and, thus, my results could be underestimating their metabolic potential compared to what they could be capable to do in a few decades from now. Column microcosm experiments using beach sediment from Tupirvik and simulating tidal conditions were

developed by colleagues from my research group and were run at 8 °C for 32 days and a second version was run for 92 days at 4 °C (Chen et al., 2024). These conditions could be more representative of the temperatures and summer durations that could occur in the NWP towards the second part of the century. However, these results are not directly comparable since in the column experiment the ULSFO was placed on the top of the sediment column and was not covered with more sediment. A combination of the method used to deploy the *in situ* mesocosms and the column design from this study in which circular netting fragments coated with fuel are added to the sediment and then covered with more sediment could be useful to understand how different temperature increases impact hydrocarbon biodegradation rates.

5.3. Conclusion

During talks other members of our research team and I had with community leaders from the hamlet of Resolute before we started studying the beaches around their home, one of the elders told us that "it is not a matter of 'if', it is a matter of 'when'" as he was speaking about the possibility of a ship accident somewhere in the Arctic and causing an oil spill. Without the proper regulation and contingency plans, the Arctic is at risk of a severe environmental catastrophe. This thesis aimed to help deal with this issue by providing a description of how the hydrocarbon biodegradation process occurs *in situ* on a NWP beach in order to understand if we can rely mainly of the native microbial community of Arctic shorelines as the main remediation strategy.

In Chapter 2, I provided a description of the baseline microbial communities of beach sediments from 9 high Arctic shorelines. I detected the presence of known hydrocarbon-degrading organisms as well as the genes they use to degrade hydrocarbons. In some cases, hydrocarbon degraders dominated the microbiome of these beaches even in the absence of large amounts of hydrocarbon on these presumably pristine beaches. Accordingly, I showed that these bacteria have alternative metabolic pathways that they could be using to survive in these oligotrophic environments where there are no abundant sources of carbon.

In Chapter 3, I studied the *in situ* natural attenuation of three types of fuels used by the shipping industry. LSFOs are more biodegradable than the legacy HFO tested under Arctic environmental conditions. Even though biodegradation occurring for all three fuels was observed, it was mostly for aliphatic compounds for which biodegradation genes were highly abundant and expressed. However, a large presence or expression of microbial metabolic pathways associated with the degradation of PAHs was detected.

In Chapter 4, I evaluated the use of long-term biostimulation as a strategy to improve the LSFO biodegradative capacity of the microbiota of the beach sediment of a NWP shoreline.

An effect of the use of N and P fertilizers on the biodegradation activity was not observed. Strong changes in the microbial community of fueled samples was detected and serves as evidence that a longer incubation time might allow for the colonization of a more specialized microbial community. However, this did not lead to a pronounced improvement in the biodegradation of the tested fuels compared to the short term experiment in Chapter 4.

This thesis provides valuable details on the molecular mechanisms of the hydrocarbon biodegradation process as it would occur on a NWP shoreline. I also showed the first evidence of the biodegradability of the new generation of LSFOs and how they are an improvement over the HFOs they are meant to replace. Nonetheless, my results should also serve as a warning for the Canadian government and other stakeholders that will be involved in a potential shipping route through the NWP. These entities will need to evaluate how we implement hydrocarbon remediation in the high Arctic and to improve their preparedness as it is very likely that we will not be able to fully rely on the native microorganisms in the case of a fuel spill in the NWP. Discussions should also be had on whether the time and cost savings that the NWP provides are worth the potential environmental damage that a spill on one of these beaches would create, and if we should reconsider using the NWP for global transport altogether.

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